Zootaxa 1725: 37–47 (2008)
www.mapress.com/zootaxa/

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

Species delimitation in the *Drosophila aldrichi* subcluster (Diptera: Drosophilidae) using DNA sequences

DEODORO C. S. G. OLIVEIRA[1], MELISSA LEONIDAS[2], WILLIAM J. ETGES[3], PATRICK M. O'GRADY[4] & ROB DeSALLE[2]

[1]*Department of Biology, University of Rochester, Rochester, NY, USA. E-mail: dolivei2@mail.rochester.edu*
[2]*Division of Invertebrate Zoology, American Museum of Natural History, New York, NY, USA. E-mail: desalle@amnh.org*
[3]*Department of Biological Sciences, University of Arkansas, Fayetteville, AR, USA; email: wetges@uark.edu*
[4]*Division of Organisms and the Environment, University of California, Berkeley, CA, USA. E-mail: ogrady@nature.berkeley.edu*

Abstract

DNA sequence data has been successfully used to verify current species-level taxonomic hypotheses based on morphology and other characters. Setting species boundaries in the *Drosophila repleta* group has been challenging because this group contains several cryptic taxa and morphologically polymorphic populations. Mitochondrial (*cox1* and *nad2*) and nuclear (*sina* and *Marf)* genes were employed to assess species limits for two traditionally recognized, closely related, and taxonomically problematical species, *D. aldrichi* and *D. wheeleri.* Both tree-based and character-based methods were used to show that *D. wheeleri* is indeed a distinct species; however, our data shows that *D. aldrichi* is a paraphyletic assemblage of two lineages as previously suggested based on patterns of reproductive isolation. One lineage is sister to *D. wheeleri* and includes populations originating from southern and western Mexico (western-*aldrichi*). The second, basal group also contains flies from southern Mexico, along with populations from the northern and eastern regions within the species boundaries traditionally described as *D. aldrichi* (eastern-*aldrichi*). The populations of *D. aldrichi* that were introduced into Australia were found to be included in the eastern-*aldrichi* group. Our results, particularly those based on the rapidly evolving mtDNA sequences, confirm the presence of at least two cryptic species previously referred as "*D. aldrichi*".

Key words: *Drosophila repleta* group, cactophilic *Drosophila*, cryptic species, molecular phylogenetics

Introduction

Many species in the *Drosophila repleta* species group are not readily identifiable using external morphological characters. As a result, male genitalia, reproductive isolation, and polytene chromosome inversions have been used to determine species limits and to discover cryptic species. However, this approach is unwieldy and in some instances species resolution is still problematical. This is the case for *D. aldrichi* Patterson & Crow and *D. wheeleri* Patterson & Alexander. These species are homosequential for polytene chromosome banding (Wasserman 1954), and there are no obvious morphological differences in male genitalia suggesting that their status as valid species is questionable (Vilela 1983). Nevertheless, patterns of reproductive isolation, often in the form of F_1 male sterility, have been indicative of multiple species as seen in reciprocal crosses between *D. aldrichi* and *D. wheeleri* (Patterson & Alexander 1952). Furthermore, Wasserman (1992) suggested that *D. aldrichi* likely consists of a number of cryptic species based on incompatible crosses among several different populations. Krebs and Barker (1994) also proposed that an introduced population of *D. aldrichi* in Australia and a population of *D. aldrichi* from northwestern Mexico were different species based on observed hybrid sterility.

Accepted by D. Bickel: 31 Jan. 2008; published: 12 Mar. 2008

Drosophila aldrichi and *D. wheeleri* belong to the *repleta* species group of New World cactophilic *Drosophila*. These two closely related allopatric species are associated with various species of *Opuntia*, their cactus hosts (Patterson 1943; Ruiz & Heed 1988). *Drosophila wheeleri* is restricted to southern California, USA, and northern Baja California, Mexico. While *D. aldrichi* has a broader distribution and is found in southern Texas, USA, the lowlands of both costs of mainland Mexico (Patterson & Wagner 1943; Patterson & Mainland 1944; Wasserman 1992), it has more recently been collected in two isolated locations in Baja California Sur (Etges and Heed, unpublished data). *Drosophila aldrichi* was also introduced in Australia along with its cactus hosts (Mulley & Barker 1977). Phylogenetically, these taxa belong to the *mulleri* subgroup, the most species-rich of the five subgroups in the *repleta* group (the other subgroups are *fasciola*, *repleta*, *hydei*, and *mercatorum*; Vilela 1983; Wasserman 1992). Their closest relatives are *D. mulleri* Sturtevant and *D. nigrodumosa* Wasserman & Fontdevila; these four species were included in the *mulleri* cluster, together with *D. mayaguana* Vilela, *D. parisiena* Heed & Grimaldi, and *D. straubae* Heed & Grimaldi (Wasserman 1982; Heed & Grimaldi 1991). Heed *et al.* (1990) proposed that *D. aldrichi* and *D. wheeleri* were distinct enough to merit another grouping, the *aldrichi* subcluster, based on allozyme differences.

We used nuclear and mtDNA sequences to examine species boundaries of *D. aldrichi* and *D. wheeleri*. Tree and character-based methods for testing species limits were used (e.g. Sites & Marshall 2003). The aims of this study were to (1) test the hypothesis that *D. wheeleri* is a distinct species from *D. aldrichi* at the DNA sequence level, (2) examine whether *D. aldrichi* is composed of multiple distinct lineages, and (3) examine the genetic affinities of the native New World *D. aldrichi* populations with those introduced into Australia.

