The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2013, ••, ••–••. With 3 figures

Genetic differentiation, speciation, and phylogeography of cactus flies (Diptera: Neriidae: *Odontoloxozus*) from Mexico and south-western USA

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Received 27 March 2013; revised 24 April 2013; accepted for publication 24 April 2013

Nucleotide sequences from the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, comprising the standard barcode segment, were used to examine genetic differentiation, systematics, and population structure of cactus flies (Diptera: Neriidae: *Odontoloxozus*) from Mexico and south-western USA. Phylogenetic analyses revealed that samples of *Odontoloxozus* partitioned into two distinct clusters: one comprising the widely distributed *Odontoloxozus longicornis* (Coquillett) and the other comprising *Odontoloxozus pachycericola* Mangan & Baldwin, a recently described species from the Cape Region of the Baja California peninsula, which we show is distributed northward to southern California, USA. A mean Kimura two-parameter genetic distance of 2.8% between *O. longicornis* and *O. pachycericola*, and eight diagnostic nucleotide substitutions in the COI gene segment, are consistent with a species-level separation, thus providing the first independent molecular support for recognizing *O. pachycericola* as a distinct species. We also show that the only external morphological character considered to separate adults of the two species (number of anepisternal bristles) varies with body size and is therefore uninformative for making species assignments. Analysis of molecular variance indicated significant structure among populations of *O. longicornis* from three main geographical areas, (1) Arizona, USA and Sonora, Mexico; (2) Santa Catalina Island, California, USA; and (3) central Mexico (Querétaro and Guanajuato), although widely-separated populations from Arizona and Sonora showed no evidence of structure. A TCS haplotype network showed no shared haplotypes of *O. longicornis* among the three main regions. The potential roles of vicariance and isolation-by-distance in restricting gene flow and promoting genetic differentiation and speciation in *Odontoloxozus* are discussed. *Biological Journal of the Linnean Society*, 2013, ••, ••–••.

ADDITIONAL KEYWORDS: dispersal – haplotype network – *O. longicornis* – *O. pachycericola* – population structure.

INTRODUCTION

The insect order Diptera is well-represented in the specialized microhabitat formed of necrotic tissue in columnar cacti from the Sonoran Desert of North America. Five species of *Drosophila* (Drosophilidae), referred to collectively as the 'cactophilic *Drosophila*', are common inhabitants of Sonoran Desert cactus necroses, and indepth studies on these species have provided important insights into evolutionary processes and speciation in the harsh and variable desert environment (Pfeiler & Markow, 2011; O'Grady & Markow, 2012). Also associated with cactus necroses

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are flies in the genus *Odontoloxozus* (family Neriidae), commonly referred to as 'cactus flies' (Olsen & Ryckman, 1963; Ryckman & Olsen, 1963; Mangan, 1979, 1984). Although not as abundant or as well studied as the cactophilic *Drosophila*, neriid flies form an important component of the community of cactophilic arthropods, which includes approximately 40 arthropod species, mostly insects, comprising 23 families and ten orders (Castrezana & Markow, 2001). Because the host cacti form discrete patches for the resident arthropods, genetic studies can be highly informative about the role of ecological and geographical variables in their evolution. Thus, our laboratories are undertaking broader studies aiming to examine evolutionary relationships, speciation, and population biology in this interesting group of cactophilic flies along with other cactophilic arthropod species.

The Neriidae is a relatively small family of dipterans, containing approximately 110 species and 19 genera found worldwide, mainly in the tropics (Cresson, 1938; Mello & Ziegler, 2012). Four genera are known from North America (including Central America): *Odontoloxozus* Enderlein, *Glyphidops* Enderlein, *Nerius* Fabricius and *Cerantichir* Enderlein (Buck, 2010), with only a single species, *Odontoloxozus longicornis* (Coquillett), currently recorded for the USA (Olsen & Ryckman, 1963). An older record of *Glyphidops* (*Oncopsia*) *flavifrons* (Bigot), reported as *Nerius flavifrons*, from Tucson, Arizona (Hubbard, 1899), probably represented *O. longicornis* (Olsen & Ryckman, 1963). *Odontoloxozus longicornis* is widely distributed in North America, being found from south-western USA to Costa Rica (Cresson, 1930, 1938; Ryckman & Olsen, 1963). *Odontoloxozus pachycericola* Mangan & Baldwin from the Cape Region of the Baja California peninsula, Mexico, is the only other member of the genus currently known from the Americas. The previously recognized *Odontoloxozus peruanus* Hennig from Central and South America (Peru and Bolivia) has been reassigned to the genus *Cerantichir* (Buck, 2010).

Within the Sonoran Desert, *O. longicornis* has been found in necrotic tissue of a variety of columnar cacti, including saguaro (*Carnegiea gigantea*), cardón (*Pachycereus pringlei*), etcho (*Pachycereus pecten-aboriginum*), pitahaya agria (*Stenocereus gummosus*) organ pipe (pitahaya dulce; *Stenocereus thurberi*) and senita (*Lophocereus schottii*) (Ryckman & Olsen, 1963; Mangan & Baldwin, 1986; T.A.M., pers. observ.). Although *O. longicornis* has been collected and reared on other substrates, especially papaya (Ryckman & Olsen, 1963; Steyskal, 1965), it is most commonly associated with the rotting cactus microhabitat for breeding and feeding in the wild (Ryckman & Olsen, 1963). A population of *O. longicornis*, which is also present on Santa Catalina Island off the southern California (USA) coast, utilizes rotting pads of prickly pear cactus (*Opuntia* spp., including *Opuntia littoralis*). In addition, populations of *O. longicornis* are found throughout mainland southern California, and have been reported as far north as Santa Clara County (Ryckman & Olsen, 1963). The eastern limit of its range in the USA is southern Texas (Ryckman & Olsen, 1963; Mangan & Baldwin, 1986). Only minor differences in coloration and size occur in populations of *O. longicornis* from central Mexico and Costa Rica compared to populations from south-western USA, and it is considered that these populations represent *O. longicornis* (Cresson, 1930, 1938; Olsen & Ryckman, 1963). Genetic comparisons of *O. longicornis*, however, have only been conducted on two populations from the mainland Sonoran Desert (Pfeiler *et al*., 2009a). These preliminary studies suggested little genetic divergence and high gene flow of *O. longicornis* between populations at Tucson, Arizona, USA, and Guaymas, Sonora, Mexico. The relationships among the Sonoran Desert populations and those from southern California, USA, and central Mexico, however, have not been studied.

