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A mitochondrial DNA analysis of vicariant speciation in two lineages in the *Drosophila mulleri* subgroup

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ABSTRACT

Hypothesis: Biogeography and patterns of host cactus use in two ancestral-derived species pairs of cactophilic *Drosophila* suggest that recent divergence and speciation in both lineages were triggered by the same ecological/geological event in North America, the northward spread of the Sonoran Desert, isolating western coastal populations from the main distribution of each ancestral species.

Organisms: Two pairs of species in the *Drosophila repleta* species group: *D. aldrichi* and *D. wheeleri* of the *D. mulleri* cluster, and *D. longicornis* and *D. mainlandi* of the *D. longicornis* cluster.

Analytical methods: We analysed sequences of the mitochondrial cytochrome oxidase subunit II (*cox2*) and NADH dehydrogenase subunit 3 (*nad3*) from both species pairs, as well as members of relevant outgroups, to determine whether molecular evidence is consistent with concurrent speciation in both lineages. Based on long-term collecting records, we documented patterns of host cactus use throughout the ranges of all four species.

Results: Pairwise sequence comparisons between members of each species pair showed ~1% sequence difference. This difference was no greater than pairwise intraspecific comparisons within *D. aldrichi* consistent with recent evidence that *D. aldrichi* may be composed of more than one species. The interspecific differences we observed could also represent ancient polymorphisms, rather than species-specific divergences. We estimated an upper limit on the time of divergence by constructing a linearized tree based on transversion substitutions for nine species in the *D. repleta* species group. The results suggest that these species pairs arose no more than 0.2 million years ago, and may be much more recent.

Keywords: cactus, *Drosophila mulleri* subgroup, *Drosophila repleta* group, mtDNA, Sonoran Desert, speciation.

† This paper is dedicated to the memory of Bill Heed.

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INTRODUCTION

Gauging the antiquity of current biogeographical assemblages generally requires historical information from fossil, tectonic or other direct means, including estimates of genetic divergence of extant species distributed across geographic landscapes with well-studied climatic histories. Assessing concordance among species distributions experiencing similar climatic or other vicariant events has provided great insight into the understanding of micro-evolutionary events that have shaped current species distributions (Riddle *et al.*, 2000; Douglas *et al.*, 2006; Riddle and Hafner, 2006). Biotic assemblages in the southwestern deserts of North America have been the subject of intensive comparative phylogeographic analysis because of the well-studied geological and climatic histories of these regions. The recent history of the southwestern United States and northwestern Mexico may be conceptualized in terms of the origin and expansion of the Sonoran Desert scrub and other arid lands and deserts that surround it, as interpreted from zoogeographic (Morafka, 1977) and botanical evidence (Axelrod, 1979; Thorne, 1986; Betancourt *et al.*, 1990). These studies have shown that current biotic communities are very recent associations, not only because of their constant latitudinal (and longitudinal) movement and elevational progression and retreats along mountain slopes, but by the ever-present addition and deletion of individual species to and from them. This dynamic activity has led to a number of examples of disjunct distributions among communities and species.

Among the less enigmatic, but most well-studied species associations in and around the Sonoran Desert are the cacti and *Drosophila* species that inhabit them (Heed, 1978; Heed and Mangan, 1986; Ruiz and Heed, 1988). Two pairs of sister species in the *mulleri* subgroup of the *repleta* species group in the genus *Drosophila*, *D. aldrichi*/*D. wheeleri* and *D. longicornis*/*D. mainlandi*, show vicariant species distributions. The two members of each pair are currently separated by the Gulf of California, the mountain ranges of northern Baja California and southern California, as well as the Sonoran and Mojave Deserts (Fig. 1). The Gulf, in its present location, is a hindrance to migration only in the southern part of the ranges of these species. To the north of the Gulf, isolation of the western member of each pair is due to mountain ranges in southern California, northern Baja California, and deserts. These mountains are not high enough to hinder dispersal; therefore, of the three possible barriers to gene exchange of a postulated once continuous distribution, we consider the deserts to be most important (Fig. 1).

Deserts can be both barriers to faunal exchange for some insects, as well as areas of survival for others (Howden, 1969). The *Drosophila* fauna endemic to the Sonoran Desert very likely originated from four independent lineages from the more tropical parts of Mexico, and most likely evolved *in situ* with columnar cacti, their host plants (Gibson, 1982; Heed, 1982; Etges *et al.*, 1999). By contrast, the two pairs of sister species reported here evolved with prickly pear cacti in the genus *Opuntia* (Patterson and Stone, 1952; Wasserman, 1992). Prickly pears reach their arid limits in the Sonoran Desert region (Turner *et al.*, 1995) because they are sensitive to long periods of drought (Shreve, 1951), even though they can tolerate short-term, high-temperature conditions (Gibson and Nobel, 1986). Although these cactophilic drosophilids are not as host plant specific as are the endemic desert species because they use a variety of different *Opuntia* species, the exclusion of most *Opuntia* species from the more arid parts of the desert regions apparently forms an effective barrier between the eastern and western members of each pair.

What is most interesting is the coincident distribution and commonality of larval feeding

