CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INTITUTO POLITÉCNICO NACIONAL

UNIDAD IRAPUATO

UNIDAD DE GENÓMICA AVANZADA

"Early events in speciation: cryptic species of *Drosophila aldrichi*"

Tesis que presenta

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Para obtener el grado de:

Maestra en ciencias

En la especialidad de:

Biología Integrativa

Directora de Tesis

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Irapuato, Guanajuato **Septiembre, 2016**

El presente trabajo fue realizado en el laboratorio de Genómica de Biodiversificación de la Unidad de Genómica Avanzada del CINVESTAV Irapuato, bajo la dirección de la Dra. Therese Ann Markow.

El trabajo se realizó con el financiamiento brindado por el Consejo Nacional de ciencia y Tecnología a través del programa de ciencia básica (donativo CB-180385) y fondos de la UGA-LANGEBIO.

AKNOWLEDGEMENTS

I thank CONACyT for the scholarship granted these past 2 years for my master's program and for the grant that supported this work (CB-180385) and LANGEBIO funds. I also thank the UC San Diego Drosophila Species Stock Center for providing the *Drosophila aldrichi* strains used in my study. I would like to thank my laboratory partners for their support and help throughout my stay in the lab, especially to Mariana Ramírez for her aid in the molecular part of my project and Alejandra Vieyra. I thank Alejandra Chávez for helping maintaining the *Drosophila* stocks. I thank my committee members, Dr. Jorge Ibarra and Dr. Sean Rovito, for their helpful comments and suggestions on the seminar presentations. Also I thank Dr. Maxi Richmond of UC San Diego for her comments and suggestions in data analysis. Finally, I express my sincere thanks to Dr. Therese Markow, not only my advisor but my friend, for allowing me to be part of her laboratory group, for her patience and guidance through this project.

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ABSTRACT

In sexually reproductive organisms, the speciation process involves the emergence of reproductive isolating barriers that prevent gene flow between diverging lineages. Studying species at early stages can give us insights into when these barriers arise during the genetic divergence and whether certain reproductive barriers appear before others. *Drosophila aldrichi* is a member of the largely cactophilic *repleta* species group of Drosophila. Widespread in North America, its morphology suggests it is one species, however, other observations suggest the presence of more than one reproductively isolated population. In this study my analysis of 1159 bp of concatenated mitochondrial genes cytochrome oxidase subunit 1 and cytochrome oxidase subunit 2 shows two distinct *D. aldrichi* lineages: one formed by Baja Calfornia Sur, Texas and Guerrero populations, and a second one with populations from the southern Mexican mainland. I also examined pre- and post-zygotic reproductive isolation among certain members of the two lineages. Baja California exhibits significant prezygotic (behavioral) and postzygotic (F1 male sterility) reproductive isolation when crossed with all mainland populations. These results suggest the presence of at least two *D. aldrichi* cryptic species in North America. While the presence of reproductive isolation among populations is consistent with the molecular data, new collections from additional parts of the *D. aldrichi* range could establish whether there are additional distinct lineages and to what degree reproductive isolation exists among them.

RESUMEN

En organismos con reproducción sexual, el proceso de especiación involucra la aparición de barreras de aislamiento reproductivo que previenen el flujo genético en linajes divergentes. El estudio de especies en etapas tempranas nos puede dar indicios acerca de cuándo surgen estas barreras durante la divergencia genética así como si ciertas barreras reproductivas aparecen antes que otras. *Drosophila aldrichi* es un miembro cactofílico del largo grupo de especies *repleta*. Ampliamente distribuida en América del Norte, su morfología sugiere que es una sola especia, no obstante, otras observaciones sugieren la presencia de más de una población aislada reproductivamente. En este estudio, el análisis de 1159 pares de bases concatenadas de los genes mitocondriales citocromo oxidasa subunidad 1 y citocromo oxidasa subunidad 2 muestran dos linajes de *D. aldrichi*: uno formado por las poblaciones de Baja California Sur, Texas y Guerrero, y el segundo formado por poblaciones del sur de la parte continental de México. A su vez, también examiné el aislamiento pre- y postzygótico entre ciertos miembros de los dos linajes. Baja California presenta un significativo grado de aislamiento precopulatorio-precigótico (etológico) y postzigótico (esterilidad en los machos F1) al ser cruzados con todas las poblaciones de la parte continental de México. Éstos resultados sugieren la presencia de al menos dos especies crípticas de *D. aldrichi* en norteamérica. Mientras la presencia de aislamiento reproductivo entre las poblaciones es consistente con los datos moleculares, nuevas colectas de partes adicionales del rango de *D. aldrichi* podrían establecer si hay linajes adicionales y qué grado de aislamiento reproductivo existe entre ellos.

INTRODUCTION

Speciation, or the origin of new species, is a fundamental process in evolutionary biology. Understanding how new species are formed, however, remains a challenging problem. Although there are over 20 different concepts (Hey, 2001), the Biological Species Concept (BSC) (Dobzhansky, 1950; Mayr, 1942) is the one most commonly used for sexually reproducing organisms. Mayr (1942) defined species as groups of interbreeding natural populations that are reproductively isolated from other such groups. In addition, Dobzhansky (1950) defined species as groups of organisms that share a common gene pool, that maintain cohesion through gene exchange between them and avoid hybridization by a reproductive isolating mechanism (barrier) or a combination of several such mechanisms.

According to the BSC, speciation is the evolution of reproductive barriers among populations that permit the maintenance of genetic and phenotypic distinctiveness of these populations (Seehausen *et al.* 2014). In order to understand how speciation occurs, we must first understand how these barriers to gene flow evolve. The isolating barriers refer to those biological characteristics of organisms that impede the exchange of genes with members of other populations (Coyne and Orr, 2004). Reproductive isolating mechanisms fall into three categories (see Table 1): premating, postmating-prezygotic and postzygotic (Dobzhansky 1937; Coyne and Orr 2004). Premating isolating barriers prevent mating among individuals from separate populations through behavioral (sexual), ecological or mechanical incompatibilities (Coyne and Orr, 2004). If mating does

occur, postmating prezygotic barriers act prior to zygote formation or fertilization (Coyne and Orr 2004, Markow 1997, Servedio 2001). Finally, postzygotic isolating barriers act after fertilization, interfering with the formation of hybrids or their viability or fertility (Table 1). Reproductive isolating barriers promote genetic isolation and thus accelerate differentiation. Nevertheless, the relationship between the degree of genetic differentiation observed between two populations and when the first reproductive isolating barriers appear remains unclear. At the same time, how rapidly such barriers evolve and whether one of these barriers tends to arise before others also are unknown.

Table 1. Classification of reproductive isolating barriers (Adapted from Coyne and Orr, 2004).