In the original description of *O. pachycericola*, Mangan & Baldwin (1986) reported that samples collected on senita and cardón near La Paz, Baja California Sur, Mexico, differed from *O. longicornis* in several traits, including adult and larval morphology, cytology, and mating behaviour. They stated that the two cryptic species '. . . are easily separated by different numbers of bristles (with dark patches at their base) on the lateral thoracic area of adults. Third stage larvae and puparia are separated by differing numbers of papillae on the anterior spiracles'. Additionally, Mangan & Baldwin (1986) showed that *O. pachycericola* hybridized in the laboratory with individuals taken from several known populations of *O. longicornis* in the Sonoran Desert and farther south at Mazatlán, Sinaloa, Mexico. These crosses produced sterile progeny, further supporting the view that they are distinct species. It is generally assumed that *O. pachycericola* is restricted to the peninsular Cape Region, although the limits of its geographical distribution and the putative genetic differences between it and *O. longicornis* have never been investigated.

In the present study, we used mitochondrial (mt)DNA sequence data from a segment of the cytochrome *c* oxidase subunit I (COI) gene to investigate genetic differentiation among populations of *Odontoloxozus* over a wide geographical area, from south-western USA to central Mexico. We provide the first molecular genetic evidence supporting the recognition of *O. pachycericola* as a distinct species-level taxon, and show that its range extends northward

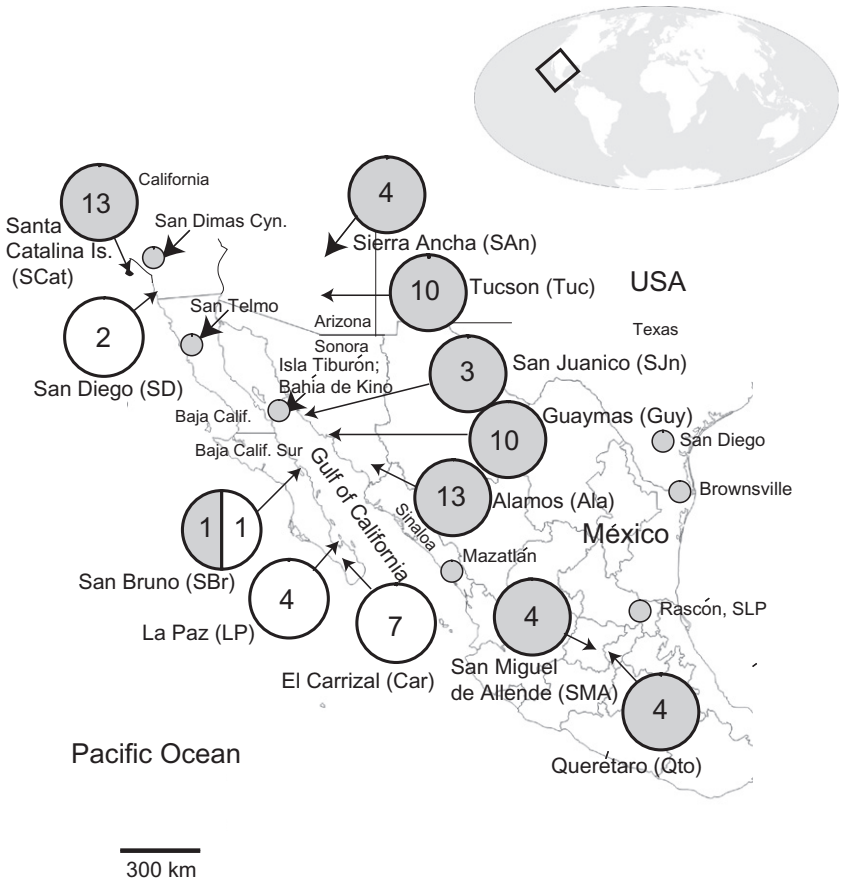

Figure 1. Map showing collecting localities for *Odontoloxozus* in Mexico and south-western USA. Large circles show the number of individuals of *Odontoloxozus longicornis* (grey) and *Odontoloxozus pachycericola* (white) collected at each locality as determined from molecular analyses. Locality abbreviations are shown in parentheses. The small grey circles indicate the localities mentioned in the text where *O. longicornis* has been identified in previous studies using morphological characters; SLP, San Luis Potosí.

from the Cape Region into southern California, USA. We also examined the single external morphological difference reported to separate adult *O. pachycericola* and *O. longicornis* and show that this character (number of anepisternal bristles) varies with body size and therefore is uninformative for taxonomic assignments. In addition, we show that substantial genetic differentiation exists in *O. longicornis* from central Mexico compared to other regions, which, taken together with minor colour differences, may indicate incipient speciation.

