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Short Communication

Mitochondrial DNA evidence for deep genetic divergences in allopatric populations of the rocky intertidal isopod *Ligia occidentalis* from the eastern Pacific

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ABSTRACT

Nucleotide sequences from the cytochrome *c* oxidase subunit I (COI) gene were used to test for genetic differentiation in the rocky intertidal isopod crustacean, *Ligia occidentalis* (Ligiidae), from the eastern Pacific. Phylogenetic analyses showed that individuals of *L. occidentalis* from southern California, USA to Manzanillo, Colima, Mexico partitioned into 15 highly-divergent clades. Mean Kimura 2-parameter (K2P) genetic distances among clades ranged from 13.2% to 26.7%. These values are similar to interspecific genetic distances found in a wide variety of crustaceans, including *Ligia* spp., suggesting that the taxon *L. occidentalis* represents a complex of cryptic species.

1. Introduction

The rock louse, *Ligia occidentalis* Dana, 1853, is a common rocky intertidal isopod distributed from Oregon, USA south to Chamela Bay, Jalisco, Mexico, including the entire Gulf of California (Espinosa-Pérez and Hendrickx, 2006). Several aspects of the life history of *L. occidentalis* suggest that dispersal capability is probably extremely limited, which leads to the prediction that substantial genetic differentiation should occur throughout its range. Development of the young is direct, occurring in brood sacs (Warburg, 1993), thus dispersal is limited to the juvenile and adult stages. Juveniles and adults, however, inhabit a narrow ecological niche, limited mainly to the upper intertidal splash zone of rocky beaches where they feed on algae and scavenge on dead animals and plants (Morris et al., 1980). Rock pools are used by these isopods to replenish water lost by dehydration, but they are unable to survive for prolonged periods in seawater. Throughout the range of *L. occidentalis*, rocky beach habitat is discontinuous, sometimes being interrupted by long stretches of sandy beaches, especially along the mainland coast of the Gulf of California (Thomson et al., 2000). In the present study we test the prediction that these physical dispersal barriers, together with life history traits, will result in restricted gene flow and substantial population structure in *L. occidentalis* by analyzing DNA sequences from a segment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene from individuals sampled over a broad portion of its range.

2. Materials and methods

2.1. Sampling

We sampled 163 *L. occidentalis* from 44 coastal localities in the eastern Pacific and Gulf of California, from Long Beach, California, USA to Manzanillo, Colima, Mexico (Fig. 1). We also included an available GenBank sequence for *L. occidentalis* from San Diego, California, USA (Accession No. AF255780). Sample size per locality varied from 1 to 17 individuals. Specimens were preserved in 95% ethanol.

2.2. Molecular analyses

Total genomic DNA was extracted from legs or thoracic muscle using the DNeasy™ (QIAGEN Inc., Valencia, CA) protocol. The polymerase chain reaction (PCR) was used to amplify a segment of the COI gene (~700 bp) with primers LCO1490f (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198r (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) using standard PCR conditions (Folmer et al., 1994). Verification of successful amplification was assessed by agarose gel electrophoresis. Sequencing reactions were performed on an Applied Biosystems (Foster City, CA) ABI 3730XL DNA sequencer at the DNA Sequencing Facility, University of Arizona, Tucson, using the amplifying primers. Sequences were proofread and