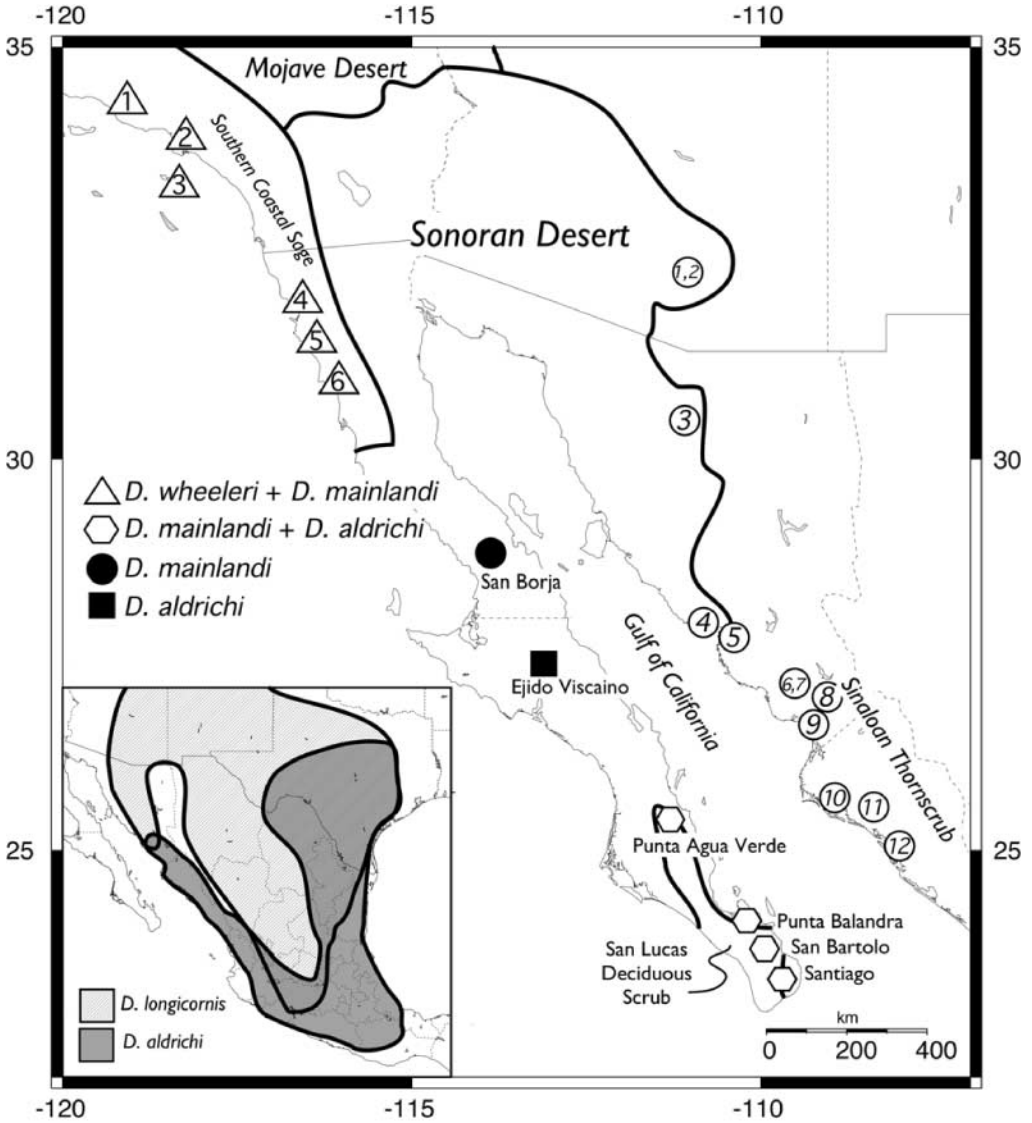

Fig. 1. The extent of the Sonoran Desert in the southwestern USA, Baja California, and northwestern Mexico; the southern Mojave Desert, and adjacent vegetation types are demarcated by thick lines (see text for details). The numbered mainland sites from Tucson, Arizona to El Dorado, Sinaloa correspond to the rearing records listed in Table 5A, and the numbered sites from Camarillo, California to San Telmo, Baja California correspond to the rearing records in Table 5B. *Drosophila aldrichi* and *D. mainlandi* also originated from locations described in the text from baited collections in the remainder of Baja California. INSET: In mainland Mexico and the southern USA, *D. longicornis* is chiefly a resident of the central plateau, while *D. aldrichi* is more abundant in the tropical coastal lowlands.

sites of the two derived western isolates, *D. wheeleri* and *D. mainlandi*, associated with several species of *Opuntia* in coastal southern California (as described by Benson and Walkington, 1965), several of the Channel Islands, and northern Baja California (Fig. 1). All these areas are described as Diegan Coastal Sage and the southern part of the Ventura Coastal Sage (Axelrod, 1978). This parallel biogeography and pattern of host plant use led us to explore the possibility that the vicariant event was concurrent for the two parental species. Evidence that the separation of *D. aldrichi* and *D. wheeleri* was a relatively recent event comes from crossing experiments, where fertile female and sterile male hybrids have been obtained in both reciprocal crosses (Patterson and Alexander, 1952) and high genetic similarity based on allozyme data (Heed *et al.*, 1990). Comparable crosses were carried out between *D. longicornis* and *D. mainlandi* as part of this study.

A current classification of the species studied here is given in Table 1. The *Drosophila mulleri* subgroup is the largest of five defined subgroups within the *D. repleta* species group and is made up of about 48 species (Wasserman, 1992; Oliveira *et al.*, 2005). The subgroup as a whole appears to represent a relatively recent radiation, based on morphological, cytological, and DNA sequence studies. The subgroup is further subdivided into complexes, based primarily on cytological similarities, and the complexes into clusters. This hierarchical classification helps to clarify the diversity of species in the subgroup, and is the most recent estimate of phylogenetic affinity among these species (Durando *et al.*, 2000; Oliveira *et al.*, 2005).

Here, we first examine sequences of two mitochondrial genes from representatives of these four species, and compare them with sequences from several outgroup members of the *D. repleta* group (Table 1). The main hypotheses are: (1) that the mtDNA sequence divergence between the members of each species pair is small, and indicative of very recent speciation; and (2) that the level of mtDNA divergence is similar in both species pairs, suggesting that speciation was initiated in both lineages at about the same time. We then present the results of long-term collection records mapping the distributions of the

Table 1. Classification of the species included in this study (after Wasserman, 1992; Oliveira *et al.*, 2005)

Group	Subgroup	Complex	Cluster	Species
D. repleta (~100 species)				
	D. hydei (7 species)			*D. hydei*
	D. mulleri (48 species)	*D. mulleri* (22 species)	*D. mulleri* (8 species)	*D. mulleri* *D. aldrichi* *D. wheeleri*
		D. longicornis (13 species)	*D. longicornis* (4 species)	*D. longicornis* *D. mainlandi* *D. spenceri*
			Other (4 species)	*D. hamatofila*

Note: The species diversity of each lineage is shown below the names.

vicariant species pairs to view the phylogenetic results from a biogeographic perspective in an effort to understand the forces driving species diversification.