Reproductive isolating barriers
I. **Premating isolating barriers.** Isolating barriers that prevent mating and thus, impede the formation of the hybrid zygote. A. **Behavioral isolation ("ethological" or "sexual" isolation).** Members of different species fail to court or mate due to lack of attraction. B. **Ecological isolation.** Species occupy different habitats within the same area or breed at different times. C. **Mechanical isolation.** Incompatibility of reproductive structures prevents copulation between two species.

Reproductive isolating barriers (cont'd)

II. **Postmating, prezygotic isolating barriers.** Isolating barriers that act after copulation and sperm transfer and prevent fertilization.

A. **Gametic isolation.** Problems with transfer or storage of gametes limit fertilization.

B. **Sperm competition.** Females from one species are exposed to sperm from males of multiple species; conspecific sperm precedence has fertilization advantage.

III. **Postzygotic isolating barriers (hybrid sterility and inviability).**

A. **Extrinsic.** Isolation depends either on the influence of the external environment (ecological niche) or interactions with other individuals

1. **Ecological inviability.** Hybrids are inviable because they are not adapted to either of the parent's habitat.

2. **Behavioral sterility.** Hybrids have reduced fertility due to behavioral factors and fail to obtain mates.

B. **Intrinsic.** Isolation that includes developmental problems in hybrids that are independent of the environment.

1. **Hybrid inviability.** Hybrid survival is affected due to developmental difficulties.

2. **Hybrid sterility.** Hybrids fail to produce viable gametes or have developmental problems in their reproductive system.

Genus *Drosophila*

Flies of the genus *Drosophila* have provided popular model systems to study the role of isolating mechanisms in evolution as we have well-established phylogenetic relationships of hundreds of species for which we also know the resource ecology and geographic distributions. Coyne and Orr (1989, 1997) performed meta-analyses on published studies of laboratory premating and postmating isolation between closely related *Drosophila* species pairs whose genetic distances were determined from allozyme data. They found that 1) both prezygotic (assortative mating) and postzygotic (hybrid inviability and sterility) increase gradually with genetic distance; 2) postzygotic isolation evolves more rapidly in males than in females: hybrid sterility or inviability usually affects males first ("Haldane´s Rule", Haldane 1922) and female sterility appears when taxa are older; 3) among recently diverged populations, premating isolation appears to be a stronger barrier to gene exchange than postzygotic isolation (Coyne and Orr 1989, 1997) when the two populations are sympatric (overlap geographically). Further studies (Noor 1995; Higgie *et al* 2000) confirmed that *Drosophila* species pairs have stronger premating isolation in sympatry than in allopatry (geographically separated). The species pairs in the previously mentioned studies, however, were already considered as full species (have already undergone speciation). That being the case, we cannot be certain that premating isolation is a stronger barrier to gene exchange than postzygotic isolation or that it arises earlier.

Studying populations that are in early stages of speciation should provide better insights into the origin of these species barriers. One example is the cactophilic species, *Drosophila mojavensis*. Endemic to the Sonoran Desert, it is composed of four geographically distinct subspecies, each of which utilizes necroses of different cactus species (Pfeiler *et al.* 2009). Laboratory studies show the presence of two reproductive isolation barriers between the four distinct populations: premating (behavioral) and postmating prezygotic isolation when paired (Knowles and Markow 2001). Another example is *D. willistoni* and its subspecies *D.willistoni quechua*. These subspecies are morphologically indistinguishable, nonetheless they also show a small degree of premating isolation (behavioral). Crosses between female *D. willistoni quechua* and male *D. willistoni willistoni* yield sterile males in the F1 generation, while its reciprocal cross yields fully fertile hybrid males (postzygotic isolation). Female offspring of both crosses are fertile (Dobzhansky 1975). The level of genetic divergence between these *D. willistoni* subspecies is unknown, leaving it unclear at what point the sterility arose.

Present study

Drosophila aldrichi is a cactophilic member of the *mulleri* complex of the *repleta* species group that provides a good opportunity to examine early events in speciation. Widespread in Mexico and the southwestern part of the United States, it also has been reported from Central and South America (Markow & O'Grady

2005). In addition, the species was accidently introduced into Australia in the 1930s and the populations have expanded across that continent (Mulley & Barker 1997) (Figure 1). *Drosophila aldrichi* breeds primarily in the decaying pads of *Opuntia* cactus species, although anecdotal reports of association with columnar cacti exist as well (Oliveira *et al*. 2012). Long assumed to be one species, because there are no observable phenotypic differences among populations (Figure 2), several reports suggest the possible existence of cryptic species. For example, Richardson (1982) mentioned that crosses between strains of *D. aldrichi* from Texas and Sonora yield sterile male offspring, although he provided no data. Subsequently Wasserman (1992) also reported that crosses among different strains of *D. aldrichi*, including those collected at the same locality, could not interbreed, but again, no data were shown. Finally, Krebs and Barker (1994) found evidence that crossing *D. aldrichi* from Australia and *D. aldrichi* from Sinaloa, Mexico, produced fertile female and sterile male hybrids in both reciprocal crosses. They pointed out that the exact North American origin of the Australian *D. aldrichi* was unknown and because several decades is not likely to be sufficient time to produce reproductive isolation between the North American and the Australian *D. aldrichi*, it is likely that *D. aldrichi* already existed as multiple species in North America. None of these former studies provides any quantitative assessment of hybrid sterility or measures of sperm motility in the F1 males.

Figure 1. *Drosophila aldrichi* distribution in North America and Australia (Markow & O'Grady 2005, Mulley & Barker 1997) and its host cactus, *Opuntia spp*.

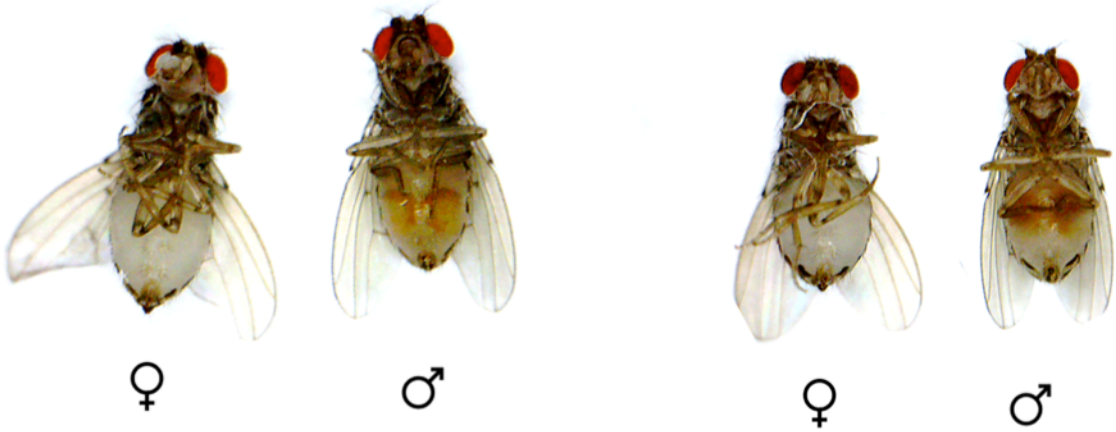


Figure 2. *D. aldrichi* female and male from Baja California (left) and from Oaxaca (right).