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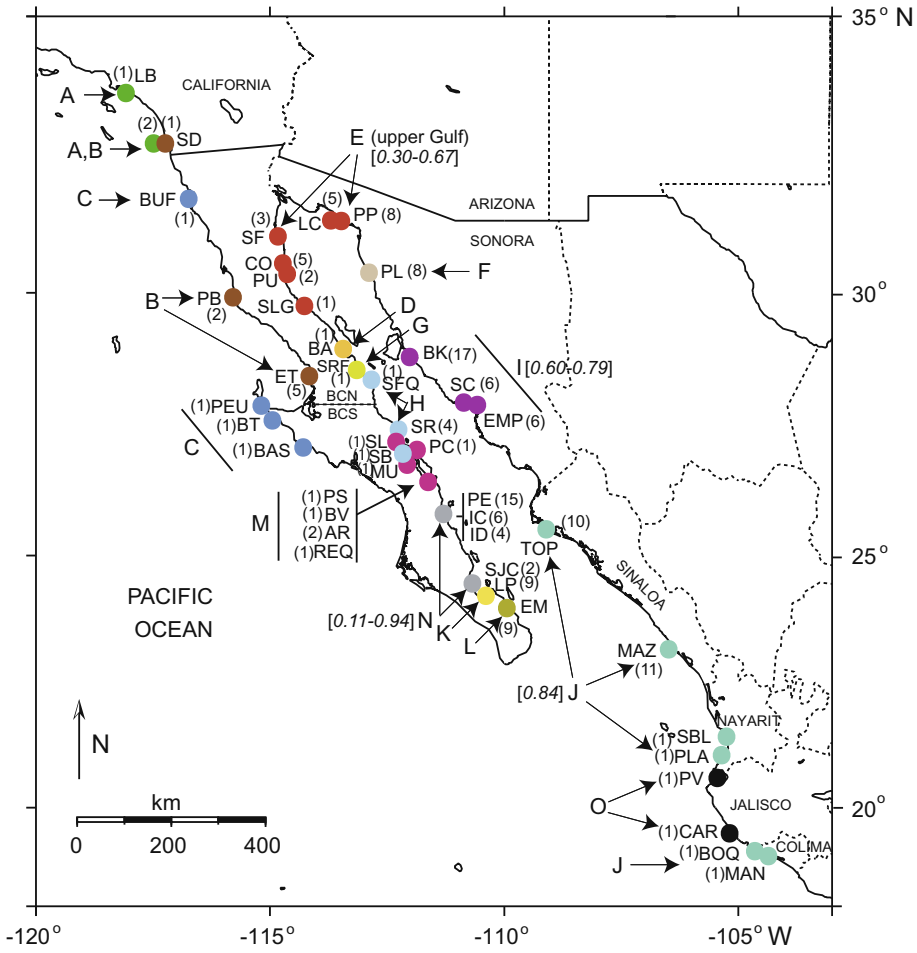

Fig. 1. Map showing collecting localities for *Ligia occidentalis* in the eastern Pacific and Gulf of California. The number of individuals sequenced per locality is shown in parentheses. Locality abbreviations: LB, Long Beach; SD, San Diego; BUF, La Bufadora; PB, Punta Baja; ET, El Tomatal; PEU, Punta Eugenia; BT, Bahía Tortuga; BAS, Bahía Asunción; EM, Ensenada de los Muertos; LP, La Paz; SJC, San José de la Costa; IC, Isla Carmen; ID, Isla Danzante; PE, Puerto Escondido; PS, Punta Sueño; BV, Buenaventura; AR, Armenta; REQ, El Requesón; MU, Mulegé, SB, San Bruno; PC, Punta Chivato; SL, San Lucas; SR, San Rosalía; SFQ, San Francisquito; SRF, San Rafael; BA, Bahía de los Angeles; SLG, San Luis Gonzaga; PU, Puertecitos; CO, El Coloradito; SF, San Felipe; LC, La Choya; PP, Puerto Peñasco; PL, Puerto Lobos; BK, Bahía de Kino; SC, San Carlos; EMP, Empalme; TOP, Topolobampo; MAZ, Mazatlán; SBL, San Blas; PLA, Platanito; PV, Puerto Vallarta; CAR, Punta Careyes; BOQ, La Boquita; MAN, Manzanillo. State abbreviations on the Baja California peninsula: BCN, state of Baja California; BCS, Baja California Sur. Clades assignments (A to O) for localities are shown using the color scheme in Fig. 2. Italicized numbers in brackets shown for clades I, E, J and N denote values obtained in pairwise comparisons of Φ_{ST} for populations within each clade where $N \geqslant 4$.