MATERIALS AND METHODS

Sources of the flies for the molecular analyses

Most of the taxa analysed here were derived from stocks maintained in Tucson, Arizona. Many of them are available from the Tucson species stock centre. In a few cases, single wild-caught flies were analysed. For each species, the stock designation and origin is given: *D. mulleri* (A950 – Flamingo, Everglades, Florida), *D. aldrichi* (five lines: W8 – Tehuantepec, Oaxaca; A917 – South of Ejido Viscaino, Baja California; A976/A978 – Santiago, Cape and Punta Agua Verde, Baja California [pooled]; A987 – Rio Grande Village, Big Bend National Park, Texas; A990 – Las Bocas, Sonora), *D. wheeleri* (three lines: A751 – Arcadia, California; A956 – Santa Catalina Island, California; and A754 – Ejido Uruapan, Baja California), *D. spenceri* (A882 – Playa Cocharit, Mexico), *D. longicornis* (A909 – Punta Gorda Island, Empalme Bay, Guaymas, Sonora; A892 – Alamos, Sonora; and two wild individuals from Tucson, Arizona), *D. mainlandi* (A956 – Catalina Island, California; A978 – Punta Agua Verde, Baja California; one wild-caught from Oxnard, California). The other two taxa were collected for this study: *D. hamatofila* was collected from fermenting bananas in Tucson, Arizona, while *D. hydei* was collected over fermenting tomatoes in Coquitlam, British Columbia, Canada.

DNA extraction, PCR, and sequencing

DNA was extracted from individual females using the technique described in Beckenbach *et al.* (1993). Briefly, each fly was ground in the presence of protease K, extracted with phenol, then with chloroform/isoamyl alcohol, and ethanol precipitated. The pellet was washed once or twice with 70% EtOH and redissolved in 50 µl H_2O for use in the polymerase chain reactions (PCRs).

Each fly was analysed for two mitochondrial genes, cytochrome oxidase II (*cox2*) and NADH dehydrogenase subunit 3 (*nad3*). Two methods of amplification and sequencing were employed over the course of this study. Initially, amplification and sequencing of *cox2* was carried out using the methods described in Liu and Beckenbach (1992) and Beckenbach *et al.* (1993). Later, *cox2* was analysed by double-strand amplification of the gene in two overlapping fragments using primers TL-J3033 (5′-TAATATGGCAGATTAGTGCA) and C2-N3665 (5′-CCACAAATTTCTGAACATTG) for the first portion, and C2-J3396 (5′-ACAATTGGTCATCAATGATA) and TK-N3796 (5′-ACTATTAGATGGTTTAAGAG) for the second half of the gene. Primer designations are J for the coding strand and n for the non-coding strand. Primers are numbered according to the 3′ position in the *Drosophila yakuba* mitochondrial sequence (Clary and Wolstenholme, 1985). Primers used for the *nad3* gene are as follows: TG-J5584 5′-AGTATATTTGACTTCCAATC ($tRNA^{GLY}$) and TN-N6160 5′-TCAATTATATCATTAACAGTGA ($tRNA^{ASN}$). Products were sequenced from both strands for at least one member of each species. A short compression in the *D. aldrichi cox2* gene was resolved by sequencing both strands for all individuals in that region.

In the initial stages of this study, *cox2* products were amplified asymmetrically and single-strand sequenced as described in Beckenbach *et al.* (1993). Later, we switched to cycle

sequencing with ^{33}P-labelled dideoxy sequencing using Thermal Sequenase (Amersham), following the manufacturer's recommended procedures. Double-strand template was gel purified using the freeze–squeeze technique (Thuring *et al.*, 1975; Tautz and Renz, 1983): PCR products were run out on an agarose gel with tris acetate buffer, the band cut out and placed at −20°C overnight. They were then spun for 5 min in a microfuge, and the liquid squeezed out during centrifugation used directly for sequencing.

The primary reason for sequencing two different regions of the mitochondrial genome was to provide improved discrimination for closely related species by increasing the number of sites. The *nad3* gene was chosen in part because it is one of the most variable of the mitochondrial genes, at least for higher-level phylogenetic comparisons (Clary and Wolstenholme, 1985). The *cox2* gene was chosen to allow comparison with the drosophilid *cox2* sequence database (Beckenbach *et al.*, 1993; Spicer, 1995; Spicer and Pitnick, 1996). The *cox2* gene has a higher GC content (25.6–26.9% vs. 20.2–21.6% for *nad3*), reflecting differences in amino acid composition, but the two genes have very similar levels of variation – mostly within 1% of each other.

Data analysis

No gaps were observed in either gene, or in any of the sequences. Therefore, alignment and homology of all sites were unambiguous. Initial analysis was carried out using ESEE (Cabot and Beckenbach, 1989). Trees were constructed using three different methods: neighbour-joining, maximum parsimony, and maximum likelihood. Maximum likelihood was conducted using DNAML and DNAMLK in the PHYLIP package (Felsenstein, 2005). Bayesian analysis was conducted for 500,000 generations with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Neighbour-joining (Saitou and Nei, 1987) and parsimony were carried out using the MEGA4 program (Kumar *et al.*, 2004). In the neighbour-joining analysis, distances were corrected using the Kimura-2-parameter method (Kimura, 1980). Rate constancy was tested using the NJBOOT package of programs (Takezaki *et al.*, 1995).

The two genes, *nad3* (351 nucleotides, 117 codons) and *cox2* (687 nucleotides, 229 codons), were analysed separately, and then combined into a concatenated sequence for each taxon. This procedure is appropriate for mtDNA molecules, which are primarily maternally inherited with little evidence of recombination. Except where noted, all analyses gave comparable results, so only the analyses of the concatenated sequences are given.

Host plant data

Fermenting joints (pads) of various species of prickly pear cacti, *Opuntia* sp., were collected in the field and placed in 5-gallon jars in the laboratory at the University of Arizona. Emerging adults were aspirated from the jars and aged on laboratory media and later counted and identified. Species identification of the cactus vouchers was requested in cases of doubtful identity.

Hybridization tests

Drosophila longicornis from Navojoa, Sonora (stock no. A893) was crossed to *D. mainlandi* from Santa Catalina Island (stock no. A956) in two sets of reciprocal small mass matings (10–20 individuals each sex). No larvae were observed after 2 weeks, so the replicate mass matings were pooled for each reciprocal. F_1 progeny appeared after a further 2 weeks.