MATERIAL AND METHODS

SAMPLES

The sample consisted of 76 *Odontoloxozus* collected as larvae or adults from rotting tissue of a variety of cactus species, and artificial baits made from bananas and live yeast, from four localities in the south-western USA, including Santa Catalina Island, and eight localities in Mexico, including the Baja California peninsula (Fig. 1). Flies from the Sonoran Desert [the Sierra Ancha (Gila County) and Tucson, AZ; San Juanico and Guaymas, Sonora and Baja California Sur] and Santa Catalina Island were collected on cactus species or using artificial baits. One of the collecting localities, Alamos, Sonora, is located in a tropical dry forest biome approximately 220 km south-east of the southern limit of the Sonoran Desert near Guaymas. This population shows close genetic similarity with other populations from the mainland Sonoran Desert and is included within this region in the present study. Flies from central Mexico [San Miguel de Allende, Guanajuato and the northern outskirts (Juriquilla) of Querétaro, Querétaro] were collected on cacti (*Stenocereus queretaroensis* and *Myrtillocactus geometrizans*) or artificial baits. Although our samples sizes were relatively small, this can be attributed more to the ecology of the flies rather than our collection effort. As noted by

Ryckman & Olsen (1963), *Odontoloxozus* '. . . is not readily collected in the field by conventional means . . .'. Most of the records given in Ryckman & Olsen (1963) were obtained by rearing out adults from sections of rotting cacti taken in the field, a procedure that often is impractical when conducting field work for extended periods.

Coquillett (1904) originally described *O. longicornis* as *Nerius longicornis* (type locality: San Diego, Duval County, Texas). The type series included material from Brownsville, Texas, Tucson, Arizona, and Los Angeles County, California. Our samples of *O. longicornis* were collected at (Tucson), or near (Santa Catalina Island), two of the localities in the type series (Fig. 1). In addition, our samples of *O. pachycericola* included flies collected at two Cape Region sites (La Paz and El Carrizal, BCS) (Fig. 1) located near the type locality of El Centenario, BCS, 15 km west of La Paz (Mangan & Baldwin, 1986).

Counts and measurements

Counts of anepisternal bristles were conducted on adult *Odontoloxozus* as described by Mangan & Baldwin (1986). Thoracic length, used as a proxy of adult body size, was measured to the nearest 0.25 mm with an ocular micrometer.

DNA extraction and sequence analysis

Total genomic DNA was extracted from tissue samples using the DNeasy (Qiagen Inc.) protocol. The polymerase chain reaction (PCR) was used to amplify a segment of the COI gene, also known as the COI barcode segment (Ratnasingham & Hebert, 2007), using the primer pairs LCO1490f/HCO2198r and standard assay conditions (Folmer *et al*., 1994). Sequencing reactions were performed on an Applied Biosystems ABI 3730XL DNA sequencer at the DNA Sequencing Facility, University of Arizona, using the PCR primers. Sequences were proofread and aligned in ClustalX, version 1.81 (Thompson *et al*., 1997) followed by manual editing. Sequences were trimmed to remove ambiguous sites, resulting in a final segment of 639 bp. This COI segment corresponds to nucleotide positions 1515–2153 in the complete mitochondrial genome of *Drosophila yakuba* (GenBank accession number NC001322). Aligned sequences were translated in MEGA, version 5.0.5 (Tamura *et al*., 2011). No frameshifts or stop codons were found in any of the sequences, and mean CG content was 33.7%, suggesting that our sequences represent mtDNA and are not nuclear mitochondrial pseudogenes (numts), which have been reported for the COI gene in insects (Song *et al*., 2008). The sequences of all COI haplotypes have been deposited in GenBank under accession numbers FJ532245–FJ532254 and KC660943–KC660967.

Calculations of Kimura (1980) two-parameter genetic distances (d) among sequences were carried out in MEGA. Calculations of genetic diversity indices were performed in DnaSP, version 5.00.07 (Librado & Rozas, 2009). Neutrality tests, Tajima's (1989) D and Fu's (1997) F_S, were performed in ARLEQUIN, version 3.5.1.3 (Excoffier & Lischer, 2010). Fu's Fs test is also useful for detecting signatures of population expansions, which lead to large negative values in the test statistic (Fu, 1997; Ramos-Onsins & Rozas, 2002). The significance of F_S at the 0.05 level is indicated when $P < 0.02$ (Excoffier & Lischer, 2010).

Genetic differentiation and population structure

Relationships among COI haplotypes were initially assessed with Bayesian inference implemented in MrBayes, version 3.1 (Huelsenbeck & Ronquist, 2001). The model of nucleotide substitution that best fit the data set, determined with Modeltest, version 3.7 (Posada & Crandall, 1998) using the Akaike information criterion, was TrN + I (Tamura & Nei, 1993). Bayesian analyses were run under the parameters of the above model (nst = '2'; rates = 'propinv') for 5 000 000 generations, sampled every 250th generation (20 000 trees sampled), using the default random tree option to begin the analysis. The neriid *Glyphidops dispar* (Cresson) was used as the outgroup. We also constructed haplotype networks for *O. longicornis* and *O. pachycericola* using statistical parsimony implemented in TCS, version 1.21 (Clement, Posada & Crandall, 2000). The connection limit among haplotypes was set to the default value of 95%, unless indicated otherwise. Because genetic differentiation among COI haplotypes within species was low, the results of only the more informative haplotype networks are presented.

Analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) performed in ARLEQUIN was used to test for population structure in *O. longicornis* for populations with $N \geq 4$. The calculation of the significance of pairwise comparisons of the fixation index Φ_{ST} using a Bonferroni correction (Rice, 1989) was based on 10 000 permutations of the data matrix. Pairwise estimates of the number of migrants per generation (N_m) among populations assumed to be in mutation–drift equilibrium were also calculated in ARLEQUIN. Samples from the nearby localities (approximately 50 km apart) of San Miguel de Allende and Querétaro (Fig. 1) were combined for the AMOVA.