aligned in either Sequencher version 4.1 (GeneCodes Corp., Ann Arbor, MI) or ClustalX 1.81 (Thompson et al., 1997) followed by manual editing. Sequences were trimmed to remove ambiguous sites or missing data, resulting in a final segment size of 610 bp. Aligned sequences were translated in MEGA version 4.0 (Tamura et al., 2007). No stop codons or indels were found in the translated gene segment. In addition, this segment showed high amino acid sequence homology with COI in other species of Ligiidae available in GenBank. Thus we are confident that our sequences represent mitochondrial DNA and are not nuclear pseudogenes which are known for a variety of organisms, including crustaceans (Williams and Knowlton, 2001). Calculations of genetic distances among sequences (uncorrected *p*-distances and Kimura 2-parameter [K2P] distances [Kimura, 1980]) were carried out in MEGA. Calculations of genetic diversity indices and neutrality tests [Tajima's (1989) *D*] were performed in DnaSP version 5.00.04 (Librado and Rozas, 2009). Analysis of molecular variance (AMOVA, Excoffier et al., 1992) performed in ARLEQUIN version 3.1 (Excoffier et al., 2005) was used to test for population structure within identified clades of *L. occidentalis* (see Section 3) for localities with samples sizes $\geqslant$4. The calculation of significance of pairwise comparisons of the fixation index Φ_{ST} was based on 10,000 permutations of the data matrix and was assessed with a sequential Bonferroni correction for multiple comparisons (Rice, 1989). 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. The COI gene segment was also sequenced in a specimen of *L. exotica* from Fort Johnson, Charleston, South Carolina, USA. Voucher specimens from all collection localities were taken and preserved in 95% ethanol. Sequences of all unique COI haplotypes have been deposited in GenBank under the following Accession Nos. GU270878–GU270929.

2.3. Phylogenetic analyses

Relationships among COI haplotypes from the entire *L. occidentalis* data set were initially assessed with the neighbor-joining (NJ) algorithm of Saitou and Nei (1987) carried out in MEGA using a matrix of both uncorrected *p*-distances and K2P distances. In addition to the new sequence for *L. exotica*, we used the following GenBank sequences from the family Ligiidae as outgroups: *Ligia italica* (DQ182858); *L. oceanica* (DQ442914); *L. hawaiensis* (AY051325); *L. perkinsi* (AY051332); and *Ligidium beieri* (DQ182790). These initial analyses revealed that 84 ingroup haplotypes partitioned into 15 terminal clades (not shown), 13 of which were highly-supported (86–99%) by bootstrap analyses using 1000 pseudorepli-

cates (Felsenstein, 1985). Clades were assigned subjectively based on clustering in the terminal nodes and by assuming a maximum value of 12% for within-clade K2P distances. To decrease analysis time, a subset of haplotypes comprised of representatives from each locality was then selected for further phylogenetic analyses using Bayesian inference and maximum parsimony (MP). For those localities with multiple samples (see Fig. 1), the two most common haplotypes were selected.

Bayesian analyses were implemented in MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). Two separate runs were conducted with identical results. The model of nucleotide substitution that best fit the data set, determined with Modeltest 3.7 (Posada and Crandall, 1998) using the Akaike Information Criterion, was GTR+I+Γ. Bayesian analyses were run under the parameters of this model (nst = "6"; rates = "invgamma") for 1,000,000 generations, sampled every 250th generation (4000 trees sampled), using the default random tree option to begin the analysis. Clade support, expressed as posterior probabilities, was estimated utilizing a Markov chain Monte Carlo (MCMC) algorithm. Log-likelihood values from four simultaneous MCMC chains (three hot and one cold) stabilized at about 25,000 generations. The first 100 trees, therefore, were discarded from the analysis (burnin = 100). The MP analyses were carried in MEGA using the CNI heuristic search option and 100 random additions of sequences. Relative support for tree topology was obtained by bootstrapping using 500 pseudoreplicates.

3. Results

Haplotype diversity (h) in the 610 bp COI segment varied from 0.389 to 1.000 among the 15 assigned clades (see below) of *L. occidentalis*; nucleotide diversity (π) ranged from 0.00070 to 0.04536 (Table 1). None of the values for Tajima's D were significant. Mean G + C (guanine + cytosine) content ranged from 39.1% to 44.4% which falls within the range of values for isopods (Costa et al., 2007).