RESULTS

Molecular analyses

A total of 1038 nucleotide sites were compared from representatives of eight species. A total of 223 variable sites (45 first, 13 second, and 165 third codon positions) were observed. Aligned sequences of *cox2* and *nad3*, for one member of each stock/species, have been deposited in GenBank (accession numbers: EU555337–EU555374). Within the *D. mulleri* subgroup (i.e. excluding the outgroup species, *D. hydei*), 182 variable sites were found (33, 11, and 138 at the first, second, and third codon positions respectively). Corrected pairwise sequence divergences and number of differences between species pairs are given in Table 2. Corrected pairwise divergences among the *D. mulleri* group ranged from less than 1% to just over 10%, indicating relatively recent divergence of these species. The outgroup species, *D. hydei* (in the *D. hydei* species group), showed corrected divergences of 11–14% for all members of the *D. mulleri* group.

The two members of each species pair, *D. longicornis*/*D. mainlandi* and *D. aldrichi*/*D. wheeleri*, are very closely related, with just over 1% divergence between members of each pair (Table 2). This divergence is only slightly greater than that observed in some intraspecific comparisons (Tables 3, 4). There were some differences between individuals of the widespread species, *D. aldrichi*, that were as great as the *D. aldrichi*/*D. wheeleri* interspecific comparisons (Table 4). These results are consistent with a very recent separation of these species, and the hypothesis that the western and eastern populations of *D. aldrichi* comprise at least two different species (Richardson, 1982; Krebs and Barker, 1994; Oliveira *et al.*, 2008).

A neighbour-joining tree was based on the concatenated sequences (Fig. 2). Maximum likelihood (assuming a molecular clock) and the Bayesian analysis (Fig. 3) produced identical topologies, except for some intraspecific differences within *D. aldrichi* and *D. longicornis*. When a clock is assumed, the outgroup species, *D. hydei*, falls naturally into a basal position. Three of the methods used here (corrected Kimura-2-parameter neighbour-joining distances, neighbour-joining using generalized Jukes-Cantor corrections of synonymous codon differences, and maximum likelihood) gave identical unrooted topologies for both the *cox2* and concatenated sequences. Maximum parsimony, when rooted by *D. hydei*, placed *D. hamatofila* outside of the combined *D. mulleri*/*D. longicornis* clusters consistent with its uncertain phylogenetic affinity within the *D. repleta* group (Wasserman, 1992). Trees based on the *nad3* sequences reversed the placement of *D. spenceri* and *D. hamatofila* in all analyses. It should be noted that the *cox2* sequences are almost twice the length of *nad3*, giving an almost two-fold weighting of *cox2* in the concatenated sequences.

Monophyly of the *D. mulleri* cluster (see Table 1) was supported in 98% of bootstrap samples using neighbour-joining (Fig. 2). The *D. longicornis* cluster was less well resolved based on these two mtDNA gene regions. *Drosophila spenceri* has been considered a member of the cluster, based on cytology and morphology (Wasserman, 1982), but in a later review, Wasserman (1992) excluded *D. spenceri* from this cluster. *Drosophila hamatofila* has been placed outside of the *D. mulleri* complex (see Table 1, 'other') (Wasserman, 1992). Our results indicated that *D. hamatofila* separated from the lineage leading to the *D. longicornis* cluster after the separation of the two clusters. Thus the mtDNA results are consistent with inclusion of *D. hamatofila* within the *D. longicornis* complex (Fig. 2), consistent with the topology of the *D. longicornis* species complex proposed by Oliveira *et al.* (2005).

Table 2. Pairwise comparisons of the *cox2* and *nad3* sequences estimated in this study

	D. hydei	*D. hamatofila*	*D. spenceri*	*D. longicornis*[a]	*D. mainlandi*[a]	*D. aldrichi*[a]	*D. wheeleri*[a]	*D. mulleri*
D. hydei	—	12.78	13.93	12.58	12.86	11.07	11.05	12.10
		(6.58)	(7.24)	(6.80)	(6.69)	(5.84)	(5.82)	(5.93)
D. hamatofila	12.8	—	10.09	9.19	9.83	8.89	8.96	9.87
	12.7		(4.01)	(3.85)	(3.91)	(4.13)	(4.11)	(4.43)
D. spenceri	14.0	10.4	—	8.86	9.25	9.69	9.76	10.42
	13.7	9.4		(3.59)	(3.49)	(4.34)	(4.32)	(4.43)
D. longicornis[a]	12.1	10.2	8.8	—	1.24	9.66	9.50	9.73
	13.6	7.5	9.2		(0.10)	(4.18)	(4.16)	(4.43)
D. mainlandi[a]	12.5	10.8	9.1	1.1	—	10.15	10.05	9.91
	13.6	8.1	9.6	1.6		(4.24)	(4.22)	(4.32)
D. aldrichi[a]	11.0	9.4	9.9	9.1	9.5	—	1.08	5.70
	11.1	8.0	9.2	11.1	11.4		(0.17)	(1.53)
D. wheeleri[a]	10.9	9.4	9.8	8.7	9.3	0.9	—	5.70
	11.2	8.0	9.7	11.3	11.6	1.4		(1.67)
D. mulleri	12.0	10.3	10.3	9.1	9.3	6.3	5.9	—
	12.4	9.1	10.7	11.3	11.3	4.6	5.3	

Note: The upper triangle gives percent divergence corrected by the generalized Jukes-Cantor correction for all changes and for transversions (in parentheses) for concatenated sequences (1038 bases). Corrected percent divergences for the two genes separately are given below the diagonal (*cox2*, upper).

[a] Means for the 3–5 individuals sequenced for these species.