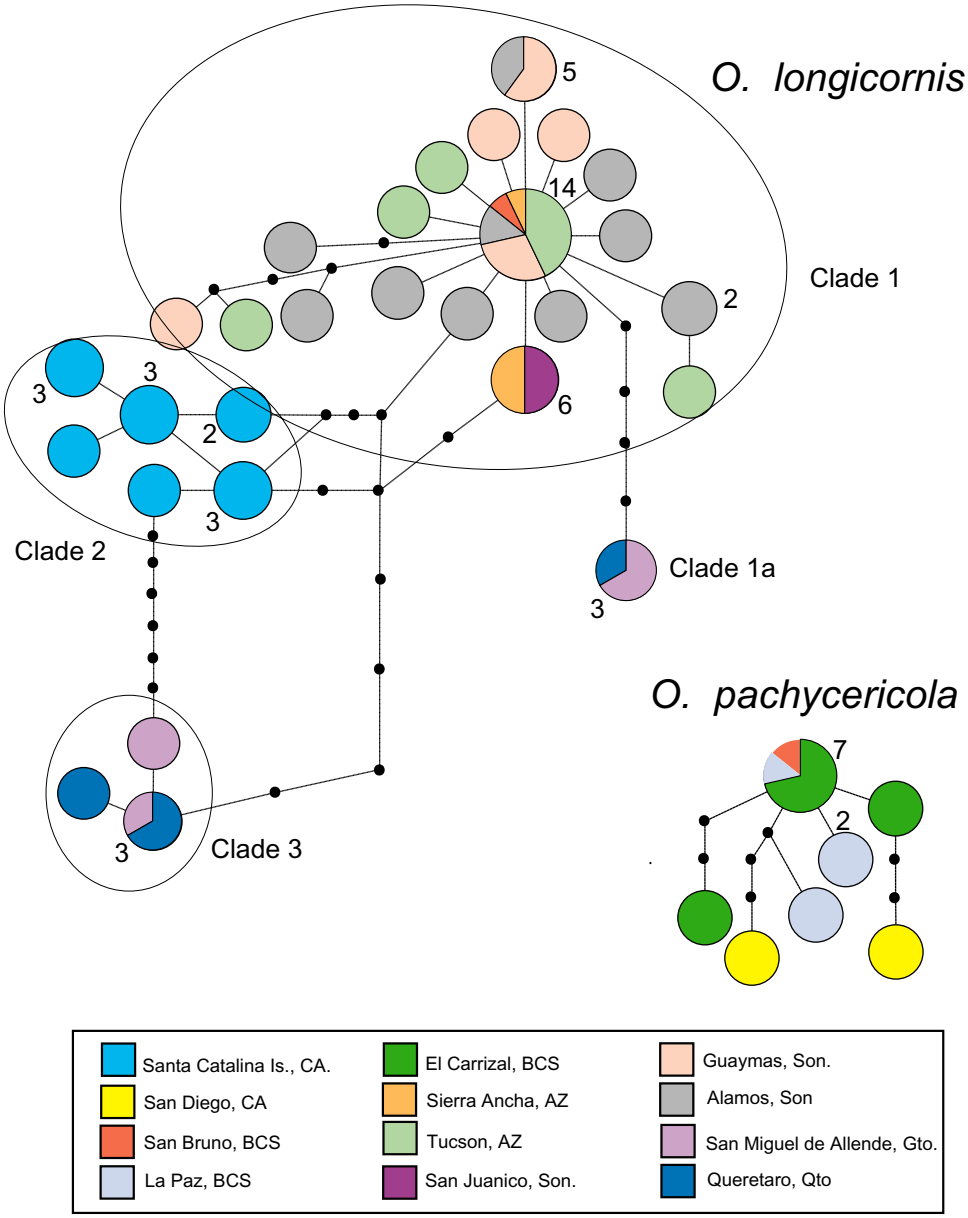

Figure 2. TCS haplotype networks for the cytochrome *c* oxidase subunit I (COI) gene in *Odontoloxozus longicornis* and *Odontoloxozus pachycericola* based on a 95% connection limit. Geographical distributions of haplotypes are colour-coded. Each line segment represents a single mutation. Inferred intermediate haplotypes that were not sampled are shown as black dots. The size of the circles is proportional to haplotype frequency. Numbers next to the circles represent number of individuals with that haplotype, if greater than one.

RESULTS

GENETIC DIFFERENTIATION

Relationships among COI haplotypes in *Odontoloxozus* based on Bayesian analysis revealed two main partitions (not shown): one comprised individuals from the peninsular Cape Region, which were assigned to *O. pachycericola*, and the other comprised the widespread *O. longicornis*. Within *O. longicornis*, three clades (clades 1, 2, and 3) and one subclade (clade 1a) were resolved.

The TCS analyses showed that haplotypes of *O. longicornis* and *O. pachycericola* resolved in separate networks at the 95% connection limit, as expected for distinct species-level taxa (Fig. 2). In addition to being present in the peninsular Cape Region, haplotypes of *O. pachycericola* were also found at San Bruno in northern Baja California Sur

and at San Diego, California. The TCS haplotype network in *O. longicornis* revealed a pattern congruent with the partitioning of clades based on the Bayesian tree. Clade 1 of *O. longicornis* was distributed throughout the Sonoran Desert and adjacent regions, being found from the Sierra Ancha north-east of Phoenix, Arizona, to Alamos in southern Sonora, although it was absent from the Cape Region (Figs 1, 2). A single individual of clade 1 was also found on the Baja California peninsula at San Bruno. The fourteen singleton haplotypes in clade 1 were all closely related, forming a star-like pattern consistent with evidence for a population expansion reported previously (Pfeiler *et al*., 2009a). Clade 2 consisted of six haplotypes entirely restricted to Santa Catalina Island. The mean genetic distance between clades 1 and 2 was low ($d = 0.9\%$). The three haplotypes comprising clade 3 from central Mexico were also unique and not closely related to those in the other populations, being separated by seven mutational steps. Clade 3, although connected to the other *O. longicornis* haplotypes at the 95% connection limit, formed a separate haplotype network when the connection limit was increased to 98%, suggesting that another distinct cryptic lineage of *Odontoloxozus* may be present in central Mexico. Also, five mutational steps separated the central Mexico clade 1a from the widespread clade 1. Individuals from clades 1a and 3 were found to occur sympatrically in central Mexico (Querétaro and San Miguel de Allende). The mean genetic distances among all clades of *O. longicornis* were low ($d \leq 1.5\%$).