Bayesian and MP phylogenetic trees yielded similar topology (Fig. 2) and confirmed the extensive genetic differentiation among haplotypes of *L. occidentalis* observed in the initial NJ tree. Applying the criteria described in Section 2.3, 15 terminal clades (clades A to O) were again resolved, and most of these (12 of 15) were highly-supported (97–100%). Several of the deeper nodes, however, were poorly supported, especially in the MP tree. For each locality where multiple individuals were sampled, only a single clade was represented. The exception seen at San Diego (Fig. 1) resulted from our incorporating a GenBank sequence for *L. occidentalis* which clustered with clade B whereas the new specimens from San Diego, along with the specimen from Long Beach, were assigned to clade A. The 15 clades of *L. occidentalis* partitioned into four major groups: (1) outer Pacific coast of the Baja California (Baja) peninsula and southern California, USA; (2) northern and central Gulf of California; (3) central and southern Gulf; and (4) Pacific coast of mainland Mexico south of the Gulf (Fig. 2). Clade O from the Pacific coast of Mexico resolved in a basal position in the ingroup. *Ligia exotica* unexpectedly clustered with *L. occidentalis*, resolving in a basal position with the three clades (A to C) from the outer Pacific coast of the Baja peninsula and southern California.

Genetic differentiation among clades, measured as K2P distances, ranged from 13.2% (between clades B and C from the Pacific coast of the Baja peninsula and adjacent San Diego, California) to 26.7% (between clade A from southern California and clade E from the northern Gulf of California). The overall average K2P distance between clades was 20.7%. Within-clade distances ranged from 0.2% to 10.8%.

The substantial genetic differentiation among clades suggested that third codon position substitutions were probably saturated in the relatively fast evolving COI gene. When we plotted the number of transitions and transversions at each codon position against K2P distances using DAMBE (Xia and Xie, 2001), third position transitions and transversions were saturated as predicted (not shown). We deleted third codon positions and found that all phylogenetic trees (NJ, Bayesian and MP) were still able to resolve the 15 clades, but statistical support for the nodes was generally lower than shown in Fig. 2.

In four of the assigned clades (clades I, E, J and N), sample sizes from different localities were sufficient ($N \geqslant 4$) to conduct AMOVA to assess the degree of within-clade population structure. All pairwise comparisons of Φ_{ST} (Fig. 1) were significant using a Bonferroni correction, except for the comparison between the nearby localities of Isla Carmen (IC) and Isla Danzante (ID) in clade N ($\Phi_{ST} = 0.11$; $P = 0.41$). Pairwise values for the number of migrants per generation (N_m) among populations within clades were generally low ($N_m = 0.03–0.37$). The only exceptions were seen between populations at IC and ID in clade N ($N_m = 4.00$) and between the two populations separated by about 12 km at La Choya (LC) and Puerto Peñasco (PP) from clade E ($N_m = 1.18$).

4. Discussion

Based on the limited dispersal capability, lack of a pelagic larval stage and restricted ecological niche of *L. occidentalis* we expected

Table 1
Summary of genetic diversity indices and Tajima's D neutrality tests in the 610 bp COI gene segment in clades of *Ligia occidentalis* in which $N \geqslant 4$.

Clade	N	k	K	h (±SD)	π (±SD)	Tajima's D	G + C (%)
A	3	47	2	–	–	–	42.4
B	8	45	4	0.750 ± 0.139	0.02450 ± 0.01166	−1.23 n.s.	40.9
C	4	52	4	1.000 ± 0.177	0.04536 ± 0.01763	−0.81 n.s.	41.9
D	1	–	1	–	–	–	42.1
E	24	46	17	0.971 ± 0.019	0.01372 ± 0.00309	−1.31 n.s.	44.4
F	8	7	4	0.643 ± 0.184	0.00410 ± 0.00124	−0.35 n.s.	43.9
G	1	–	1	–	–	–	44.3
H[a]	5	62	5	1.000 ± 0.126	0.04189 ± 0.02342	−1.26 n.s.	41.7
I	29	10	9	0.732 ± 0.073	0.00363 ± 0.00039	−0.41 n.s.	40.2
J	25	38	13	0.913 ± 0.036	0.01669 ± 0.00222	0.04 n.s.	41.3
K	9	1	2	0.389 ± 0.164	0.00070 ± 0.00030	0.16 n.s.	41.8
L	9	4	3	0.417 ± 0.191	0.00146 ± 0.00072	−1.61 n.s.	41.3
M	8	57	8	1.000 ± 0.063	0.03150 ± 0.01160	−0.84 n.s.	41.6
N	27	40	9	0.815 ± 0.051	0.01958 ± 0.00284	0.53 n.s.	42.9
O	2	14	2	–	–	–	39.1