Furthermore, sexual isolation was assumed to be absent among populations of this species because the crosses among the different strains did produce hybrid offspring. Matings were never observed directly, however. The fact that sterility was observed in reciprocal crosses, and not just in one direction, suggests the existence of significant divergence between the tested strains by Krebs and Barker (1994).

Beckenbach *et al.* (2008) suggested the existence of two divergent clades of *D. aldrichi* in North America, based on molecular phylogenetic studies of 688 bp of the mitochondrial genes *CO2* and 354 bp *nad3*. Molecular sequence data of combined *CO1* and *nad2* from Oliveira *et al*. (2008) also suggest the existence of two *D. aldrichi* lineages. While the above-mentioned crosses and the molecular phylogenetic patterns strongly point to the existence of cryptic species of *D. aldrichi*, it nonetheless remains untested whether the same populations that show reproductive isolation also belong to different lineages. Here, I studied the nature of reproductive isolation among different strains of *D. aldrichi* from six populations throughout Mexico in the context of their evolutionary relationships based upon sequences of two mitochondrial genes.

HYPOTHESIS

Drosophila aldrichi is a group of two or more cryptic species. One or more reproductive isolating mechanisms should be observed among populations. Populations that are the most diverged genetically will exhibit the strongest reproductive isolation.

AIMS

GENERAL AIM

The general aim is to determine if there is evidence that *D. aldrichi* is more than one species.

SPECIFIC AIMS

1. To use molecular sequence data to determine the genetic relationships among different *D. aldrichi* populations.
2. To determine what type (s) of reproductive isolating (RI) mechanism (s) exist among *D. aldrichi* lineages and localities.
3. To determine if the populations that belong to different lineages show greater reproductive isolation than those within the same lineage.

MATERIALS AND METHODS

Strains of *D. aldrichi*

Drosophila aldrichi strains were obtained from the Drosophila Species Stock Center in UCSD, the Etges laboratory collection from the University of Arkansas and from more recent collections made by our laboratory from different localities in Mexico (Figure 3, Table 2). Collected flies were keyed to species using Markow and O'Grady (2005) under a stereo microscope (Figure 4) and the identifications of all flies used in the study were also verified by molecular techniques (mt*CO2* amplification). Flies were reared in potato-prickly pear culture medium with live yeast at 24 ± 1 °C with a 12-hour photoperiod.

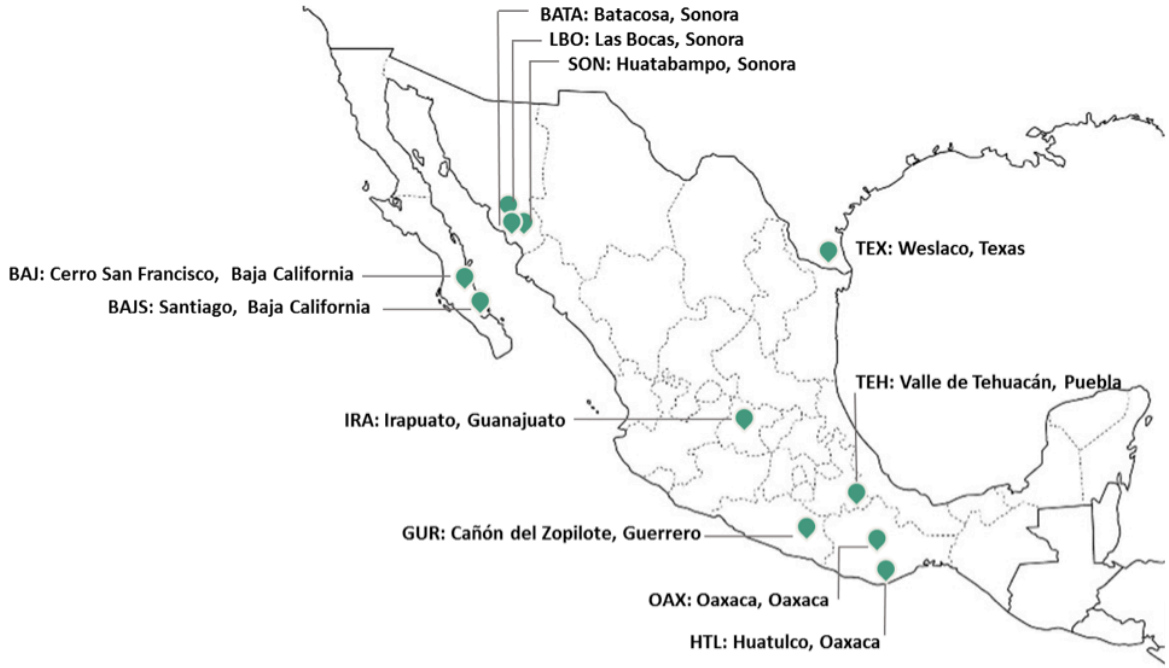


Figure 3. Geographic location of *D. aldrichi* populations used in the present study

Table 2. Collection localities of *D. aldrichi* used in the present study. Some stocks were only used in the molecular studies because living collections were not available for studying reproductive isolation.

Locality	Strain number	Reference	Abreviation
Cerro San Francisco, Baja California Sur	15081-1251.15	UC San Diego Drosophila Stock Center	BAJ
Oaxaca, Oaxaca	15081-1251.13	UC San Diego Drosophila Stock Center	OAX
Cañón del Zopilote, Guerrero	15081-1251.12	UC San Diego Drosophila Stock Center	GUR
Valle de Tehuacán, Puebla	TEH-99	Etges Laboratory Stock Collection	TEH
Huatulco, Oaxaca	HTL-02	Etges Laboratory Stock Center	HTL
Huatabampo, Sonora	HTB-0515	Collected from wild	SON
Las Bocas, Sonora	LB09	UC San Diego Drosophila Stock Center	LBO
Irapuato, Guanajuato	IRA-1214	Collected from wild	IRA
Weslaco, Texas	15081-1251.01	UC San Diego Drosophila Stock Center	TEX
Batacosa, Sonora	BATA	Collected from wild	BATA
Santiago, Baja California	15081-1251.10	UC San Diego Drosophila Stock Center	BAJS

Figure 4. *Drosophila aldrichi* morphological characters. The key feature of the *repleta* species group of *Drosophila* is the presence of a pale brown mesonotum with dark/brown spots that fuse on both sides of the mid-dorsal line (a, left). *Drosophila aldrichi* is characterized by the triangular areas in posterolateral corners about the same shade or lighter than apical bands on median portions of the

tergites (a, right). It has vermilion eyes and males have deep orange testes (b) (Markow & O´Grady 2005).