Table 3. Intra- and interspecific sequence divergences for concatenated *nad3* and *cox2* genes in *D. longicornis* and *D. mainlandi*

	D. longicornis				*D. mainlandi*		
	A909	A892	Tuc-1	Tuc-2	A978	A956	Oxnard
D. longicornis							
Sonora – A909	—	0.87	0.68	0.77	1.36	0.97	1.16
Sonora – A892	9	—	0.19	0.48	1.56	1.16	1.36
Arizona – Tuc-1	7	2	—	0.29	1.36	0.97	1.16
Arizona – Tuc-2	8	5	3	—	1.46	1.07	1.26
D. mainlandi							
Baja California – A978	14	16	14	15	—	0.39	0.39
California – A956	10	12	10	11	4	—	0.39
Oxnard, CA	12	14	12	13	4	4	—

Note: Jukes-Cantor corrected distances are given in the upper triangle; lower triangle gives the total nucleotide differences. Geographical locations and stock numbers are described in the text, and Tuc-1 and Tuc-2 are wild individuals from Tucson, AZ.

Table 4. Intra- and interspecific sequence divergences for concatenated *nad3* and *cox2* genes in *D. aldrichi* and *D. wheeleri*

	D. aldrichi					*D. wheeleri*		
	A917	A976	A987	A990	W8	A754	A951	A956
D. aldrichi								
Baja California – A917	—	0.97	1.07	0.48	1.07	1.07	1.26	1.17
Baja California – A976	10	—	1.07	0.68	0.39	0.97	1.17	1.07
Texas – A987	11	11	—	0.97	0.87	0.97	1.07	1.17
Sonora – A990	5	7	10	—	1.07	1.07	1.17	1.17
Oaxaca – W8	11	4	9	11	—	0.87	1.07	0.97
D. wheeleri								
Baja California – A754	11	10	10	11	9	—	0.19	0.10
California – A951	13	12	12	13	11	2	—	0.10
California – A956	12	11	11	12	10	1	1	—

Note: Jukes-Cantor corrected distances are given in the upper triangle; lower triangle gives the total nucleotide differences. Geographical locations and stock numbers are described in the text.

Host plant data and species distributions

A total of 19,890 individuals from nine *Drosophila* species were reared from *Opuntia* pads collected over 24 years from Tucson, Arizona to El Dorado, Sinaloa, Mexico (Fig. 1; Table 5A). Of these individuals, 51% consisted of *D. longicornis* and 26% were *D. aldrichi*. The majority of *D. longicornis* records came from the introduced *O. ficus-indica* collected within the city of Tucson. However, in the desert surrounding Tucson, *D. hamatofila* was the

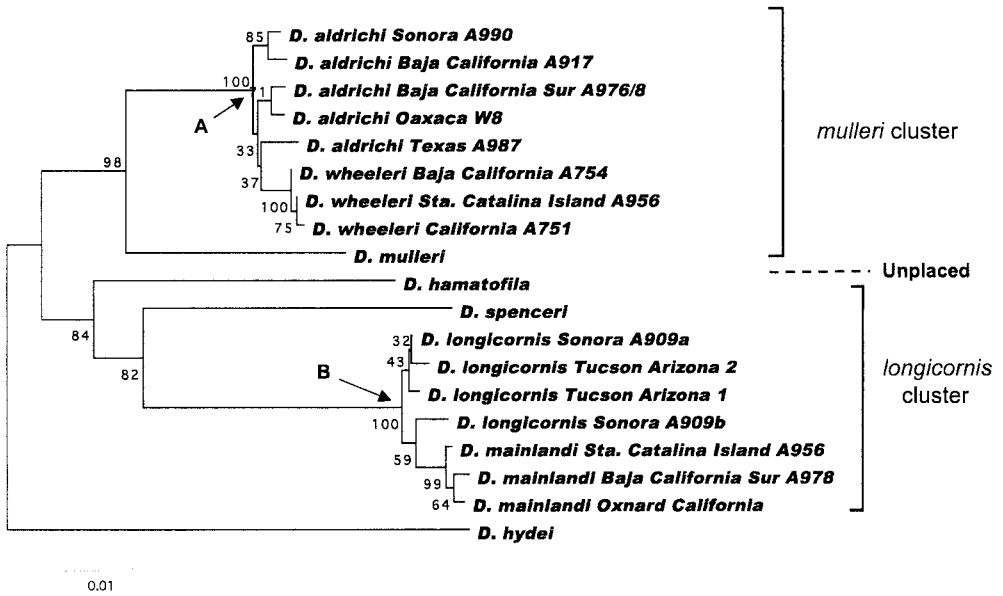

Fig. 2. Molecular phylogeny of the species analysed in this study. The tree is a neighbour-joining tree using concatenated complete *cox2* and *nad3* sequences, with Jukes-Cantor distance corrections. Numbers associated with the nodes are bootstrap results. Nodes associated with the two vicariant species pairs are indicated (A and B).

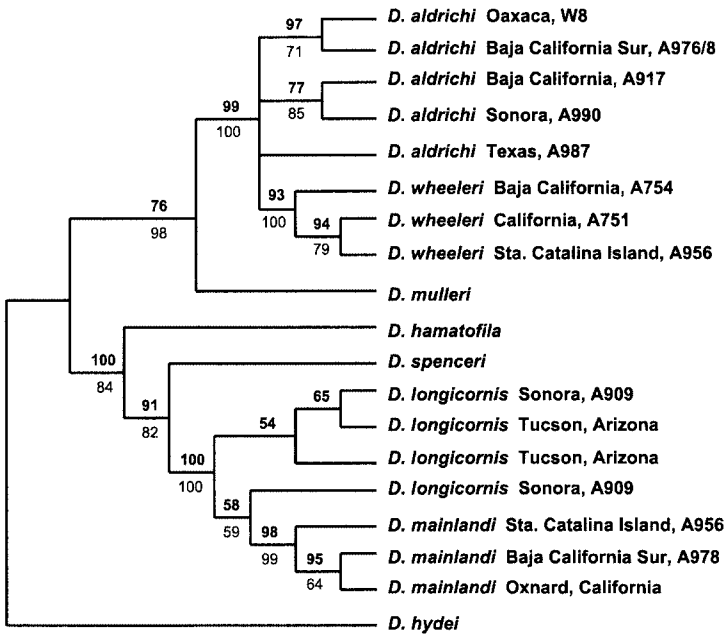

Fig. 3. Bayesian reconstruction of the phylogenies. Numbers above the internal branches are credibility scores; those below are bootstrap results from the neighbour-joining tree, for comparison.