Material and methods

Taxon Sampling and DNA Sequencing

Multiple samples of *D. aldrichi* (18) and *D. wheeleri* (5) were examined in the present molecular work (Table 1). In some cases two samples originated from the same collection were included, and in all cases polymorphism was found in at least one gene (Table 1). Ten outgroup taxa were included to reflect a range of divergence (Wasserman 1992; Durando *et al.* 2000). The most closely related species are the ones in the *mulleri* cluster, i.e. *D. mulleri*, *D. nigrodumosa*, and *D. mayaguana*. The next group contains, *D. mainlandi* Patterson, *D. buzzatii* Patterson & Wheeler, *D. navojoa* Ruiz, Heed & Wasserman, *D. mojavensis* Patterson, and *D. arizonae* Ruiz, Heed & Wasserman. The latter species are also members of the *mulleri* subgroup, but recognized as members of different species clusters (Wasserman 1992). A more distant related species, *D. repleta* Wollaston, is in the *repleta* subgroup. While these nine outgroup species belong to the *repleta* group, another cactophilic species, *D. acanthoptera* Wheeler, belonging to the *nannoptera* group, was included to root the phylogeny.

DNA was extracted from a single adult fly using the procedure described in the DNAeasy tissue kit (Qiagen). Direct PCR of total DNA extractions were performed with standard methods. Primers for the two mitochondrial fragments, *nad2* (NADH dehydrogenase subunit 2) and *cox1* (cytochrome oxidase subunit 1), were described in Oliveira *et al.* (2005); primers for the two nuclear genes, *sina* (seven in absentia) and *Marf* (mitochondrial assembly regulatory factor), were from Bonacum *et al.* (2001). Cycle sequencing was performed using BigDye terminators (Perkin-Elmer) and the amplified products were run on an Applied Biosystems 3700 DNA Analyzer. Sequences were edited in Sequencher 4.0 (Gene Codes Corp.). All sequences generated in this study were submitted to GenBank and accession numbers can be found in Table 1.

Genetic divergence and Phylogenetic Analysis

MacClade (Maddison & Maddison 2000) was used to visually inspect alignments, to identify diagnostic characters, and to translate the sequences. The two mtDNA genes and the nuclear gene *sina* did not require

alignment. Amplified sequences of *Marf* spanned an intron, and in order to make alignments unambiguous, the intronic region was excluded from the analysis. Alignments are available from the authors upon request. Combined gene analysis was performed in a Maximum Parsimony (MP) framework using PAUP* 4.0b10 (Swofford 2002). When all data were combined into a single analysis, a total of 1547 characters resulted, 189 of which were parsimony-informative (PI) as follows: *sina* 397 characters; 24 PI; *Marf* (exon) 258 characters, 22 PI; *cox1* 372 characters, 70 PI; *nad2* 520 characters, 71 PI. A mtDNA tree (*cox1* + *nad2*) and a nuclear tree (*sina* + *Marf*) were also obtained under a MP framework. Support at each node was assessed using bootstrap proportions (Felsenstein 1985, 1988) and jackknife (33% deletion; Farris *et al.* 1996) with 100 bootstrap or jackknife replicates. Decay indices (Bremer 1988) were calculated using TreeRot.v2b (Sorenson 1999). Uncorrected pairwise divergence was estimated with PAUP* 4.0b10 (Swofford 2002).

TABLE 1. Samples used in this study and GenBank accession numbers.

Taxon[a]	Voucher number[b] and other identification numbers	Collection locality	Genbank accession number[c, d]			
			sina	*Marf*	*cox1*	*nad2*
Ingroup						
D. aldrichi (E)	119125; W5; 15081-1251.2	Acatlan, Puebla, Mex.	EU341605 (1)	EU341653 (3)	EU341664 (2)	EU341689
D. aldrichi (E)	119128; 15081-1251.5	Dixalea, Queensland, Australia	EU341606 (1)	EU341649 (4)	EU341666	EU341691
D. aldrichi (E)	119129; 15081-1251.6	Dixalea, Queensland, Australia	NA	EU341644	EU341667	EU341692
D. aldrichi (E)	119123; E51.3 POOL; 15081-1251.0	Francisco Medrano, Tamaulipas, Mex.	EU341604 (1)	EU341646 (1)	EU341662	EU341687
D. aldrichi (E)	119126; W8; 15081-1251.3	Tehuantepac, Oaxaca, Mex.	EU341618	EU341640 (3)	EU341665 (2)	EU341690 (2)
D. aldrichi (E)	101848; W8	Tehuantepec, Oaxaca, Mex.	EU341617	EU341643	EU341660 (2)	EU341686 (2)
D. aldrichi (E)	119124; E5.1; 15081-1251.1	Weslaco, Texas, USA	EU341612	EU341648	EU341663	EU341688 (2)
D. aldrichi (E)	119133; A1018; 15081.1251.12	Zopilote Canyon, Guerrero, Mex.	EU341609 (1)	EU341641 (5)	EU341671 (2)	EU341694 (2)
D. aldrichi (E)	119141; A1018	Zopilote Canyon, Guerrero, Mex.	EU341614	EU341642 (5)	EU341675 (2)	EU341693 (2)
D. aldrichi (W)	105437; A1026	Huatulco, Oaxaca, Mex.	EU341602 (1)	EU341645 (3)	EU341661 (1)	EU341696 (1)
D. aldrichi (W)	119138; A1026	Huatulco, Oaxaca, Mex.	EU341610 (1)	EU341651 (2)	EU341672 (1)	EU341700 (1)
D. aldrichi (W)	101825; A809	Ixtlahuacan del Rio (Guadalajara), Jalisco, Mex.	EU341619	EU341639 (1)	EU341659	EU341695 (1)
D. aldrichi (W)	119139	Ixtlan del Rio, Nayarit, Mex.	EU341613	EU341654	EU341673	EU341701
D. aldrichi (W)	101824; A804	Las Barrancas (Guadalajara), Jalisco, Mex.	EU341603 (1)	NA	EU341658	NA
D. aldrichi (W)	119132; A990; 15081.1251.11	Las Bocas, Sonora, Mex.	EU341608 (1)	EU341647 (3)	EU341670	EU341699

to be continued.