N, number of sequences; k = number of variable sites; K, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; G + C, guanine plus cytosine content; n.s., not significant at the 0.05 level.

[a] One individual of the six from this clade had missing sequence data and was omitted from the analysis.

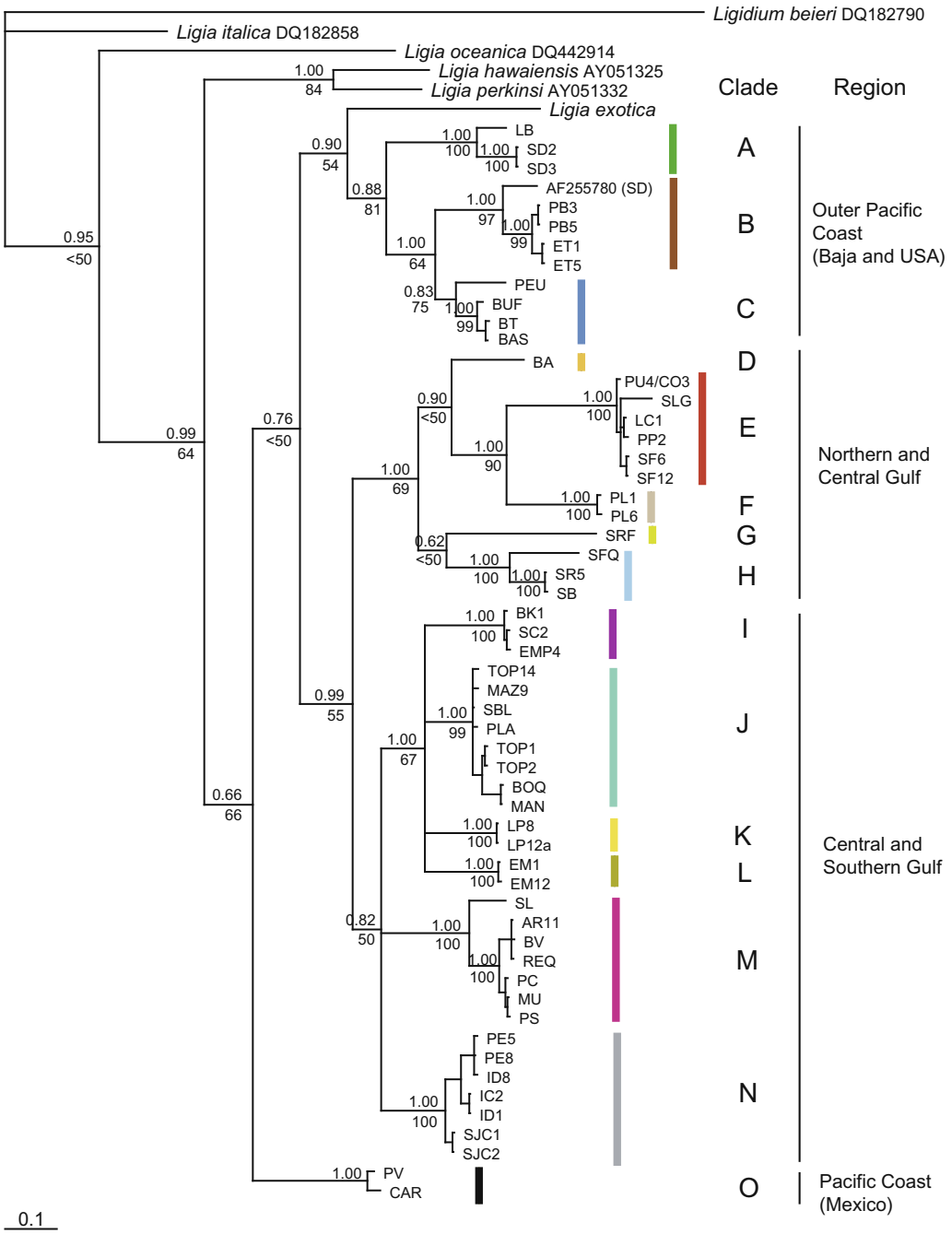

Fig. 2. Bayesian 50% majority rule consensus tree showing relationships among haplotypes of *Ligia occidentalis*, and selected sequences of *Ligia* available in GenBank, based on analysis of a 610 bp segment of the COI gene. The tree was rooted with *Ligidium beieri* (family Ligiidae). Clade support expressed as posterior probabilities is shown above branches. Bootstrap support values for the MP tree (length = 1494; CI = 0.317; RI = 0.714; 291 variable sites; 249 parsimony informative sites) is shown below the branches. Scale shows substitutions per site. Branch terminals are labeled with sample identification number and locality abbreviation (see Fig. 1). Colored bars represent assigned clades (A to O) that are colored coded to localities shown in Fig. 1. Vertical lines on the far right show the geographic partitioning of clades into four major groups.

to find a high degree of genetic differentiation among populations of this widely-distributed rocky intertidal isopod. The significant population structure found within four of the identified clades of *L. occidentalis* was consistent with our prediction, but the overall degree of genetic diversification revealed in the phylogenetic analyses was unexpected. Phylogenetic trees constructed with different algorithms (NJ, MP and Bayesian inference) were congruent and showed 15 deep lineages (clades A to O) within the currently recognized taxon, *L. occidentalis*. The large interclade K2P distances found for the COI gene (13.2–26.7%) are more typical of those seen between species. For example, in seven well-defined species of *Ligidium* (Ligiidae) mean K2P distances range from 14.4% to 23.3% for the COI gene (Klossa-Kilia et al., 2006). Within the subphylum Crustacea, Costa et al. (2007) showed that mean congeneric K2P distance based on COI sequence data from 54 species and 31 genera of decapod crustaceans was about 17%, and increased to about 25% when the orders Cladocera and Amphipoda were included. The K2P distances among the six species of *Ligia* shown in Fig. 2, including the GenBank sequence for *L. occidentalis*, ranged from 14.9% (between *L. hawaiensis* and *L. perkinsi*) to 30.3% (between *L. exotica* and *L. oceanica*), with an average of 24.6%. Thus the genetic divergences found among clades of *L. occidentalis* are similar to