Odontoloxozus pachycericola differed from *O. longicornis* (all clades combined) at eight diagnostic nucleotide positions in the 639-bp COI segment (Table 1). The diagnostic nucleotide positions for *O. pachycericola* were: 40(A), 154(C), 226(T), 358(G), 400(C), 457(T), 571(C), and 578(T). Within the *O. longicornis* lineage, two diagnostic substitutions were found in clades 2 and 3, and three diagnostic substitutions characterized clade 1a.

Genetic diversity indices and the results of neutrality tests (Tajima's D and Fu's F_S) for COI in *O. longicornis* and *O. pachycericola* are shown in Table 2. Haplotype diversity was high, and nucleotide diversity was low, in both species. Except for *O. longicornis* clade 1, Tajima's D was not significant. A significant value for Fu's F_S was seen only for clade 1 in *O. longicornis* and when all clades were combined. The mean genetic distance between *O. longicornis* and *O. pachycericola* was $d = 2.8\%$.

Table 1. Diagnostic nucleotide sites in the mitochondrial cytochrome *c* oxidase subunit I (COI) gene fragment in *Odontoloxozus pachycericola* and *Odontoloxozus longicornis*

		Odontoloxozus longicornis			
COI site	*Odontoloxozus pachycericola*	Clade 1	1a	2	3
40	A*	T	T	T	T
82	C	C	C	T*	C
154	C*	T	T	T	T
193	T	T	C*	T	T
226	T*	C	C	C	C
235	C	C	C	C	T*
358	G*	A	A	A	A
382	T	T	C*	T	T
400	C*	T	T	T	T
421	T	T	T	T	C*
457	T*	C	C	C	C
571	C*	T	T	T	T
578	T*	C	C	C	C
622	T	T	T	C*	T
631	A	A	G*	A	A

*Denotes diagnostic nucleotide substitutions, all of which were synonymous. All sites are at the third codon position, except site number 578 (first codon position).

Counts and measurements

The mean number of anespisternal bristles in adult *O. longicornis* from Alamos, Sonora (clade 1) agreed closely with mean values reported by Mangan & Baldwin (1986) for flies from Bahía de Kino, Sonora and Mazatlán, Sinaloa (Table 3). Counts for *O. pachycericola*, however, although showing a mean value less than seen in *O. longicornis*, were more variable than reported by Mangan & Baldwin (1986) and overlapped those of *O. longicornis*.

The incongruence between our molecular results and the reported diagnostic external morphological character for separating adult *O. pachycericola* and *O. longicornis* prompted us to examine this character in more detail. We counted the number of anepisternal bristles in a sample of flies of both species that differed in body size. The results obtained (Fig. 3) revealed that the number of bristles was significantly related to body size as measured by thorax length. In this analysis, we combined data for both species to produce a single linear regression. Figure 3 shows that only the two smallest individuals of the nine *O. pachycericola* identified by molecular data possessed the number of bristles ($N = 17$–25) considered diagnostic for this species (Mangan & Baldwin, 1986).

Population structure

The AMOVA showed no significant structure among populations of *O. longicornis* from Guaymas, Tucson,

Table 2. Summary of genetic diversity indices and results of neutrality tests (Tajima's *D* and Fu's F_S) in the cytochrome *c* oxidase subunit I (COI) gene segment in *Odontoloxozus longicornis* and *Odontoloxozus pachycericola*

Species	*N*	*L*	*k*	*K*	*h* (±SD)	π (±SD)	Tajima's *D*	Fu's F_S
Odontoloxozus longicornis	62	639	35	28	0.930 ± 0.021	0.0072 ± 0.0007	–1.29	–13.96*
Clade 1**	44	639	25	19	0.873 ± 0.039	0.0035 ± 0.0006	–2.06*	–13.02*
Clade 2	13	639	5	6	0.872 ± 0.054	0.0023 ± 0.0003	–0.39	–2.05
Clade 3	5	639	2	3	0.700 ± 0.218	0.0013 ± 0.0005	–0.97	–0.83
Odontoloxozus pachycericola	14	639	10	7	0.758 ± 0.116	0.0035 ± 0.0009	–1.15	–1.76

N, number of sequences; *L*, sequence length (bp); *k*, number of variable sites; *K*, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity. *Significant at the 0.05 level ($P \leq 0.001$ for Fu's F_S). **Includes the three individuals from clade 1a (Fig. 2).

Table 3. Comparison of number of anepisternal bristles in adults of *Odontoloxozus pachycericola* and *Odontoloxozus longicornis*

Species	Locality	Mean	Range	SD	*N*	Reference
Odontoloxozus pachycericola	BCS; San Diego, California	31.78	18–43	8.90	9	Present study
Odontoloxozus pachycericola	La Paz, BCS	20.82	17–25	3.12	28	Mangan & Baldwin (1986)
Odontoloxozus longicornis (clade 1)	Alamos, Sonora	42.67	30–56	10.95	6	Present study
Odontoloxozus longicornis (clade 3)	Querétaro, Querétaro	26			1	Present study
Odontoloxozus longicornis	Mazatlán, Sinaloa	43.80	Not given	6.13	20	Mangan & Baldwin (1986)
Odontoloxozus longicornis	Bahía de Kino, Sonora	46.59	Not given	7.28	23	Mangan & Baldwin (1986)
Interspecific hybrids		23.42	Not given	4.31	7	Mangan & Baldwin (1986)

N, number of individuals; BCS, Baja California Sur, Mexico.

Alamos, and the Sierra Ancha (Table 4). The population from Santa Catalina Island, however, showed significant structure compared to all other populations ($\Phi_{ST} \geq 0.75$). The population from central Mexico (Qto/SMA) also showed significant structure compared to all other populations ($\Phi_{ST} \geq 0.72$). Overall Φ_{ST} was 0.67 ($P < 0.0001$), indicating that only 33% of the total variability occurred within populations. The sample size for *O. pachycericola* was too small to obtain a reliable estimate of population structure over the geographical range of this species.