Table 1. (continued)

Taxon[a]	Voucher number[b] and other identification numbers	Collection locality	Genbank accession number[c, d]			
			sina	*Marf*	*cox1*	*nad2*
D. aldrichi (W)	119131; A976/ A978; 15081.1251.10	Santiago, Baja California Sur, Mex.	NA	NA	EU341669	EU341697
D. aldrichi (W)	119130; 810.5; 15081-1251.9	Tuxtla Gutierrez, Chiapas, Mex.	EU341607(1)	EU341650 (4)	EU341668	EU341698
D. aldrichi (W)	119140; A1027	Zapotitlan de Salinas, Puebla, Mex.	EU341615	EU341652 (2)	EU341674 (1)	EU341702 (1)
D. wheeleri	102358; A751; 15081-1501.1	Arcadia, California, USA	NA	EU341657	EU341681 (3)	EU341704 (3)
D. wheeleri	102360; A756	Ensenada, Baja California Norte, Mex.	NA	EU341655 (5)	EU341682 (3)	EU341703 (3)
D. wheeleri	102361; A757	San Telmo, Baja California Norte, Mex.	NA	NA	EU341683	EU341706
D. wheeleri	102363; A826; 15081-1501.3	Santa Catalina Island, California, USA	NA	NA	EU341684	NA
D. wheeleri	102367; 562	El Cajon, California, USA	EU341616	EU341656	EU341685	EU341705
outgroup						
D. nigrodumosa	102319	Merida, Venezuela	EU341627	EU341633	EU341679	EU341710
D. mulleri	102305	Fond Parisien, Haiti	EU341625	EU341638	DQ202072	DQ202112
D. arizonae	106307	San Luis Potosi, Mexico	EU341620	EU341636	EU341676	EU341707
D. mojavensis	106302	Punta Onah, Sonora, Mexico	EU341624	EU341637	EU341677	EU341708
D. navojoa	105433	Chamela, Jalisco, Mexico	EU341626	EU341635	EU341678	EU341709
D. buzzatii	102049	Cochabamba, Bolivia	EU341621	EU341631	DQ202051	DQ202091
D. mainlandi	102275	Santa Catalina Island, California, USA	EU341622	EU341632	DQ202065	DQ202105
D. mayaguana	102279	Great Inagua Island, Bahamas	EU341623	EU341634	DQ202067	DQ202107
D. repleta	102340	Santa Catalina Island, California, USA	EU341628	EU341630	EU341680	EU341711
D. acanthoptera	101823	Tehuantepec, Oaxaca, Mexico	EU341611	EU341629	DQ202050	DQ202090

[a]The letter between the parentheses indicate if the sample falls in the eastern-*aldrichi* (E) lineage or western-*aldrichi* (W) lineage, see text for details.

[b]Ambrose Monell Cryo Collection barcode number (AMNH).

[c]Numbers between parentheses indicate samples that posses the same haplotype.

[d]Underlined sequences are from Oliveira *et al.* (2005).

Results

Tree-based analysis

Species limits for *D. aldrichi* and *D. wheeleri* were investigated in an MP analysis combining all four genes, two nuclear genes (*sina* and *Marf*) and two mitochondrial genes (*cox1 and nad2*), that yielded a surprising result: although the five samples of *D. wheeleri* were recovered as a monophyletic group, *D. aldrichi* was rendered paraphyletic and formed two distinct phyletic groups with nine terminals each (Fig. 1). Trees

obtained by partitioning nuclear and mitochondrial data indicated that the resolution in the combined analysis was given by the mtDNA sequences, i.e. the mtDNA tree was basically the same as the combined tree (Fig. 2). Nuclear genes were unable to resolve the branching pattern within the *aldrichi* subcluster. Both nuclear and mtDNA data produced robust support for the relationships among the ingroup flies (Fig. 1 and Fig. 2).