Phylogenetic studies

The barcode region of the mitochondrial cytochrome oxidase subunit 1 gene (mtCO2, Folmer *et al*. 1994) and cytochrome oxidase subunit 2 (mtCO1, Simon *et al.* 1994) (see Table 3) were sequenced to determine the evolutionary relationships between flies from different locations (Table 2). A total of 1159 base pairs (bp) were analyzed for the concatenated dataset: 560 bp from mtCO1 and 638 bp from mtCO2.

Table 3: Genes and primers used for phylogenetic analysis.

Gene	Primer	Sequence (5´- 3')	Reference
CO1	LCO1490-F	GGTCAACAAATCATAAAGATATTGG	**Folmer *et al*. 1994**
	HCO2198-R	TAAACTTCAGGGTGACCAAAAAAT	
CO2	TL2-J-3037-F	ATGGCAGATTAGTGCAATGG	**Simon *et al*. 1994**
	TK-N-3785-R	GTTTAAGAGACCAGTACTTG	

Total genomic DNA was isolated from individual adult flies using a DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA). Whole flies were used for DNA extraction. The manufacturer's protocol was followed with the following modifications: flies were ground individually with sterile pestles for each specimen in 1.5 mL tubes with tissue lysis buffer and proteinase K and incubated at 56 °C for 20 minutes to digest the exoskeleton and tissue. A second lysis buffer was added to lyse cells and cellular components and samples were incubated at 56 °C for 10 min. Molecular Biology grade ethanol (96%) was added and samples were poured in a spin column for centrifugation at 8 000 rpm. Flow-through liquid was discarded and washing buffer was added prior centrifugation at 8 000 rpm. The previous step

was repeated, adding a second washing buffer. Finally, the spin column was transferred to a clean 1.5 mL tube and DNA was centrifuged with 30 µL of elution buffer. To determine the evolutionary relationship of the different populations, two mitochondrial genes were used for polymerase chain reaction (PCR) and sequencing: CO1 and CO2. PCR amplifications for CO1 were performed using the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 45 sec, 72 °C for 1 min and 72 °C for 7 min. *CO2* amplification was performed using the same conditions, only differing in the annealing temperature: 52 °C for 45 sec. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA) and sequenced. Forward and reverse sequencing reactions for both CO1 and CO2 were performed on an Applied Biosystems (Foster City, CA) ABI 3730XL DNA sequencer at the LANGEBIO core DNA sequencing facility. Sequences were aligned and corrected with Geneious® version R9.1 (Kearse et al., 2012). Selection of best-fit partitioning schemes and models of molecular evolution was performed using PartitionFinder version 1.1.1 (Lanfear *et al*., 2012) and concatenated gene analysis was performed in a Bayesian inference of phylogeny (BI) framework using MrBayes version 3.2.6 (Huelsenbeck & Ronquist, 2001) with 2 runs and 4 chains in each run for 1 x 10^7 generations sampling every 1000 generations. Both CO1 and *CO2* were split by codon in order to select the appropriate substitution model. Three partitions were selected, one for each position of the codon in both genes. Substitution models for each partition were as follows:

1. First codon position: General Time Reversible (GTR).
2. Second codon position: Felsenstein (F81).
3. Third codon position: Hasegawa-Kishino-Yano (HKY85+G).

A haplotype network was built using statistical parsimony implemented in TCS (Clement *et al.* 2000) using PopART version 1.7 (http://popart.otago.ac.nz) in order to analyze the relationships between haplotypes in *D. aldrichi* populations. Uncorrected pairwise genetic distances of the combined CO1 and CO2 were calculated using PAUP* version 4.0 (Swofford 2002).

Reproductive isolation

Six locations were selected for the reproductive isolation tests (Table 4, Figure 4). Populations were selected based on their evolutionary relationships established in the phylogenetic analyses, the availability of living strains for experiments, and upon their geographical localities. Six populations were used: BAJ, OAX, SON, GUR, TEH and HTL.

Table 4. Collection localities and abbreviations of *D. aldrichi* strains used in the reproductive isolation tests.

Locality	Strain number	Reference	Abreviation
Cerro San Francisco, Baja California Sur	15081-1251.15	UC San Diego Drosophila Stock Center	BAJ
Oaxaca, Oaxaca	15081-1251.13	UC San Diego Drosophila Stock Center	OAX
Cañón del Zopilote, Guerrero	15081-1251.12	UC San Diego Drosophila Stock Center	GUR
Valle de Tehuacán, Puebla	TEH-99	Etges Laboratory Stock Collection	TEH
Huatulco, Oaxaca	HTL-02	Etges Laboratory Stock Center	HTL
Huatabampo, Sonora	HTB-0515	Collected from wild	SON

Premating isolation was measured using standard multiple-choice tests for *Drosophila* (Ehrman & Petit 1968). Male and female adult flies were separated upon eclosion and were kept in separate vials for 10 days to make sure they were sexually mature. In order to distinguish the strains, flies were dusted with microfluorescent powder (R-103-G119 from the U.S. Radium Corporation) 24 hours prior to the start of the experiment (Markow *et al*. 1983), using 2 different colors to distinguish each population. Matings were performed in the morning, which is the typical mating time in nature (Hardeland 1972). Ten pairs of sexually mature virgin flies, five from each of two strains, were placed in a clear plexiglass mating chamber and observed for one hour (Figure 5). The colors of the mating pairs were recorded and approximately 10 replicates were conducted for each set of two strains with colors alternated between replicates.

Figure 5. a) Mating chamber for multiple-choice tests. b) Graphical representation of the multiple choice tests.

Chi square tests were performed for departures from random mating in the multiple-choice tests. In addition, the isolation index (I) (Merrell 1950) was calculated as well as female and male isolation indices (I_1, I_2) from each strain for the multiple choice tests according to following:

$$I= [(n_{11}+n_{22}) – (n_{12}-n_{21})]/n$$

$$I_1= (n_{11}-n_{12})/(n_{11}+n_{12})$$

$$I_2= (n_{22}-n_{21})/(n_{22}+n_{21})$$

Where n_{11} is the number of homotypic matings (females from Strain 1 and males from Strain 1), n_{12} is the number of heterotypic matings (females from Strain 1 and males from Strain 2 and *vice versa*) and n is the total number of matings. Standard errors (SE) of these indices were calculated by:

$SE= \sqrt{((1-I_2)/n)}$ (Malagolowkin-Cohen et al. 1965).

Isolation indices are statistically significant if the index is twice as large as the standard error (Malogolowkin-Cohen *et al*. 1965, Zouros and D'Entremont 1980).