DISCUSSION

Speciation and geographical distribution of *O. pachycericola*

In the present study, we provide the first molecular evidence, based on a 639-bp segment of the COI barcode region, supporting the species status of *O. pachycericola*. Although the pitfalls and limitations of using DNA barcodes in species identifications have been widely discussed (Goldstein & DeSalle, 2010; Yassin *et al.*, 2010), we show that eight diagnostic nucleotide substitutions in the COI barcode segment separate the cryptic *O. pachycericola* and *O. longicornis* (Table 1), and thus provide important characters for species determination. We also show that the only external morphological character reported to separate adult *O. pachycericola* and *O. longicornis*, a lower number of thoracic bristles with dark patches at their bases (anepisternal spots) (Mangan & Baldwin, 1986), varies with body size in both species and thus is uninformative for making species assignments. The only other external morphological character that separates the two species is a lower number of papillae on the anterior spiracles of third-instar larvae of *O. pachycericola* (Mangan & Baldwin, 1986). Although morphological variation of genitalia is routinely used in insect taxonomy for distinguishing taxa at both the species and subspecies level (Pfeiler *et al.*, 2009b; Richmond, Johnson & Markow, 2012), no interspecific differences in male or female genitalia have been found in *O. pachycericola* and *O. longicornis* (Mangan & Baldwin, 1986). Thus,

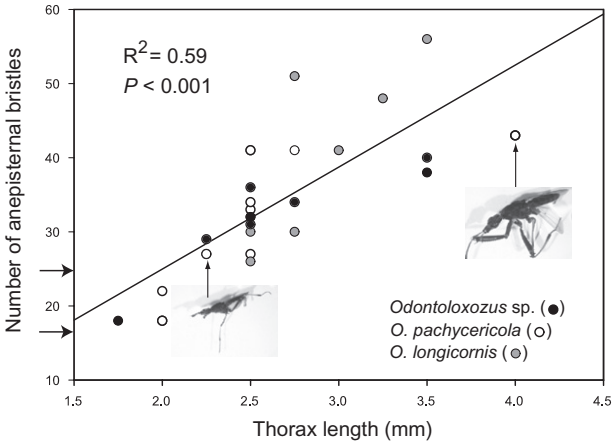

Figure 3. Linear regression of number of anepisternal bristles against thorax length in 24 specimens of *Odontoloxozus*. Species assignments were based on diagnostic nucleotide substitutions in the cytochrome *c* oxidase subunit I (COI) gene segment (Table 1). Values for *Odontoloxozus* sp. (black dots) represent specimens from Santa Catalina Island that had not been analyzed for COI. Horizontal arrows show range of values (17–25) for number of bristles reported as diagnostic in the original description of *Odontoloxozus pachycericola* (Mangan & Baldwin, 1986). Insets are photographs shown to scale of two male specimens of *Odontoloxozus pachycericola* from El Carrizal, Baja California Sur (vertical arrows indicate corresponding data points for each fly) to emphasize the differences in body size.

COI barcodes are presently the only diagnostic characters capable of separating adults of the two species of *Odontoloxozus*.

The unexpected finding of two individuals of *O. pachycericola* at San Diego in southern California, together with a single specimen from northern Baja California Sur (San Bruno), greatly expands the known range of this species, which had been assumed to be restricted to the Cape Region of Baja California Sur (Mangan & Baldwin, 1986). In contrast to the findings of Mangan & Baldwin (1986), who did not detect zones of sympatry for *O. pachycericola* and *O. longicornis*, we recorded single individuals of each species at San Bruno (Fig. 1), probably as a result of secondary contact after speciation (see below). Each of the two San Bruno flies possessed the common haplotype for that species (Fig. 2). Additional sampling along the Baja California peninsula and in southern California will be required to assess the sympatric distribution of the two species, although there is evidence suggesting that *O. longicornis* may be widely distributed on the peninsula and islands of the Gulf of California. Mangan & Baldwin (1986) examined morphology of larvae of *Odontoloxozus* from San Esteben ($N = 8$) and Tiburón islands ($N = 2$) in the upper Gulf of California and assigned them to *O. longicornis*. They also examined four adults from San Telmo in the northern region of the Baja California peninsula (Fig. 1), which were identified as *O. longicornis*, although this assignment is now questionable based on our morphological results. Although we lacked samples of *O. longicornis* from mainland southern California, Mangan & Baldwin (1986) concluded that spiracles shown in larval figures from specimens collected in Los Angeles County (Olsen & Ryckman, 1963) were characteristic of *O. longicornis*.

Our results support the conclusions of Mangan & Baldwin (1986) suggesting that speciation in *O. pachycericola* was allopatric and that host plant specializations are unlikely explanations for the genetic isolation of *O. pachycericola* and *O. longicornis*. Four species of columnar cacti known to be hosts of *Odontoloxozus*, cardón, etcho, organ pipe, and senita occur in both the Cape Region and mainland Sonora (Turner, Bowers & Burgess, 1995). A more likely explanation is that genetic diversification leading to speciation resulted from vicariance and reduced gene flow owing to the geographical separation of the peninsula from the mainland during the formation of the Gulf of California during the late Miocene and Pliocene (Carreño & Helenes, 2002; Ledesma-Vázquez & Carreño, 2010). Recent work has implicated the formation of the Gulf of California as being an important factor in genetic diversification and speciation of a variety of arthropods dependent upon the necrotic cactus microhabitat (Pfeiler & Markow, 2011). Our results are also consistent with a scenario in which speciation of *O. pachycericola* occurred in the Cape Region of the Baja California peninsula, with subsequent northward migration into southern California, USA. Finding two unique haplotypes of *O. pachycericola* in the two individuals from San Diego (Fig. 2) also suggests that these individuals were not introduced from the peninsular Cape Region by recent human transport. The lack of records for *O. pachycericola* from the mainland Sonoran Desert or the Midriff Islands in the upper Gulf of California suggests that this species may be unable to utilize these islands as 'stepping stones' (Pfeiler & Markow, 2011) to disperse from the peninsula to mainland Sonora.