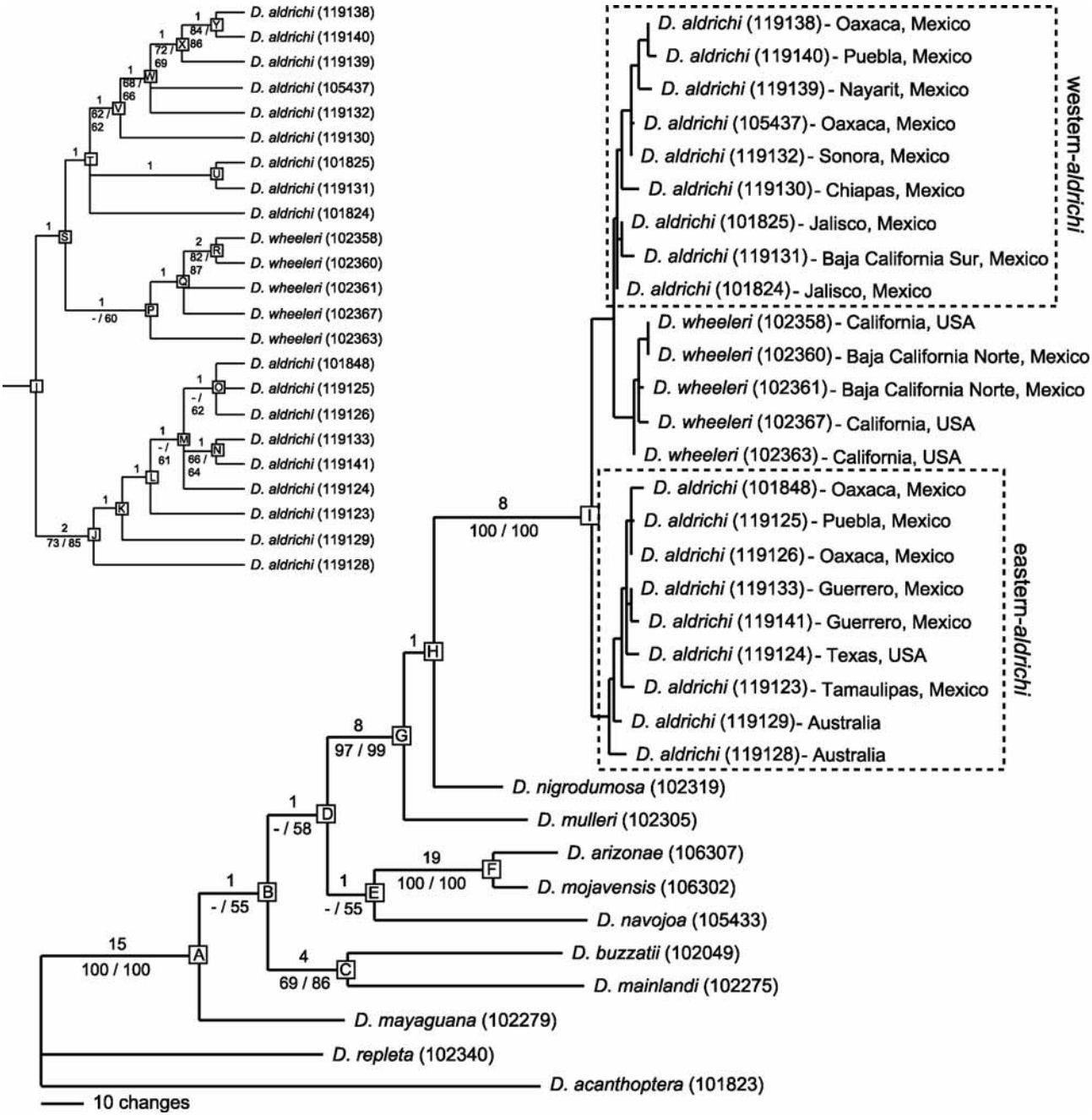

FIGURE 1. Strict consensus tree of 54 most parsimonious trees in the combined analysis of all four genes – length = 693; CI = 0.693; RI = 0.718; RC = 0.497. Decay index is shown above the nodes. Numbers below the nodes are Bootstrap / Jackknife support values higher than 50 %. Each node is identified by a letter to relate with Fig. 2. The smaller cladogram highlights branching and support values for the ingroup.

The basal clade in the *aldrichi* subcluster included samples collected in the southern and eastern parts of the distribution of *D. aldrichi* (Fig. 3). Samples from populations distributed in southern and northward regions along the Pacific coast were included in the group sister to *D. wheeleri*. Distributions of the two clades overlapped in southern Mexico, but for simplicity they are referred to here as eastern-*aldrichi* and western-

aldrichi respectively. The eastern-*aldrichi* lineage was well supported by the mtDNA sequences (Fig. 1 and Fig. 2). In contrast, support for monophyly of the western-*aldrichi* group, *D. wheeleri*, and the clade grouping them were quite weak. This is consistent with the basal position of the eastern-*aldrichi* clade, and we hypothesize that it diverged earlier and thereby accumulated a larger number of diagnostic mtDNA sites.

Phylogenetic analyses also placed the Australian populations of *D. aldrichi* in the eastern-*aldrichi* lineage. Interestingly, the two Australian samples branched off basally within this lineage. A possible explanation could be the founder effect, which potentially can increase genetic distinctiveness.

The topology for the ten outgroup species, in total and in the mtDNA trees (Fig. 1 and Fig. 2a), was consistent with previous phylogenetic inferences (Wasserman 1992; Durando *et al.* 2000). The placement of *D. mayaguana* as the most basal terminal in the *mulleri* subgroup, separated from the *mulleri* cluster, was the only unexpected result, because it has been grouped in the *mulleri* cluster (Heed *et al.* 1990; Wasserman 1992; O'Grady *et al.* 2002). For the most part, the two nuclear genes produced poor resolution of the branching pattern for the more divergent outgroup species (Fig. 2b).