interspecific distances among *Ligia* spp. and among *Ligidium* spp., as well as among crustaceans in general, suggesting that at least 15 genetically distinct cryptic species have been lumped under *L. occidentalis*. We found no obvious external morphological differences in voucher specimens from these clades, although more detailed examination of the vouchers my reveal subtle differences. However, even in distinct species of the related genus *Ligidium* little morphological variation is found (Klossa-Kilia et al., 2006).

Our conclusion that *L. occidentalis* represents a species complex is based on a phylogenetic species concept in which evolutionary lineages are diagnosed and defined as distinct species based on phylogeny (Cracraft, 1983; Egge and Simons, 2006). Here we assume that the COI gene trees we present accurately reflect the species phylogeny. The presence of two distinct clades (clades A and B; K2P distance = 17.8%) at San Diego, California also suggests the possibility of reproductive isolation in sympatry, but because the clade B sequence was obtained from GenBank, we do not know whether individuals from both clades actually occur together on the same rocky beach. Interestingly, Burton and Lee (1994) also found two highly divergent lineages of the intertidal copepod *Tigriopus californicus* at San Diego (15.4% uncorrected *p*-distance). For *L. occidentalis*, more extensive sampling will be required to determine not only if clades A and B occur in sympatry, but also to assess the degree of genetic divergence within California. We sampled from only two localities (Long Beach and San Diego) and thus samples from most of the California coast are lacking. Even if additional cryptic species are not found in California, our results unstabilize the taxonomy of *L. occidentalis* because the type locality is listed only as "California, USA" and thus it is unclear whether the name *L. occidentalis* should be assigned to clade A or B. In addition, our findings indicate that numerous nomenclatural changes will need to be made to the taxon *L. occidentalis* (sensu lato).