Postmating reproductive isolation was measured by reciprocal crosses among the six populations. Virgin adult flies were collected and separated by sex after eclosion using light CO_2 anesthesia and were held on fresh medium for 10 days. Ten virgin females and 10 virgin males were placed in vials with culture medium according to the combinations below:

Mating combinations	
Virgin females ♀ (n=10) Strain 1 X males ♂ (n=10) Strain 1	Homotypic
Virgin females ♀ (n=10) Strain 2 X males ♂ (n=10) Strain 2	
Virgin females ♀ (n=10) Strain 1 X males ♂ (n=10) Strain 2	Heterotypic
Virgin females ♀ (n=10) Strain 2 X males ♂ (n=10) Strain 1	

All possible mating combinations were tested for each location and the number of hybrid progeny and sex ratio was recorded. Hybrid males were stored in fresh food vials for 12 days to assure that they had reached sexual maturity. As sperm motility is the standard criterion to assess fertility/sterility in *Drosophila* studies (Coyne and Orr, 1997), mature male hybrid offspring were dissected, removing their testes with dissection tweezers. The presence of motile sperm was scored under the microscope (Dark field, 100X. Nikon Microphot-FX light/epi-fluorescence microscope). Sperm were scored as either motile (at least one or more sperm moving) or nonmotile.

RESULTS

Evolutionary Relationships

A total of 1159 bp of concatenated CO1 and CO2 were analyzed for each *D. aldrichi* strain. Phylogenetic relationships from Bayesian analysis are shown in Figure 6. Results indicate the presence of two *D. aldrichi* lineages: (A) containing the peninsular strains from Baja California along with mainland Guerrero and Texas, and (B) with the remaining mainland strains. Nodes separating the two lineages are well supported, while the relationships within the B lineage are less clear. The TCS haplotype network with a parsimony connection limit of 95% shows 8 different *D. aldrichi* haplotypes (Figure 7). The Mexican mainland strains, with the exception of Guerrero, appear more closely related as nucleotide substitutions range between 0 and 3. Oaxaca, Huatulco and Las Bocas form a haplogroup, and Batacosa forms another with Tehuacan. Texas and Guerrero appear separated from the latter by 14 and 19 nucleotide substitutions, respectively. Both Texas and Guerrero appear more closely related to Baja California strains, although they remain separated by up to 18 nucleotide substitutions.

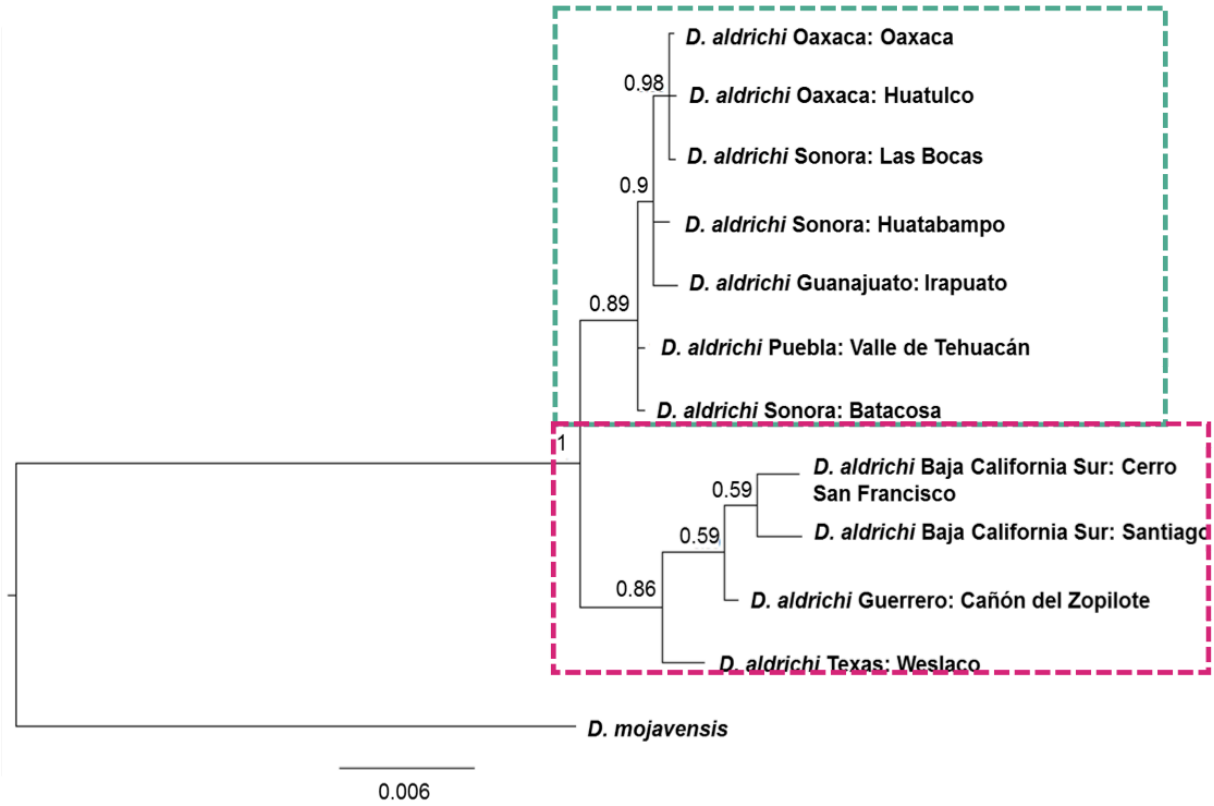
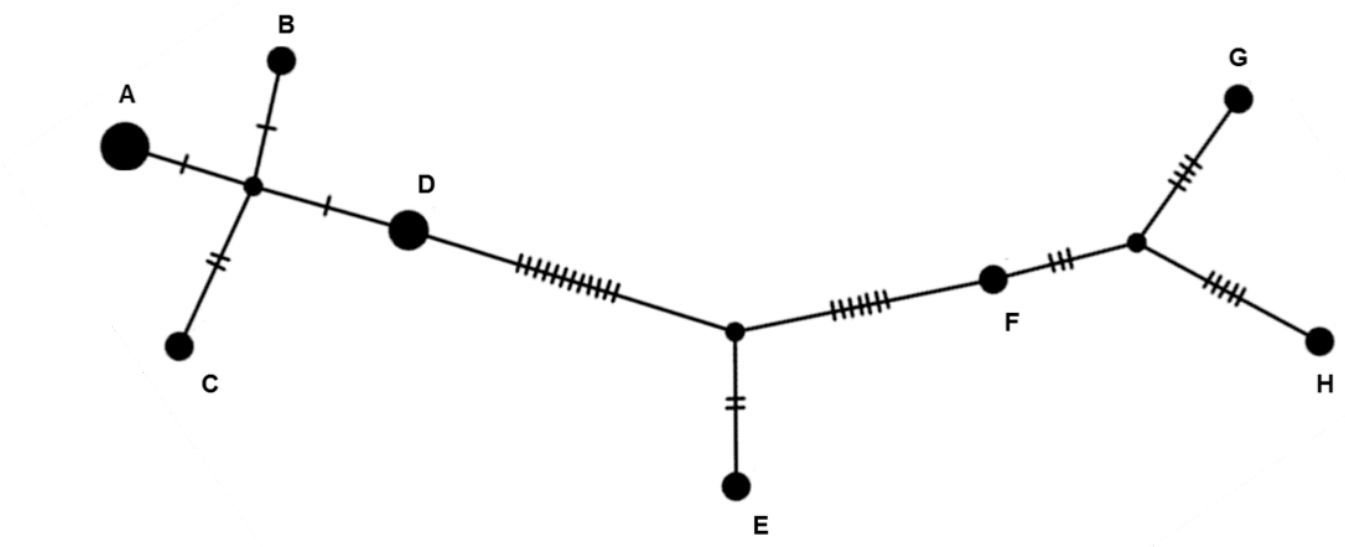


Figure 6. Phylogenetic analysis of *D. aldrichi* by Bayesian inference (BI). Consensus BI tree of concatenated CO1 and CO2. Numbers above nodes are posterior probabilities estimated by the Bayesian analysis.