Table 5. Rearing records from *Opuntia* pads from two north–south transects: (A) from Tucson, Arizona to El Dorado, Sinaloa (1962–1986), and (B) to the west of the Sonora/Mojave Deserts from Camarillo, California to San Telmo, Baja California (1979–1991)

A. Locality (collections)	Lat. °N	*D. longicornis*	*D. aldrichi*	Other[a]	Cactus sp.
1. Tucson, AZ (7)	32°15′	9,557	—	435	*O. ficus-indica*
2. Vicinity of Tucson, AZ (8)	32°15′	138	—	1931	*O. phaeacantha*
3. Magdalena, Sonora (1)	30°38′	44	—	21	N.D.[c]
4. Guaymas, Sonora (1)	27°56′	152	52	—	*O. wilcoxii*
5. Playa Cochorit, Sonora (1)	27°52′	84	—	26	*O. violacea*
6. Navojoa, Sonora (1)	27°04′	—	1137	72	*O. ficus-indica*
7. Navojoa, Sonora (5)	27°04′	60	1299	347	*O. wilcoxii*
8. Alamos, Sonora (3)	27°01′	142	1401	163	'Velutina complex'
9. Las Bocas, Sonora (2)	26°24′	—	858	629	*O. wilcoxii*
10. Los Mochis, Sinaloa (1)	25°47′	2	111	1	*O. wilcoxii*
11. Los Hornos, Sinaloa (1)	25°18′	—	59	116	N.D.[c]
12. El Dorado, Sinaloa (1)	24°20′	—	175	890	*O. wilcoxii*
B. Locality (collections)	**Lat.° N**	***D. mainlandi***	***D. wheeleri***	**Other[b]**	**Cactus sp.**
1. Camarillo, CA (1)	34°13′	30	176	23	N.D.[c]
2. Palos Verde Peninsula, CA (1)	33°46′	163	49	—	*O. oricola*
3. S. Catalina Island, CA (2)	33°26′	59	76	69	*O. demissa*
4. Ensenada, Baja (1)	31°53′	—	72	23	*O. ficus-indica*
5. Ejido Uruapan, Baja (1)	31°37′	91	76	1	*O. ficus-indica*
6. San Telmo, Baja (2)	30°58′	62	1,975	738	*O. oricola*

Note: Other species ([a, b]) collected by locality are listed as percentages of total flies collected.

[a] *D. hamatofila*: (1) 0.31, (2) 0.99, (3) 1.0; *D. arizonae*: (1) 0.43, (2) 0.01, (5) 0.35, (6) 0.10, (7) 0.05, (8) 0.01, (9) 0.03; *D. pseudoobscura*: (1) 0.26; *D. mojavensis*: (5) 0.65; *D. meridiana*: (7) 0.02; *D. navojoa*: (6) 0.90, (7) 0.77, (8) 0.04, (9) 0.79, (10) 1.0, (12) 0.99; *D. 'from Sonora'*: (7) 0.15, (8) 0.95, (9) 0.17, (11) 1.0, (12) 0.01.

[b] *D. simulans*: (1) 0.78, (3) 0.02; *Gitona* sp.: (1) 0.17; *D. hydei*: (1) 0.04; *D. hamatofila*: (3) 0.57, (4) 0.17, (5) 1.0, (6) 0.96; *D. mojavensis*: (3) 0.41, (4) 0.83, (6) 0.03; *D. nigrohydei*: (6) 0.01.

[c] Species not determined.

abundant species using *O. phaeacantha*, a widespread native prickly pear in the southwest (Table 5A). The number of *D. longicornis* reared from the pears was never very high south of Tucson and its southern range limit extends to the now tropical Navojoa/Alamos region of southern Sonora, Mexico, where *D. aldrichi* (and *D. navojoa*, a member of the *D. mojavensis* cluster of species) was very abundant. Therefore, as of 1986, 27°N latitude was approximately the northern limit of the latter two species and the southern limit for *D. longicornis* in this particular region along the coast.

A total of eight species and 3683 individuals were reared from *Opuntia* pads collected in several trips over a period of 12 years from Camarillo, California to San Telmo, Baja California (Fig. 1; Table 5B). *Drosophila wheeleri* represented 66% of the total, while *D. mainlandi* represented 11%; thus 77% of the drosophilids emerging from the pears of this region were one of these two species. Santa Catalina Island records from November 1991 showed that 1233 *D. mainlandi* and 167 *D. wheeleri* were collected over various fruits used as bait.

In summary, the data in Table 5A illustrate that *D. longicornis* is at the western and southern edge of its distribution (in this particular region) coming from the Chihuahuan Desert province of the Mexican Plateau and that it narrowly overlaps with *D. aldrichi*, whose range protrudes northward along the tropical Mexican west coast. By contrast, Table 5B shows that their two cismontane sister species, *D. mainlandi* and *D. wheeleri*, co-exist in the same prickly pears wherever they are found in the mild Mediterranean climate of southwestern California and northwestern Baja California. That the prickly pears, and their hybrids, are different species (except for the Mission Cactus, *O. ficus-indica*) in this coastal area compared with the Arizona, Sonora, and Sinaloa pears, and their hybrids, is of great interest also. The extent to which a change in host plants contributes to differentiation can be extensive (Etges, 1993; Etges *et al.*, 2007). The influence of this factor in the present case has not been studied.

Early collections by one of us (W.B.H.) in the Cape region of Baja California (San Lucas Deciduous Scrub) produced a few individuals of *D. mainlandi* and a few with a questionable identification of *D. wheeleri*, but no cultures were established for either species. Later collections (1986–1996) by a number of investigators provided culturable material for two species, *D. mainlandi* and *D. aldrichi*, but the presence of *D. wheeleri* was not confirmed. It is now apparent that *D. aldrichi* is either a resident species in the Cape or a recent introduction.

Other localities north of the Cape also contained these species, albeit in very low numbers (Babcock *et al.*, 1997). The most northern locality for *D. mainlandi* was San Borja at 28°48′N latitude (P.M. O'Grady, collector) and for *D. aldrichi*, Ejido Vizcaino at 27°30′N latitude (W.J.E., collector). The significance of these findings can be realized only by more detailed fieldwork in southern Baja California, especially in light of the discovery of *D. aldrichi* in Tucson, Arizona in 1992 in association with *Opuntia ficus-indica* (W.B. Heed, unpublished data), and *D. wheeleri* in Punta Onah, Sonora in 2007 (W.J. Etges, unpublished data).