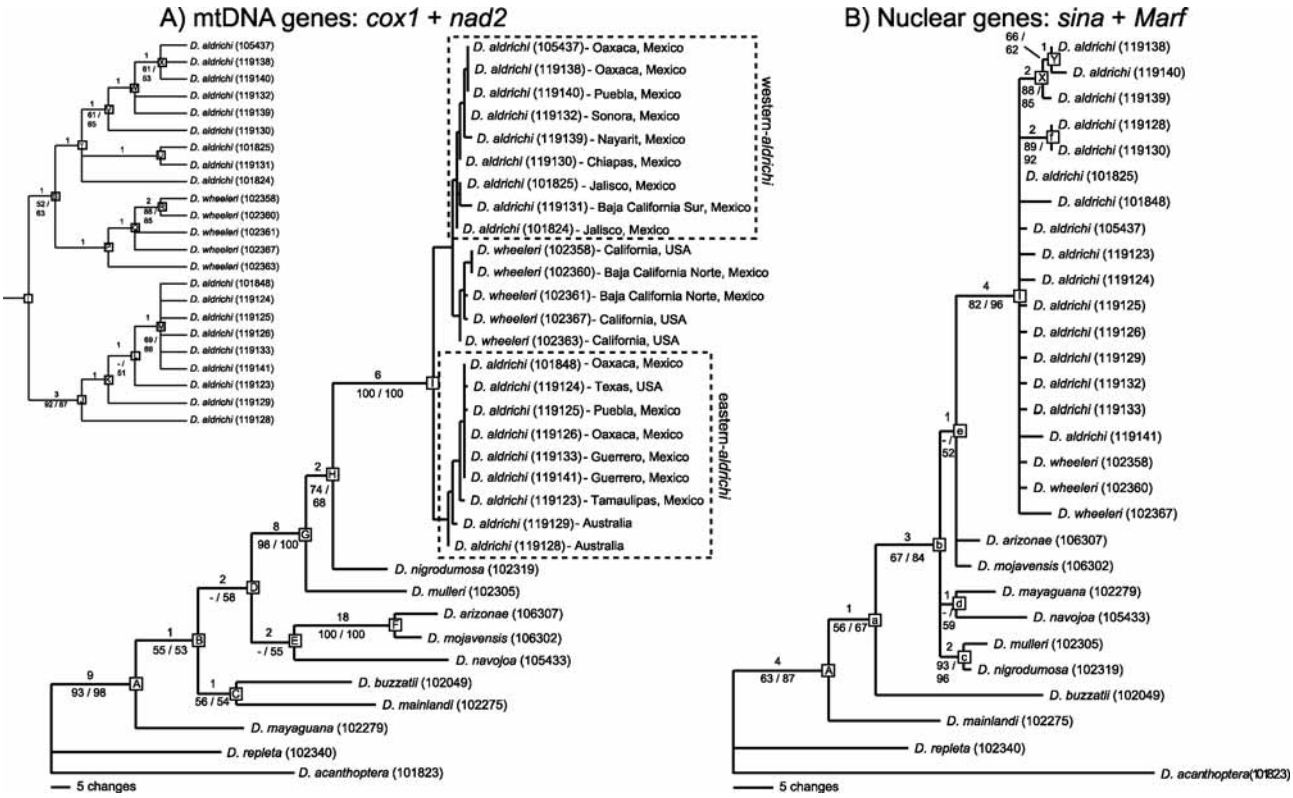

FIGURE 2. (A) Strict consensus tree of 700 most parsimonious trees in the combined mtDNA analysis – length = 479; CI = 0.683; RI = 0.753; RC = 0.514. Decay index is shown above the nodes. Bootstrap / Jackknife support values are presented below the node. Each node is identified by a letter to relate with Fig. 1. The smaller cladogram highlights branching and support values in for the ingroup. (B) Strict consensus tree of 13288 most parsimonious trees in the combined nuclear analysis – length = 194; CI = 0.789; RI = 0.606; RC = 0.563. Decay index is shown above the nodes. Bootstrap / Jackknife support values are presented below the node. Each node is identified by a letter to relate with Fig. 1.

Character-based analysis

A total of eight characters made it possible to diagnose eastern-*aldrichi* from both western-*aldrichi* and *D. wheeleri*, four in each mitochondrial gene (Table 2). Only one *cox1* character and one *nad2* character differentiated *D. wheeleri* from western-*aldrichi*, a result consistent with their respective positions and support in the phylogenetic analysis (Table 2). For *nad2*, there were two fixed amino acid differences that differentiated eastern-*aldrichi* from western-*aldrichi* and *D. wheeleri*, and one fixed amino acid replacement that distinguished *D. wheeleri* from the western-*aldrichi* clade (Table 2). In addition, there were a few segregating poly-

morphisms in the protein sequences of *nad2* among samples of the *aldrichi* subcluster. No amino acid variation was found for *cox1* in the *aldrichi* subcluster. Since relatively small regions of these genes were sequenced, these results show the diagnostic potential of mtDNA genes to resolve species limits for these flies.