A: Oaxaca: Oaxaca
Oaxaca: Huatulco
Sonora: Las Bocas
B: Sonora: Huatabampo
C: Guanajuato: Irapuato
D: Puebla: Valle de Tehuacán
Sonora: Batacosa

E: Texas: Weslaco
F: Guerrero: Cañón del Zopilote
G: Baja California Sur: Cerro San Francisco
H: Baja California Sur: Santiago

Figure 7. TCS haplotype network from *D. aldrichi* mitochondrial CO1 and CO2 under the 95% parsimony criterion. Each circle represents a unique haplotype. Bars represent mutational steps between haplotypes.

Genetic distance calculated between all pairs of 11 populations is shown in Table 5. Pairwise comparison values ranged from 0 between Oaxaca–Huatulco, Oaxaca–Las Bocas, Huatulco–Las Bocas and Tehuacan–Batacosa, up to 0.019 between Guerrero–Irapuato. Other than the Baja populations (0.0069 and 0.006), Guerrero comparisons showed the greatest genetic distance values, compared with the rest of the populations.

Table 5. Uncorrected pairwise genetic distances of 11 populations of *D. aldrichi*.

	OAX	HTL	LBO	SON	IRA	TEH	BATA	BAJ	BAJS	GUR	TEX
OAX		**0.0000**	**0.0000**	**0.0017**	**0.0026**	**0.0017**	**0.0017**	**0.0147**	**0.0155**	**0.0181**	**0.0138**
HTL			**0.0000**	**0.0017**	**0.0026**	**0.0017**	**0.0017**	**0.0147**	**0.0155**	**0.0181**	**0.0138**
LBO				**0.0017**	**0.0026**	**0.0017**	**0.0017**	**0.0147**	**0.0155**	**0.0181**	**0.0138**
SON					**0.0026**	**0.0017**	**0.0017**	**0.0147**	**0.0155**	**0.0181**	**0.0138**
IRA						**0.0026**	**0.0026**	**0.0155**	**0.0164**	**0.0190**	**0.0147**
TEH							**0.0000**	**0.0147**	**0.0138**	**0.0164**	**0.0121**
BATA								**0.0147**	**0.0138**	**0.0164**	**0.0121**
BAJ									**0.0078**	**0.0069**	**0.0129**
BAJS										**0.0060**	**0.0138**
GUR											**0.0078**
TEX											

Premating reproductive isolation

Results of the multiple choice mating tests and deviations from random mating for all combinations are presented in Table 6. Premating behavioral isolation was found among crosses between Baja California flies and all of the other localities. In most crosses with flies from the Baja California strain, there was a strong tendency towards positive assortative mating, as replicates show an excess of homotypic relative to heterotypic matings, that is, matings between females and males with their own population. On the other hand, no premating behavioral isolation was found between crosses with flies from the mainland localities. In fact, negative assortative mating was observed among some mainland populations. In some cases, negative isolation indices were significant, as in crosses between Guerrero and Tehuacan or Huatulco, Tehuacan and Oaxaca, and Tehuacan and Huatulco, indicative of outcrossing. It is of interest, however, that in crosses between Baja

and Guerrero, the isolation observed was much less than in the crosses between Baja and the other strains. Only one of the isolation indices was significant for the Baja-Guerrero crosses.

Table 6. Multiple choice test results. X^2 tests were conducted to detect deviations from random mating. I(SE) is the joint isolation index. Significant sexual isolation exists whentheindex is twice as large as the SE (Malogolowkin-Cohen *et al*. 1965, Zouros and D'Entremont 1980). I_1 indicate isolation due to females, I_2 isolation due to males.

Populations		N	AxA	AxB	BxA	BxB	X^2	I (SE)	I_1(SE)	I_2 (SE)
A	B									
BAJ	GUR	81	26	20	13	22	4.38	0.19(0.11)	0.26(0.11)*	0.13(0.11)
BAJ	OAX	81	30	15	9	27	14.55*	0.41(0.10)*	0.33(0.10)*	0.5(0.10)*
BAJ	HTL	63	25	11	5	22	16.68*	0.49(0.11)*	0.39(0.12)*	0.63(0.10)*
BAJ	TEH	77	22	15	13	27	6.48	0.27(0.11)*	0.19(0.11)	0.35(0.11)*
BAJ	SON	73	23	13	10	27	10.67	0.37(0.11)*	0.28(0.11)*	0.46(0.10)*
GUR	OAX	81	24	24	17	16	2.8	-0.01(0.11)	0.00(0.11)	-0.03(0.11)
GUR	TEH	72	13	24	17	18	3.44	-0.14(0.12)	-0.30(0.11)*	0.03(0.12)
GUR	HTL	63	10	21	19	13	5.0	-0.27(0.12)*	-0.35(0.12)*	-0.19(0.12)
GUR	SON	67	15	18	19	15	0.76	-0.10(0.12)	-0.09(0.12)	-0.12(0.12)
OAX	TEH	72	12	23	20	17	2.66	-0.19(0.12)	-0.31(0.11)*	-0.08(0.12)
OAX	HTL	64	11	19	19	15	2.75	-0.19(0.12)	-0.27(0.12)*	-0.12(0.12)
OAX	SON	66	9	17	19	12	4.18	-0.24(0.12)*	-0.29(0.12)*	-0.20(0.12)
TEH	HTL	64	16	16	21	11	3.12	-0.16(0.12)	0.00(0.13)	-0.31(0.12)*
TEH	SON	83	13	24	27	18	5.65	-0.24(0.11)*	-0.30(0.11)*	-0.20(0.11)
HTL	SON	59	14	14	18	13	1.0	-0.08(0.13)	0(0.13)	-0.16(0.13)

** $p < 0.05$*

Postzygotic reproductive isolation

Sex ratios in the progeny of homotypic control crosses did not differ significantly from 1:1 and all males had motile sperm (Table 7).

Table 7. Sex ratio and sperm motility scores from homotypic crosses. Sex ratio did not differ among replicates and all males presented sperm motility.