Hybridization tests

We describe the following crosses in some detail because this is the first test of interspecific hybridization with this species. Although *D. mainlandi* can be difficult to culture in the laboratory, a total F_1 of 16 females and 20 males was produced when *D. longicornis* was the female parent and *D. mainlandi* was the male parent. When the $F_1 \times F_1$ cross failed to produce progeny, eight of the hybrid females were backcrossed to 24 *D. mainlandi* males to test for fertility. The backcrosses produced 18 flies of both sexes before the test was terminated. Similarly, the remaining eight hybrid females were backcrossed to 21 *D. longicornis* males, and produced seven flies of both sexes before the test was terminated. No immediate matings were observed in these crosses.

In the reciprocal cross of *D. mainlandi* females and *D. longicornis* males, a total of 11 F_1 females and eight males was produced. When no progeny ensued from inbreeding the F_1, all the females were backcrossed to 27 *D. mainlandi* males. In contrast to the above crosses, all hybrid females were mounted with very little courtship by *D. mainlandi* males within 10 min of the start of a trial. Many larvae were present when the test was terminated.

In summary, these tests show that *D. mainlandi* × *D. longicornis* crosses produce at least partially fertile females but sterile males in both directions. Among species within the *D. longicornis* complex, this represents one of the very few cases of observed interspecific hybridization (Etges and Heed, 2006). *Drospohila mainlandi* was described as a new species on

morphological grounds by Patterson (1943) and the standard polytene chromosome sequence of this species is identical to that of *D. longicornis*. In addition, *D. mainlandi* is unique for one heterozygous inversion, 2 l^6 (Wasserman, 1992). We also noted unusual courtship behaviour in the crosses: in pure culture, *D. mainlandi* males tend to 'ride' the females for extended periods analogous to the behaviour of *D. pegasa* as described by Wasserman and Zweig (1991). However, this behaviour was not evident in any of the above crosses, especially with *D. mainlandi*/*D. longicornis* hybrid females × *D. mainlandi* males.

The hybridization tests with *D. aldrichi* (origin not indicated) × *D. wheeleri* (from the Arboretum, Arcadia, CA) by Patterson and Alexander (1952) were more extensive than the crosses described above. *Drosophila aldrichi* and *D. wheeleri* have no inversion differences in their polytene chromosomes – they are homosequential (Wasserman, 1992), indicating a recent time of divergence. The F_1 male hybrids were completely sterile but the hybrid females were as fertile as the controls in backcrosses to both parental males. The inbred F_2 production was very low (3–9% of 100 pair matings each cross). The F_2 backcrosses were very productive only when the males were the parental type. From these data and other information, *D. wheeleri* was described as a new species (Patterson and Alexander, 1952). Richardson (1982) reported hybrid male sterility in crosses between *D. aldrichi* strains from Texas and Sonora, and that *D. wheeleri* showed similar patterns of reproductive isolation with both of these strains of *D. aldrichi*. Thus, both eastern and western forms of *D. aldrichi* (Oliveira *et al.*, 2008) are reproductively isolated from *D. wheeleri*.

DISCUSSION

Past and present distributions of the vicariant pairs

All four species – *D. aldrichi*, *D. wheeleri*, *D. longicornis*, and *D. mainlandi* – tend to be excluded from the more xeric regions of the Sonoran and Mojave Deserts, as are their host plants, certain species of *Opuntia*. Extensive, long-term collection records presented here show that the parental species, *D. aldrichi* and *D. longicornis*, overlap narrowly at the western edge of their distributions, while collections west of the deserts show extensive overlap of the two derived species, *D. wheeleri* and *D. mainlandi*. We infer that the ancestral species that gave rise to the two vicariant species pairs once ranged more or less continuously throughout the region when conditions were more mesic. If this interpretation is correct, then the isolation of the western populations of both ancestral species, leading to the differentiation and partial reproductive isolation of *D. wheeleri* and *D. mainlandi* from the parental species, likely occurred at the same time.

In this study, we have attempted to place an upper limit on the time frame required for speciation in these lineages using mitochondrial sequences to establish a molecular clock. We first examine the relationships of these species to other members of the subgroup to use published estimates of times of divergence for members of the subgroup.

Relationships within the subgroup

As noted in the Introduction, the *D. repleta* group represents a relatively recent and very extensive radiation, including approximately 100 species, and is concentrated in the drier regions of North and South America. Evidence that the radiation is recent can be drawn from cytological analyses of the polytene chromosomes (Wasserman, 1982, 1992). All members of

the group that have been analysed can be related to an ancestral arrangement using only two-break paracentric inversions or centric fusions (Robertsonian translocations). Although other types of rearrangements, such as pericentric inversions and translocations, occur only rarely over evolutionary time, empirical observations indicate that more distantly related species usually show an accumulation of these differences (Patterson and Stone, 1952; Powell, 1997).

The mitochondrial sequence variation reported here supports a recent divergence of these species. Although there are no recent geological events to calibrate these DNA sequence divergences, comparisons can be made with analyses of other species groups. The *cox2* gene has been analysed in the *D. obscura* species group, in subgenus *Sophophora* (Beckenbach *et al.*, 1993). The divergences within the *D. mulleri* subgroup, which includes about 48 species (Oliveira *et al.*, 2005; Wasserman, 1992), range up to about 10%. Such a divergence is comparable with that observed within the *D. obscura* species group, and is thought to represent a time span of 20–30 million years (Beckenbach *et al.*, 1993).

The vicariant species pairs

Intraspecific variation in these genes ranged up to 1% in *D. aldrichi* in comparisons between individuals from widely separated populations, from Baja California, western Mexico, and Big Bend National Park, Texas (Table 4). Some of the intraspecific comparisons of *D. longicornis* were as high as 0.9%. Our samples of this species included two flies from a single mixed culture from Sonora, Mexico, and two from Tucson, Arizona. Thus the variation we observed may not be representative of the species as a whole. Intraspecific variation in the two derived species, *D. wheeleri* and *D. mainlandi*, was lower, ranging from 0.1 to 0.4% (Table 3). The reduced variation in the derived species may be the result of a very recent bottleneck, or simply the much more restricted distributions of these two species.