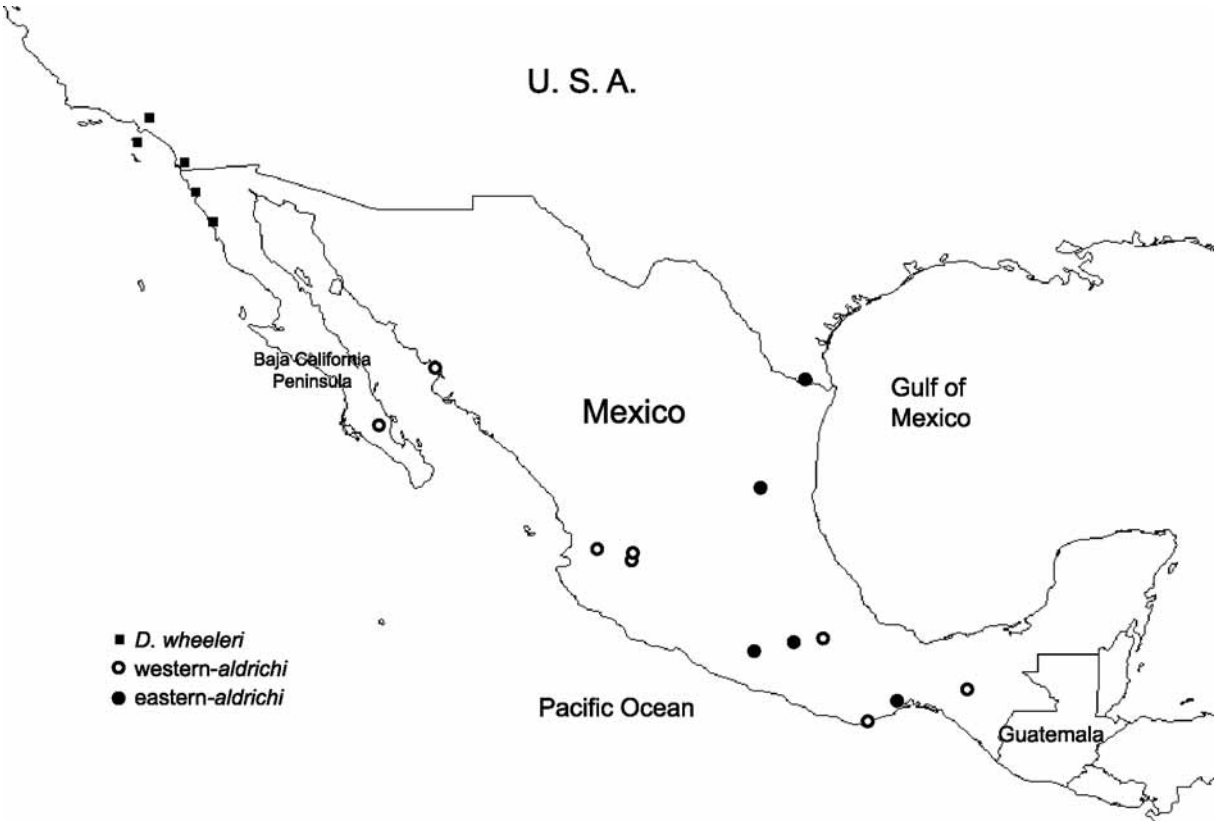

FIGURE 3. Distribution map of the samples used in the molecular analysis. Specimens collected in Australia are not shown.

TABLE 2. Diagnostic characters in the mtDNA sequences. Associated amino acid replacements are shown between parentheses. Numbers refer to the aligned nucleotide position.

	cox1					*nad2*					
	0	0	2	3	3	0	3	3	3	4	4
	0	1	5	2	7	9	1	2	2	4	6
	1	6	9	2	1	1	6	1	3	8	8
Eastern-*aldrichi*	A	C/T	C	C/T	A	T	G	T (I)	A (M)	A	C (T)
Western-*aldrichi*	G	C	T	A	C/T	C	A	C (T)	G (V)	G	C (T)
D. wheeleri	G	T	T	A	T	C	A	C (T)	G (V)	G	T (M)

Table 3 shows uncorrected p-distances for the mitochondrial haplotypes. The amount of divergence between species was quite low and overlapped to some extent with the within-species diversity. This was especially true for the western-*aldrichi* vs. *D. wheeleri* comparison. The eastern-*aldrichi* lineage was 2% divergent on average from the other two lineages and never less than 1% due to the 4 fixed characters in each gene that diagnosed eastern-*aldrichi* and accounted for about 1% of the size of the sequenced gene regions (Table 2). The single, fixed diagnostic character in each gene between *D. wheeleri* and western-*aldrichi* was often overwhelmed by segregating polymorphism in pairwise comparisons used to calculate the p-distances.

TABLE 3. Average uncorrected pairwise p-distance (%) for mtDNA genes. Range of divergence is shown between parentheses.

	Eastern-*aldrichi*	Western-*aldrichi*	*D. wheeleri*
cox1			
Eastern-*aldrichi*	0.4 (0 – 1.1)	–	–
Western-*aldrichi*	2.1 (1.2 – 3.1)	0.9 (0 – 2.4)	–
D. wheeleri	2.0 (1.3 – 2.7)	1.3 (0.5 – 1.7)	0.5 (0 – 0.5)
nad2			
Eastern-*aldrichi*	0.3 (0 – 1.4)	–	–
Western-*aldrichi*	1.8 (1.1 – 3.3)	0.03 (0 – 0.2)	–
D. wheeleri	2.0 (1.3 – 3.3)	0.3 (0.2 – 0.7)	0.2 (0 – 0.4)

The nuclear genes used in this study were not as well differentiated as the mtDNA gene regions and have not acquired mutations diagnostic at the species level. This pattern was observed before in other closely related species in the *repleta* group, and has been attributed to incomplete lineage sorting of ancestral polymorphism in nuclear genes, e.g. *D. straubae* - *D. parisiena* (O'Grady *et al.* 2002), and *D. arizonae* - *D. mojavensis* (Oliveira *et al.* 2003). Many samples of eastern-*aldrichi* and western-*aldrichi* had the same *sina* allele (Table 1). This is clearly a very conserved gene, because all sampled taxa in the *repleta* group had the same amino acid sequence, and only one replacement was found when compared to *D. acanthoptera*, a member of the distantly related *nannoptera* group (Markow & O'Grady 2006). Only one *sina* sequence for *D. wheeleri* was obtained (Table 1), even though several attempts were made to amplify this gene. Primers for this gene gave very reliable amplifications for most of the *repleta* group species and for other *Drosophila* species groups as well (Bonacum *et al.* 2001; and this study), so a possible explanation for our inability to amplify *sina* in *D. wheeleri* may be that some *D. wheeleri* alleles could have unique mutations in the primer binding sites.

The second nuclear gene, *Marf* was more polymorphic than *sina.* When only exons were considered, the same haplotypes were found in eastern and western-*aldrichi*, and one *D. wheeleri* specimen shared the same nucleotide sequence with two eastern-*aldrichi* specimens (Table 1). However, if the intronic region was included, most of the *Marf* sequences were different and only two *D. aldrichi* samples had identical sequences (119128 and 119130; data not shown). Although *Marf* did not possess replacements in the *aldrichi* subcluster, amino acid polymorphism was found among other *repleta* group species. Divergence in the protein sequence did not match the phylogenetic relationships in the group, and may have caused distortions in the nuclear gene phylogeny (Fig. 2b). For example, it was surprising that the more distantly related *D. repleta* had the same protein sequence as *D. mulleri*, *D. nigrodumosa*, and *D. mayaguana*. This might represent the ancestral state in the *repleta* group with the other species being genetically differentiated in variable degrees.