Cross			Progeny		X^2	Fertile males	
F	M	reps	F	M		N w/motile sperm	%
BAJ	BAJ	2	201	173	2.1	70/70	100%
OAX	OAX	2	75	85	0.62	70/70	100%
GUR	GUR	2	218	165	6.08*	70/70	100%
HTL	HTL	1	175	158	0.86	70/70	100%
TEH	TEH	2	228	209	0.82	70/70	100%
SON	SON	1	126	140	0.737	35/35	100%

* $p < 0.05$

For the majority of the heterotypic crosses (Table 8), sex ratio did not differ from 1:1. However in several crosses (♀OAX X ♂BAJ, ♀OAX X ♂HTL, ♀TEH X ♂OAX, ♀TEH X ♂SON and ♀HTL X ♂OAX) a significant reduction in the number of male offspring was observed.

When scoring sperm motility among populations, male sterility was observed in crosses between Baja California and all of the other localities, and it was found to be asymmetrical in degree (Table 7). In crosses where the fathers were from Baja California, male offspring effectively had no motile sperm. In only two cases did several hybrid sons of Baja fathers have motile sperm: when crossed with Guerrero males, 21% of males showed motile sperm and with Huatulco males, only 3%. On the other hand, in all reciprocal crosses (when the mothers were from Baja California), F1 males exhibited motile sperm, albeit at reduced levels, ranging

between 24 and 73%. In crosses among the mainland populations, all F1 males had motile sperm.

Table 8. Sex ratios and incidence of male sterility in interpopulation crosses.

Cross			Progeny		X^2	Fertile F1 males	
F	M	reps	F	M		N w/motile sperm	%
BAJ	GUR	2	488	465	0.55	41/70	59%
BAJ	OAX	2	137	157	1.36	36/70	51%
BAJ	HTL	2	390	377	0.22	51/70	73%
BAJ	TEH	2	159	143	0.84	17/70	24%
BAJ	SON	1	106	104	0.019	7/31	23%
GUR	BAJ	2	393	358	1.6	15/70	21%
GUR	OAX	2	261	234	1.47	70/70	100%
GUR	HTL	2	275	240	2.4	70/70	100%
GUR	TEH	2	306	291	0.37	70/70	100%
GUR	SON	1	193	164	2.35	26/26	100%
OAX	BAJ	2	284	223	7.33*	0/70	0%
OAX	GUR	2	402	431	1.01	70/70	100%
OAX	HTL	2	224	183	4.1*	70/70	100%
OAX	TEH	2	263	276	0.31	70/70	100%
OAX	SON	2	251	223	1.6	35/35	100%
TEH	BAJ	2	245	287	3.31	0/70	0%
TEH	GUR	2	230	235	0.054	70/70	100%
TEH	OAX	2	371	285	11.27*	70/10	100%
TEH	HTL	2	206	188	0.82	70/70	100%
TEH	SON	1	119	68	13.9*	28/28	100%
HTL	BAJ	2	265	283	0.6	2/70	3%
HTL	GUR	2	353	343	0.02	70/70	100%
HTL	OAX	2	321	271	4.22*	70/70	100%
HTL	TEH	2	180	174	0.10	70/70	100%
HTL	SON	1	131	116	0.91	35/35	100%
SON	BAJ	2	280	186	18.96*	0/38	0%
SON	GUR	2	178	167	0.35	35/35	100%
SON	OAX	2	68	54	1.6	35/35	100%
SON	TEH	2	194	173	1.2	35/35	100%
SON	HTL	1	113	113	-	35/35	100%

*$p<0.05$

DISCUSION

Understanding what kind of reproductive isolating barriers reduce or prevent gene flow and how these barriers evolve is one of the primary goals in speciation studies. Here, I studied the possibility of *D. aldrichi* cryptic species by analyzing evolutionary relationships as well as the isolating mechanisms existing among widespread populations in Mexico.

Using a total of 1159 bp of combined CO1 and CO2 for both a Bayesian phylogenetic analysis and the TCS haplotype network revealed considerable differentiation among populations of *D. aldrichi*. While an earlier molecular study, with fewer informative sequences, also suggested the existence of more than one lineage (Oliveira et al 2008), some of the samples in the earlier study were from different populations than the ones used in the present work. I found that flies from the Baja California peninsula, Texas and Guerrero are more closely related to each other than to the rest of the mainland populations. Only by sampling multiple wild caught individuals and performing population genetic analyses, will we know if the mainland populations, excluding Guerrero, form a panmictic population or exhibit regional differentiation. Additional sampling from more localities would reveal if additional lineages with or without reproductive isolation exist in the rest of the mainland. My data fail to support an earlier suggestion of Eastern and Western clades (Oliveira *et al.* 2008), in which flies from Oaxaca, Guerrero and Texas were said to belong to an Eastern clade. The considerable number of substitutions seen between Baja and the mainland may well reflect the barrier to gene flow created by the Sea of Cortez, but the relationship with Guerrero cannot be explained without

additional nuclear data. The geological origin of the Baja peninsula could also be correlated with the genetic differentiation among the mainland and could possibly explain the relationship with Guerrero (discussed below).

Significant behavioral isolation was found between flies from the Baja California peninsula and the mainland populations, with exception of Guerrero.. Although males appeared to court females from their own and from different populations equally (personal observation), both females and males from Baja California mated more with flies from their own population. While those from the Mexican mainland belong to the same lineage, the multiple substitutions that seem to separate them don't appear to influence reproductive isolation. Since all *D. aldrichi* strains are morphologically identical (Wasserman 1992), it is possible that Baja females are utilizing newly developed male mating signal traits, such as courtship songs (Ewing & Bennet-Clarke 1968) or epicuticular hydrocarbons (Coyne *et al.* 1994), which could be involved in the behavioral isolation in this taxon. Regardless, the behavioral isolation between Baja and mainland populations, while significant, is not complete. The incomplete sexual isolation may reflect the fact that the populations are not sympatric, consistent with the observation of Coyne and Orr (1989, 1997) that behavioral isolation is strongest between species in sympatry.

For the first time, the results of reproductive isolation tests have been tested in a phylogenetic framework. Unfortunately, there was no living stock from Texas to test for reproductive isolation. On the one hand, the isolation observed between

Baja and the other localities fits well with its evolutionary position. At the same time, the Guerrero flies, despite being in the same lineage with Baja, share similarities with those from the mainland. Although there are indications that Guerrero is less isolated from Baja than the other mainland strains, it is difficult to explain, without additional genetic, such as nuclear loci, exactly what its relationship is to the other localities.