PHYLOGEOGRAPHY OF *O. LONGICORNIS*

Odontoloxozus longicornis showed no significant population structure throughout the mainland Sonoran Desert (including Alamos, Sonora) in agreement with preliminary findings on populations from Tucson and Guaymas (Pfeiler *et al*., 2009a). Geographical distance between the northernmost collection site in the Sierra Ancha, Arizona, near the

Table 4. Pairwise comparisons of Φ_{ST} (below the diagonal) and number of migrants per generation (N_m; above the diagonal) for populations of *Odontoloxozus longicornis* based on cytochrome *c* oxidase subunit I (COI) sequences

	USA			Mexico		
	SCat (13)	SAn (4)	Tuc (10)	Guy (10)	Ala (13)	Qto/SMA (8)
SCat	–	0.11	0.15	0.15	0.17	0.16
SAn	0.818*	–	1.61	1.46	2.12	0.20
Tuc	0.770*	0.237	–	122.5	inf	0.18
Guy	0.769*	0.256	0.004	–	inf	0.18
Ala	0.750*	0.191	–0.014	–0.010	–	0.18
Qto/SMA	0.760*	0.716*	0.739*	0.738*	0.732*	–

Significant pairwise Φ_{ST} values after a sequential Bonferroni correction ($\alpha = 0.003$) are indicated by asterisks. The number of individuals from each locality is shown in parentheses. inf, N_m infinite and undefined. For locality abbreviations, see Figure 1. The nearby localities of Querétaro (Qto) and San Miguel Allende (SMA) in central Mexico were combined for the analysis and contained individuals from clade 1a ($N = 3$) and clade 3 ($N = 5$). The pairwise comparisons between Guy and Tuc are taken from Pfeiler *et al*. (2009a).

northern limit of the Sonoran Desert, and Alamos is approximately 850 km. AMOVA, however, indicated significant structure in populations of *O. longicornis* from Santa Catalina Island and from central Mexico (Table 4). The six haplotypes of *O. longicornis* from Santa Catalina Island (clade 2) were unique and were not shared with any other population (Fig. 2). The mean genetic distance between clades 1 and 2 ($d = 0.9\%$) suggests that they have been isolated since the mid-Pleistocene, assuming a standard COI molecular clock of 2.3% pairwise sequence divergence per million years in insects (Brower, 1994). The genetic divergence between clades 1 and 2 is similar to that reported for the cactophilic *Drosophila mojavensis wrigleyi* Castrezana, which also utilizes *Opuntia* as a host on Santa Catalina Island, and three other described subspecies of *D. mojavensis* Patterson and Crow from the Sonoran Desert (uncorrected *p*-distance = 1.2–1.8%) (Pfeiler *et al*., 2009b). As found for *O. longicornis*, no haplotypes of *D. mojavensis wrigleyi* were shared with the other subspecies (Reed, Nyboer & Markow, 2007; Richmond *et al*., 2013). Genetic differentiation in *D. mojavensis wrigleyi* has been attributed to ecological speciation resulting from a host plant shift after the colonization of Santa Catalina Island, rather than to a restriction of gene flow between the mainland and the island (Richmond *et al*., 2013). Because the closest mainland population of *O. longicornis* from southern California is known to use *Opuntia* as a host (Olsen & Ryckman, 1963), it is unlikely that a host plant shift occurred after island colonization from a mainland source population of *O. longicornis*. Therefore, it is most probable that the genetic divergence we found resulted from geographical isolation and an absence of gene flow owing to the 40-km wide San Pedro Channel that separates the island from mainland southern California.

Clade 3 from central Mexico was the most divergent clade of *O. longicornis* (Fig. 2). The geographical distance between Alamos and our southernmost site at Querétaro (approximately 1100 km) is similar to the distance from Alamos to the Sierra Ancha, a region in which populations show no structure. This suggests that a dispersal barrier, possibly the Sierra Madre Occidental, a major mountain range that separates populations of *O. longicornis* in northwestern Mexico from those in central Mexico, may have contributed to genetic diversification in clade 3. The mean genetic distance between clades 1a and 3 ($d = 1.5\%$), which occur sympatrically in central Mexico (Fig. 2), together with putative diagnostic interclade nucleotide substitutions (Table 1), suggests that they represent two separate genetic lineages of *O. longicornis* that have come into secondary contact. The genetic distance between the two clades is relatively large, although within the expected intraspecific value seen in haplotype networks connected at the 95% level (Chen *et al*., 2010). When the connection limit was increased from 95% to 98%, however, clade 3 resolved in a separate network.

Previous observations of minor external differences in *O. longicornis* from central Mexico and Costa Rica may be related to our genetic findings. Cresson (1930) examined flies from Higuito, Costa Rica and noted that they were larger than those from the USA. Olsen & Ryckman (1963) examined adults of *O. longicornis* reared from *Opuntia occidentalis* collected in San Dimas Canyon, Los Angeles County, California, and compared them with flies collected from several cacti,