Discussion

We have shown for the first time that *D. aldrichi* is paraphyletic relative to *D. wheeleri* based on analyses of DNA sequence variation. Although this challenges the currently accepted taxonomy, these results confirm previous hypotheses generated by studies of reproductive isolation (Richardson 1982; Wasserman 1992; Krebs & Barker 1994). We propose a new scenario that includes these phylogenetic results in light of the known species biogeographical distributions, and reproductive incompatibilities observed in mating tests made with different strains of "*D. aldrichi*".

Previous results based on laboratory crosses suggested that "*D. aldrichi*" was likely composed of more than one species. Wasserman (1992) listed a series of incompatible crosses between isofemale lines identified as *D. aldrichi*. Krebs and Barker (1994) showed that crosses between a line of *D. aldrichi* collected in Australia with a northwestern Mexican *D. aldrichi* line from Sinaloa resulted in almost completely sterile hybrid F_1 males and fertile F_1 females. Our molecular phylogeny placed Australian *D. aldrichi* in the eastern-*aldrichi* cluster and the Sinaloan line used by Krebs and Barker (1994) was likely from the western-*aldrichi* clade, indicating that their observations of hybrid sterility are consistent with the presence of two reproductive isolated clades in Mexico.

Richardson (1982) reported hybrid male sterility in crosses between *D. aldrichi* strains from Texas (eastern-*aldrichi*) and Sonora (western-*aldrichi*), and that *D. wheeleri* showed similar patterns of reproductive isolation with both eastern and western-*aldrichi*. To our knowledge, this is the only report of reproductive isolation between *D. wheeleri* and western-*aldrichi*, that our molecular data showed as a closely related group exclusive of eastern-*aldrichi*. Such concordance between genetic and reproductive isolation data is particularly meaningful, because in the original paper describing *D. wheeleri* (type specimen from California) Patterson and Alexander (1952) suggested that *D. wheeleri* had been collected in Sonora, a region where western-*aldrichi* is present. Subsequent to this publication, *D. wheeleri* has never been collected in mainland Mexico and is thought to be restricted to the southern California and northern Baja California. If indeed *D. wheeleri* and western-*aldrichi* are reproductively and geographically isolated, it strengthens the molecular results presented here and perhaps indicates a very recent speciation event. Alternatively, it is possible that *D. wheeleri* and western-*aldrichi* are actually conspecific and the molecular data only reflects population level structure.

Western-*aldrichi* and eastern-*aldrichi* are broadly distributed in southern Mexico and have been collected from Oaxaca and Chiapas (Wasserman 1992; and this study) to as far north as Hidalgo (Richardson 1982). Since these are lowland species (Patterson & Wagner 1943), western-*aldrichi* and eastern-*aldrichi* may have evolved as they diverged northwards, where the high elevation mountains and plains in the central Mexico acted as geographical barriers. It is unknown whether these reproductively isolated forms truly co-occur locally in southern Mexico, or if they are ecologically isolated perhaps by using different host cacti in different parts of their respective ranges. Early studies in Texas showed that *Opuntia linguiformis* was a major host, and northwestern Mexico populations are clearly restricted to *Opuntia* species, e.g. *O. wilcoxii* (Ruiz & Heed 1988). However, columnar cacti such as *Pachycereus weberi* are used as hosts in Guerrero, southernwestern Mexico, and *Myrtillocactus geometrizans* in Puebla (Etges, unpublished data). Further, "*D. aldrichi*" has been reported using the columnar cactus *Armatocereus sp.* (Leptocereae) in Peru (Suyo & Pilares 1987) suggesting that multiple host cactus use could have a role in the evolution of "*D. aldrichi*" cryptic species.

We conclude that the taxon "*D. aldrichi"* refers to actually two species (at least) in North America, distinguished here at the DNA sequence level as eastern-*aldrichi* and western-*aldrichi*. The reciprocal monophyly obtained for them and a relatively large number of diagnostic characters, together with data on reproductive isolation from previous studies support this proposal. Since the type specimen of *D. aldrichi* is from Texas (Patterson & Wheeler 1942), the eastern-*aldrichi* should retain the name *D. aldrichi*. The western-*aldrichi* populations thus comprise an undescribed form. Before describing western-*aldrichi*, however, studies of reproductive isolation should be repeated with reciprocal crosses between western-*aldrichi* with *D. wheeleri* and between western-*aldrichi* with *D. aldrichi* (eastern-*aldrichi*). Also, additional mtDNA gene sequences should be obtained to investigate molecular support for species delineation in the *aldrichi* subcluster. Finally, the same strains should be used in both mating tests and molecular analyses.

It is likely that the diversity in the *aldrichi* subcluster, what Wasserman (1992) called a "pack of sibling species", has not been completely sorted out. The results presented here indicate that the use of mtDNA polymorphism can be a powerful tool to uncover diversity and species limits within this group, especially when supported by evidence of reproductive isolation in controlled crosses. Since *D. aldrichi*-like flies have been reported from other places including El Salvador, Costa Rica, Colombia, Venezuela, Peru, and Brazil (reviewed in Vilela 1983 and Wasserman 1992), it is of great interest to discover if these "*D. aldrichi*" col-

lected in Central and South America belong to one of the forms delineated here, or if they comprise other, undescribed cryptic species.

Acknowledgment

This research was in part supported by NSF grant DEB 02-29105 to R. DeSalle and P. M. O'Grady. Deodoro Oliveira was an Ambrose Monell Research Fellow at the AMNH.

References

Bonacum, J., DeSalle, R., O'Grady, P., Oliveira, D., Wintermute, J. & Zilversmit, M. (2001) New nuclear and mitochondrial primers for systematics and comparative genomics in Drosophilidae. *Drosophila Information Service*, 84, 201–204.