In the mid-peninsular region, we found that clades H and M overlapped at nearby localities from Santa Rosalía to Mulegé (Fig. 1). Because the sample consisted of single individuals from most of these localities it is possible that increased sampling will reveal that both clades are sympatric in this region, probably owing to secondary contact after differentiation. Clades H and M, which are separated by a mean K2P distance of 20.7% and show six amino acid differences in the translated COI gene segment, are potentially ideal models for use in laboratory crosses to determine if the large amount of genetic differentiation among the two prevents hybridization in putative contact zones. Studies of this type have been conducted on geographically isolated populations of the intertidal copepod *T. californicus* from California, USA in which clades from central and southern California show COI divergences similar to those of clades H and M in *L. occidentalis* (Burton and Lee, 1994; Ellison and Burton, 2006). Interpopulation crosses of *T. californicus* produce fertile offspring, but hybrid breakdown, attributed to disruption of coadaptation between mitochondrial and nuclear genes, appears in the F_2 generation (Ellison and Burton, 2006). Similar hybridization studies conducted on *L. occidentalis* would complement and provide an important test of the findings on *T. californicus*.

The large K2P genetic divergences found among clades of *L. occidentalis* suggest that these clades began their separate evolutionary trajectories approximately 5.7–11.6 million years ago during the late Miocene, assuming a COI molecular clock rate of 2.3% pairwise sequence divergence per million years (Brower, 1994). Because this time frame coincides with the early stages of the complex geological events that resulted in the formation of the Gulf of California (Carreño and Helenes, 2002), it seems reasonable to assume that these geological changes, together with the highly-restricted dispersal capability of *L. occidentalis*, played a role in shaping the genetic diversification and geographic distribution of the clades that are found today. A possible genetic signature of these past geological events can be seen in the topology of the major clade groupings on the phylogenetic tree (Fig. 2). Briefly, geological and fossil evidence suggest that marine incursions (often referred to as the "proto-Gulf") occurred in the area that now includes the northern and central Gulf (including the desert region of southern California) during the mid to late Miocene, before the separation of the Baja peninsula from mainland Mexico (McDougall et al., 1999; Carreño and Helenes, 2002). These marine incursions are consistent with a vicariant formation of an outer Pacific coast group (clades A to C) distinct from the two major groups found in the Gulf. The separation of the Gulf clades into two groups, northern and central Gulf (clades D to H) and central and southern Gulf (clades I to N), is also consistent with postulated differences in geological history of the northern and southern Gulf (Carreño and Helenes, 2002). In addition, Fig. 2 shows that peninsular clades K and L from the Cape Region are more closely related to mainland clades I and J than they are to peninsular clade N (found in close proximity to the Cape Region) and clade M (central Baja peninsula) which is consistent with fossil evidence that the Cape Region remained attached to the mainland longer than the rest of the peninsula (Carreño and Helenes, 2002).

In conclusion, we suggest that life history traits (highly-restricted dispersal) and vicariance have resulted in deep genetic divergences and shaped the geographic distribution of at least 15 cryptic species of *L. occidentalis* from the eastern Pacific, a mostly terrestrial rocky intertidal isopod. Our results complement those of other workers who have found an emerging pattern of substantial genetic divergence and putative cryptic speciation among geographically separated populations of marine isopods, including *Glyptonotus antarcticus* (Held and Wägele, 2005), *Idotea balthica* (Wares, 2001; Wares et al., 2007) and *Serolis paradoxa* (Leese et al., 2008). Our findings also add to the growing list of cryptic species being discovered across metazoan taxa (Bickford et al., 2007; Pfenninger and Schwenk, 2007).

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