The interspecific pairwise divergence between members of *D. longicornis* and *D. mainlandi*, and between *D. aldrichi* and *D. wheeleri*, was also about 1% (Tables 3, 4). This level of divergence was very similar to the intraspecific differences found in *D. aldrichi*. Thus it does not appear that sufficient time has passed since the isolation of the derived species for species level differences to arise. We conclude that the portion of the tree in Fig. 2 associated with nodes A and B represent gene trees rather than species trees and that the different sequences derived from these nodes may be ancient polymorphisms (Wu, 1991; Nichols, 2001). Neighbour-joining bootstraps indicate monophyly of the derived species, but in many of the analyses both *D. longicornis* and *D. aldrichi* appear paraphyletic, including the *D. mainlandi* and *D. wheeleri* sequences respectively. Such paraphyly in *D. aldrichi* was also observed in a larger number of populations based on two mtDNA and two nuclear gene regions, consistent with levels of reproductive isolation between western versus eastern lineages of *D. aldrichi* – that is, consistent with the presence of two incipient species (Oliveira *et al.*, 2008).

Timing of the branches of the *D. mulleri* subgroup

We created a linearized tree of the eight species included in this study based on transversion differences (Fig. 4). One sequence from each species was chosen arbitrarily, and the tree constructed using maximum likelihood with a molecular clock. Transition substitutions

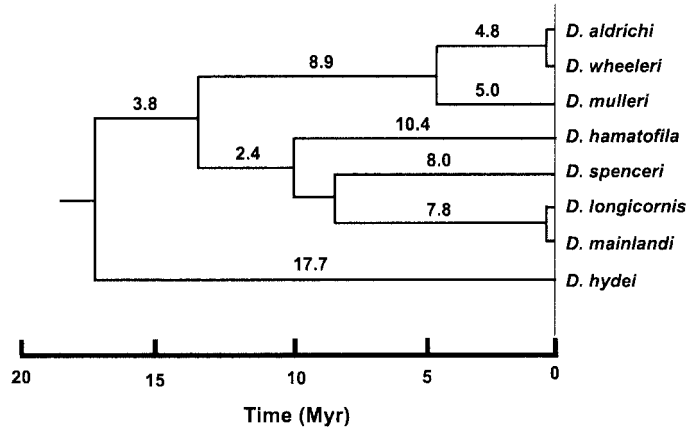

Fig. 4. Linearized tree using maximum likelihood of transversions, assuming a molecular clock. The time scale is based on an estimate of the divergence of *D. mulleri*/*D. wheeleri* of 5 million years (Russo *et al.*, 1995).

were eliminated from this analysis because they are unlikely to be linear over this time span. They are susceptible to saturation and are masked whenever a transversion occurs (Brown *et al.*, 1982). The difference in branch lengths, especially the more basal internodes, between Figs. 2 and 4 illustrates this effect. Rate constancy was tested using the 2-cluster test and branch length test (Takezaki *et al.*, 1995). The result was not significant ($Q = 7.23$, d.f. = 6).

A time scale is included in Fig. 3 based on an estimate of a 5 million year divergence between *D. mulleri* and *D. wheeleri* (Russo *et al.*, 1995). Their calibration was based on endemic drosophilids of the Hawaiian Archipelago, applied to an alcohol dehydrogenase sequence tree. Time estimates based on branch and internode lengths determined by DNAMLK are included in Fig. 3. Based on this tree, the separation of *D. hydei* from the *D. mulleri* subgroup is about 17.7 million years, compared with the 16 million years estimated by Russo *et al.* (1995). If we apply this calibration to the *D. longicornis*/*D. mainlandi* and *D. aldrichi*/*D. wheeleri* species pairs, we arrive at an estimate of about 0.2 million years. As noted above, these estimates should be regarded as an estimate of the time separating the sequences, not the species. If sequence differences reflect ancient polymorphisms, as seems likely, then we can infer that *D. mainlandi* and *D. wheeleri* originated more recently than 0.2 million years ago.

The biogeographic similarities (Fig. 1), the similarity of results of the mtDNA analyses, and recent divergence times of these two species pairs suggests that each of the derived species arose as a result of the same isolating mechanism – the northward expansion of the Sonoran Desert. Geological evidence indicates that the xeric conditions necessary to exclude prickly pear cactus from the Sonoran Desert did not develop until the end of the last glacial maximum, perhaps 13,000 years ago (Axelrod, 1979). Our results indicate that the interspecific divergence in both species pairs is no greater than some intraspecific pairwise comparisons – that is, the presence of two incipient species of '*D. aldrichi*' may complicate our understanding of the divergence of the four species considered here, but we cannot reject the hypothesis that *D. mainlandi* and *D. wheeleri* have only been isolated during the recent post-glacial episode.

Are they good species?

The two derived species are morphologically distinguishable from their eastern relatives and in all cases viable hybrids were produced in crosses with each closest relative. Both species pairs follow Haldane's rule, commonly observed in the early stages of speciation (Coyne and Orr, 2004) where hybrid females are fertile and the males are sterile. We conclude that *D. longicornis*/*D. mainlandi* and *D. wheeleri* do indeed represent good species, with *D. aldrichi* composed of at least two species each reproductively isolated from *D. wheeleri* (Richardson, 1982).

The low level of divergence from their parental species, evident in these mitochondrial DNA sequences, is not unprecedented for species with this degree of reproductive isolation: the sibling species *D. pseudoobscura* and *D. persimilis* have been shown to share *cox2* sequences (Beckenbach *et al.*, 1993). Powell (1983) ascribed the apparent sharing of mitochondrial sequences between these species to the fertility of the female hybrids, and ongoing introgression of these sequences. The latter two species are sympatric through almost the entire range of *D. persimilis*, and hybrids are sometimes encountered in nature. Although the ranges of *D. aldrichi* and *D. mainlandi* now overlap at the southern tip of Baja California (Fig. 1), *D. longicornis* and *D. wheeleri* have completely separate distributions. Thus for at least the latter pair, ongoing introgression appears unlikely.

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