Bremer, K. (1988) The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution*, 42, 795–803.

Durando, C.M., Baker, R.H., Etges, W.J., Heed, W.B., Wasserman, M. & DeSalle, R. (2000) Phylogenetic analysis of the *repleta* species group of the genus *Drosophila* using multiple sources of characters. *Molecular Phylogenetics and Evolution*, 16, 296–307.

Farris, J.S., Albert, V., Kallersjo, M., Lipscomb, D. & Kluge, A.G. (1996) Parsimony jackknifing outperforms neighbor-joining. *Cladistics*, 12, 99–124.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39, 783–791.

Felsenstein, J. (1988) Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics*, 22, 521–565.

Heed, W.B. & Grimaldi, D.A. (1991) Revision of the morphocryptic, Caribbean, *mayaguana* species subcluster in the *Drosophila repleta* group (Diptera: Drosophilidae). *American Museum Novitates*, 2999, 1–15.

Heed, W.B., Sanchez, A., Armengol, R. & Fontdevila, A. (1990) Genetic differentiation among island populations and species of cactophilic *Drosophila* in the West Indies. *In:* Barker, J.S.F., Starmer, W.T. & MacIntyre, R.J. (Eds.), *Ecological and Evolutionary Genetics of Drosophila*. Plenum Press, New York, pp. 447–490.

Krebs, R.A. & Barker, J.S.F. (1994) Crosses between *D. aldrichi* from Australia and northwest Mexico suggest that they are different species. *Drosophila Information Service*, 75, 133–134.

Maddison, D.R. & Maddison, W.P. (2000) MacClade: Analysis of phylogeny and character evolution, version 4.0. Sinauer Associates, Sunderland, Mass.

Markow, T. & O'Grady, P.M. (2006) *Drosophila: A Guide to Species Identification and Use*. Academic Press, London, 272 pp.

Mulley, J.C. & Barker, J.S.F. (1977) The occurrence and distribution of *Drosophila aldrichi* in Australia. *Drosophila Information Service*, 52, 151–152.

O'Grady, P.M., Durando, C.M., Heed, W.B., Wasserman, M., Etges, W.J. & DeSalle, R. (2002) Genetic divergence within the Drosophila mayaguana subcluster, a closely related triad of Caribbean species in the *repleta* species group. *Hereditas*, 136. 240–246.

Oliveira, D.C.S.G., Wintermute E.H., Etges W.J., Heed W.B., O'Grady P.M. & DeSalle R. (2003) Hierarchical structure in the *Drosophila mojavensis* cluster (Diptera: Drosophilidae). *Hereditas*, 139, 223–227.

Oliveira, D.C.S.G., O'Grady, P.M., Etges, W.J., Heed, W.B. & DeSalle, R. (2005) Molecular systematics and geographical distribution of the *Drosophila longicornis* species complex (Diptera: Drosophilidae). *Zootaxa*, 1069, 1–32.

Patterson, J.T. (1943) The Drosophilidae of the Southwest. *The University of Texas Publications*, 4313, 7–214.

Patterson, J.T. & Alexander, M. L. (1952) *Drosophila wheeleri*, a new member of the *mulleri* subgroup. *University of Texas Publications*, 5204, 129–136.

Patterson, J.T. & Mainland, G.B. (1944) The Drosophilidae of Mexico. *The University of Texas Publications*, 4445, 9–101.

Patterson, J.T. & Wagner, R.P. (1943) Geographical distribution of species of the genus *Drosophila* in the United States and Mexico. *The University of Texas Publications*, 4313, 217–281.

Patterson, J.T. & Wheeler M.R. (1942) Description of new species of the sub-genus *Hirtodrosophila* and *Drosophila*. *The University of Texas Publications*, 4213, 67–109.

Richardson, R.H. (1982) Phyletic species packing and the formation of sibling (cryptic) species clusters. *In*: Barker, J.S.F. & Starmer, W.T. (Eds.), *Ecological Genetics and Evolution: The Cactus–Yeast–Drosophila Model System*.

Academic Press, Sydney, pp. 107–123.
Ruiz, A. & Heed, W.B. (1988) Host-Plant specificity in the cactophilic *Drosophila mulleri* species complex. *Journal of Animal Ecology*, 57, 237–249.
Sites, J.W., Jr. & Marshall, J.C. (2003) Species delimitation: a Renaissance issue in systematic biology. *Trends in Ecology and Evolution*, 18, 462–470.
Sorenson, M.D. (1999) TreeRot, version 2. Boston University, Boston, MA.
Suyo, M.P.P. & Pilares, L. (1987) *Drosophila novemaristata*: descripcion, taxonomia y nuevas localidades peruanas. *Revista Peruana de Entomologia*, 30, 61–64.
Swofford, D.L. (2002) PAUP*: Phylogenetic Analysis Using Parsimony (and other methods), version 4.0 beta. Smithsonian Institution, Washington DC.
Vilela, C.R. (1983) A revision of the *Drosophila repleta* species group (Diptera, Drosophilidae). *Revista Brasileira de Entomologia*, 27, 1–114.
Wasserman, M. (1954) Cytological studies on the *repleta* group. *The University of Texas Publications*, 6205, 85–118.
Wasserman, M. (1982) Evolution of the *repleta* group. In: Ashburner, M., Carson, H.L. & Thompson, J.N. (Eds.), *The genetics and biology of* Drosophila, *vol. 3b*. Academic Press, London, pp. 61–139.
Wasserman, M. (1992) Cytological evolution of the *Drosophila repleta* species group. *In:* Krimbas, C.B. & Powell, J.R. (Eds.), *Drosophila Inversion Polymorphism*. CRC Press, Boca Raton, pp. 455–552.