Coyne and Orr (1989, 1997) concluded that male sterility is one of the earliest indications of speciation, and that the usual pathway of postzygotic isolation is the appearance of sterility in the heterogametic sex, usually in one direction first, followed by the appearance of sterility in the homogametic sex when taxa are older. This is consistent with Haldane´s rule (Haldane, 1922): “when in the F1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex”. Reciprocal crosses within all mainland populations showed sperm motility in both directions. On the other hand, sperm immotility in hybrid males was found in all crosses between Baja California and mainland populations. While sons from both reciprocal crosses showed a lack of motile sperm, it only was complete when the fathers were from the Baja population. On the other hand, when the mothers were from Baja, up to half of the F1 sons had motile sperm, depending upon the paternal strain. Just having motile sperm, however, does not mean that these males are fertile (Civetta & Gaudreau 2015). In all likelihood, their fertility is at least reduced if not absent. They would have to be crossed to females to assess their ability to reproduce in the face of low numbers of motile sperm.

Asymmetry in reproductive isolation is common, especially in diverging *Drosophila* populations or newly evolved species (Coyne and Orr 1989, 1997; Pesgraves & Orr 1998, Zeng & Singh 1993), but in this case, there is sterility in both directions. Asymmetry in postzygotic isolation is a common pattern in many systems including other invertebrates (Muller 1942, Oliver 1978) as well as vertebrates (Good *et al*. 2008), where interspecific reciprocal crosses produce different levels of hybrid male sterility or inviability. This general pattern can be explained by between-locus "Bateson-Dobzhansky-Muller incompatibilities" (BDMIs) in which the accumulation of epistatic interactions between alleles results in hybrid dysfunction or incompatibility (Turelli & Orr 2001). BDMI's comprise two locus interactions, with incompatibilities arising between an ancestral allele and an allele that is derived in one lineage or between alleles that are derived in two separate lineages (Seehausen *et al*. 2014). Although the accumulation of BDMIs is not well understood, Turelli and Orr (1995, 2000) suggest that X-linked incompatibilities, cytonuclear incompatibilities and maternal effects are likely to play an important role in postzygotic isolation. There was only one case in which sperm motility was not completely asymmetrical. Although partial, Guerrero hybrid males had at least some motile sperm in both crosses (59 and 21% of motile sperm). These observations, combined with the phylogenetic and haplotype data, suggest that Guerrero and Baja may share a more recent common ancestor.

The geological history of the Baja California peninsula has been subject of many hypothesis (Murphy 1983, Riddle *et al*. 2000, Grismer 1994). The most traditional geological framework is described by Riddle *et al.* (2000) in which the

Baja California peninsula began to separate from the west coast of the Mexican mainland as a result of differential movements of the Pacific and North American plates, leading to the formation of the Sea of Cortez. Given the crosses between Guerrero and Baja yield hybrids with a low degree of sterility, coupled with the phylogenetic analysis (belonging to the same lineage), it could be possible that a population from the southwestern part of Mexico was isolated when the Baja peninsula began to form. Studies suggest that the Cape region was the last part of the Baja peninsula to separate from the mainland (Helenes & Carreño 1999) and that the presence of a trans-peninsular seaway separated the northern and southern part of the peninsula (e.g. Upton & Murphy 1998, Riddle *et al*. 2000). Such events could explain why *D. aldrichi* is only present in the southern part of the Baja peninsula as well as the possible origin of this population.

Ecological factors could also underlie genetic divergence between the populations in the Baja peninsula and those in the mainland. The Baja California peninsula harbors over 80 species of cacti (Guzmán *et al*. 2007), which includes a wide variety of growth forms such as globose cacti (*Mammilaria spp.*), prickly pear (*Opuntia spp*.), and columnar cacti (*Pachycereus spp.* and *Stenocereus spp.*), among others (Prado, *et al*. 2010). In this case, it is possible that the use of multiple host cacti could be correlated with the divergence of the *D. aldrichi* lineages. As mentioned before, *D. aldrichi* utilizes *Opuntia* spp. as its host plant, although there are anecdotal reports of associations with columnar cacti *Pachycereus weberi* in Cañón del Zopilote, Guerrero and *Myrtillocactus geometrizans* in Tehuacan, Puebla (Oliveira et al. 2012). To date, there are no

rearing records for *D. aldrichi* from the Baja California peninsula, so we don't know if they are utilizing columnar or *Opuntia* cacti or any other cacti species. Regardless, observations by Heed indicate that *D. aldrichi* from Baja California utilize *Opuntia* spp. (William Heed, unpublished). Additional field studies of the resources used by *D. aldrichi* in different parts of its range, especially those areas from which the flies show reproductive isolation, would be informative as to the role of host use in the evolution of the apparently cryptic species.

The genetic distances between *D. aldrichi* populations correlate with the degree of reproductive isolation observed. As mentioned previously, both prezygotic and postzygotic reproductive isolation increase gradually with the genetic distance (Coyne and Orr 1989, 1997). This pattern is consistent in all the crosses with Baja, with most of the mainland populations showing high genetic distance and both premating and postzygotic reproductive isolation. In the case with Guerrero, the small genetic distance observed also correlates with the relatively small degree of reproductive isolation, for there is premating isolation but postzygotic isolation does not exhibit the expected asymmetrical pattern. This could also be due to the possible origin of the Baja population since they are more closely related. Also consistent with the phylogenetic data and reproductive isolation tests, genetic distance in comparisons among the mainland populations was small. In fact, some population comparisons show no differences among them (e.g. Huatulco–Oaxaca). Regardless, the genetic distance between Guerrero/Texas and the remaining mainland populations was high compared to the rest. Additionally, the genetic distances observed between Guerrero and all other

mainland populations do not correlate with the degree of reproductive isolation, since there is no behavioral or F1 male sterility among crosses.

Future studies should include sequencing of neutral nuclear genes to attain a finer resolution of the evolutionary relationships among these and additional *D. aldrichi* populations. Investigation of reproductive isolation among additional populations, such as one from Texas, also should be performed, provided by a living culture that can be established again from a new collection. The fertility of hybrid males with even a low level of motile sperm should be examined in actual crosses, and other types of reproductive isolation, such as postmating but prezygotic, would be of interest.

CONCLUSION

My study is the first to place reproductive isolation data for *D. aldrichi* in a phylogenetic framework and suggests that there are two cryptic species or they are in the process of becoming two different species. The *D. aldrichi* mtDNA phylogenetic analysis and a TCS haplotype network revealed significant genetic differentiation between the strains from the Baja California peninsula, Guerrero, and Texas and those from the mainland. The level of genetic differentiation between the strains from Baja California and the mainland is indicative that the Sea of Cortez provides a strong barrier to gene flow. Consistent with the genetic diversification, both premating and postzygotic reproductive isolation exists between *D. aldrichi* from Baja and those from mainland Mexico. Postzygotic isolation is effectively complete when males are from Baja and strong in the reciprocal cross. Behavioral isolation is significant between Baja and mainland populations though not strong enough to prevent gene flow. The existence of sperm immotility in reciprocal crosses suggests that the flies from Baja have been reproductively isolated for a longer period than originally thought, and are very likely a different species or close to becoming different species, although cryptic, from at least some of the populations from the southern mainland.

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