including *Opuntia* sp., at Querétaro, Mexico. Olsen & Ryckman (1963) stated 'These two populations are *usually* [italics added] distinguishable by the black, glossy parafrons, epicephalas, and femurs of the Querétaro population in contrast to the light-brown coloration of the corresponding areas of the San Dimas population'. Although larger sample sizes will be required for confirmation, sympatric and genetically diverged clades (1a and 3) of *O. longicornis* in central Mexico (Querétaro and San Miguel de Allende), one of which (clade 1a) is more closely related to typical *O. longicornis* (clade 1), might explain the observations of Olsen & Ryckman (1963) with respect to why the external morphological differences between the US and central Mexico populations were not always apparent. The single clade 3 fly we measured for Table 3 had much darker (almost black) femurs and parafrons relative to specimens from clade 1. Enderlein (1922) described *O. punctulatus* from material collected in Raecon [sic] (= Rascón), San Luis Potosí, Mexico, approximately 200 km north-east of our collecting localities at Querétaro and San Miguel de Allende (Fig. 1). Cresson (1930), however, compared Enderlein's (1922) description with type material of *O. longicornis* and concluded that *punctulatus* should be placed as a junior synonym of *O. longicornis*. A syntype of *O. punctulatus* is deposited in the Natural History Museum in Berlin (ZMHM; Museum für Naturkunde Berlin) (Mello & Ziegler, 2012). Thus, a name and type specimen are available if further studies show that clade 3 represents a distinct species of *Odontoloxozus*.

Comparative phylogeography of cactophilic arthropods

Genetic diversification between peninsular and mainland populations of a number of species of arthropods associated with the necrotic cactus microhabitat, including cactophilic dipterans, coleopterans and a chernetid pseudoscorpion, has now been examined (Pfeiler & Markow, 2011). The results are beginning to provide a better overall picture of how the formation of the Gulf of California may have contributed to the evolution and speciation of organisms dependent upon the ephemeral necrotic cactus resource in the Sonoran Desert. Although the 80–200-km width of the Gulf represents a potential dispersal barrier for terrestrial arthropods, the Midriff Islands in the upper Gulf (including Tiburón, San Esteben and Angel de la Guarda, and several others) form trans-Gulf 'stepping stones' that could possibly aid dispersal of desert organisms (both animals and plants) between the mainland and peninsula. Several species of columnar cacti, including those utilized by *Odontoloxozus*, are found on these islands (Turner *et al*., 1995).

Genetic differentiation among mainland and peninsular populations of arthropods (both within species and between cryptic sister species) varies widely (Pfeiler & Markow, 2011), probably as a result of various factors, including (1) the period of time peninsular and mainland populations have been physically isolated; (2) differences in dispersal ability across open waters of the Gulf; (3) sea level changes associated with Pleistocene glaciations; and (4) differences in the degree to which organisms can take advantage of the Midriff Islands during dispersal. For example, at one extreme are the highly-vagile cactophilic *Drosophila nigrospiracula* Patterson and Wheeler and *Drosophila mettleri* Heed, which show no population structure between mainland and peninsular populations (Hurtado *et al*., 2004) and which are present on the Midriff Islands (Heed, 1978). At the other extreme, substantial genetic differentiation (d = 14.6%) is found between the cryptic sister species of histerid beetles, the peninsular *Iliotona beyeri* (Schaeffer) and the mainland *Iliotona dorcoides* (Lewis), which are assumed to have evolved from a common ancestor during the early stages of tectonic separation of the peninsula from the mainland, and have apparently remained restricted to their corresponding geographical regions (Pfeiler *et al*., 2010; E. Pfeiler, S. Johnson, M. P. Richmond & T. A. Markow, unpubl. data). Other cryptic sister taxa separated by the Gulf of California show much lower genetic divergences. These include *O. longicornis* and *O. pachycericola* (d = 2.8%: present study) and clade 1 (mainland) and clade II (peninsula) of the chernetid pseudoscorpion *Dinocheirus arizonensis* (d = 2.6%; Pfeiler *et al*., 2009a). The number of diagnostic COI nucleotide differences in the two clades of *D. arizonensis* is similar to that seen between *O. longicornis* and *O. pachycericola* and thus the two clades probably represent distinct undescribed species or subspecies (Pfeiler *et al*., 2009a; Pfeiler & Markow, 2011). The similar genetic divergence seen in these two pairs of cryptic taxa is especially noteworthy given that *Odontoloxozus* and *D. arizonensis* not only share the same necrotic tissue microhabitat, but also show a symbiotic relationship termed phoresy in which the low vagility pseudoscorpion attaches to the leg of the neriid fly and is transported to a fresh host cactus necrosis when the fly disperses (Zeh & Zeh, 1992). A very low COI genetic divergence (d < 1.0) is also found between recognized peninsular and mainland subspecies of *Drosophila mojavensis*, *Drosophila mojavensis baja*, and *Drosophila mojavensis sonorensis* (Pfeiler *et al*., 2009b).

Overall, there is strong evidence supporting the role of vicariance associated with the formation of the Gulf of California in promoting speciation, although the results also suggest a complex phylogeographical

pattern that is not easy to disentangle. As noted earlier, *O. longicornis* has been collected on two of the Midriff Islands (Mangan & Baldwin, 1986) and, given its strong dispersal capability within the mainland Sonoran Desert, it is reasonable to assume that it can use these islands to disperse to the upper peninsula. The single specimen of *O. longicornis* from the peninsula possessed the common COI haplotype for this species, consistent with this assumption. Based on molecular clock considerations, *O. pachycericola* and *O. longicornis* have been genetically isolated for more than a million years. Assuming that *O. pachycericola* originated in the peninsular Cape Region, it is probable that the large over water distance between this region and the mainland, and the absence of 'stepping-stone' islands in the southern Gulf, resulted in complete, or almost complete, genetic isolation, leading to speciation.

ACKNOWLEDGEMENTS

We thank Ben G. Bitler, Sergio Castrezana, Giovanni Hanna, Luis A. Hurtado, Hiroto Kameyama, Luciano M. Matzkin, Joel Schumacher, and Tom Watts for their assistance. We also thank three anonymous reviewers for their helpful comments. This work was supported by NSF Grants DEB 00-75312 and OISE-0440648 to T.A.M., as well as UC MEXUS Grants FA 11-75 to T.A.M., and CN 12-583 to M.P.R and J.R.E. The authors state no conflict of interest.

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