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RESEARCH ARTICLE

The marine brooder *Excirolana braziliensis* (Crustacea: Isopoda) is also a complex of cryptic species on the coast of Chile

El isópodo marino *Excirolana braziliensis* (Crustacea: Isopoda) es también un complejo de especies crípticas en la costa de Chile

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ABSTRACT

Speciation is a direct consequence of isolated populations in taxa with low dispersal potential. The brooding crustacean *Excirolana braziliensis*, with a presumably wide geographic range of distribution (~16° N-41° S in the Pacific and ~19° N-35° S in the Atlantic), has been detected to correspond to cryptic species on the coast of Panama. Latitudinal variations in reproductive features in *E. braziliensis* have been attributed to phenotypic plasticity, however, the differences may be the result of divergent populations. Considering that the taxon has been reported to be a complex of cryptic species in other geographic areas and given the phenotypic differences detected along its distribution range, we hypothesized that *E. braziliensis* is a complex of species in the coast of Chile. We used partial cytochrome c oxidase subunit I (COI) sequences from 132 individuals with the diagnostic morphology of *E. braziliensis* collected along ~2200 km of coast to determine the genetic structure of *E. braziliensis*. Phylogenetic and phylogeographic analyses showed three distinct clades with 14 to 19 % of genetic divergence and high values of genetic differentiation. Intra and inter-clade divergence revealed the existence of a species complex of *E. braziliensis* on the coast of Chile, supporting growing evidence of the high abundance of cryptic species in marine invertebrate taxa.

Key words: cryptic species, genetic differentiation, genetic distance, marine peracarids, phylogenetics.

RESUMEN

La especiación es una consecuencia directa de poblaciones aisladas en taxa con bajo potencial de dispersión. El crustáceo incubador *Excirolana braziliensis* tiene un rango de distribución presumiblemente amplio (~16° N-41° S en el Pacífico y ~19° N-35° S en el Atlántico). Se ha detectado que este taxón representa un complejo de especies crípticas en la costa de Panamá. Variaciones latitudinales en características reproductivas en *E. braziliensis* han sido atribuidas a plasticidad fenotípica, sin embargo, las diferencias podrían deberse a la existencia de poblaciones divergentes. Considerando que se ha reportado que este taxón es un complejo de especies crípticas en otras áreas geográficas y dadas las diferencias fenotípicas detectadas a lo largo de su rango de distribución, hipotetizamos que *E. braziliensis* es un complejo de especies en la costa de Chile. Utilizamos secuencias parciales del gen citocromo c oxidasa subunidad I (COI) de 132 individuos con la morfología diagnóstica de *E. braziliensis* recolectados a lo largo de ~2200 km de costa para determinar la estructura genética de *E. braziliensis*. Análisis filogenéticos y filogeográficos revelaron tres clados que presentaron una divergencia genética entre 14-19 % y valores de diferenciación altos. La divergencia intra e interclados indican que existe un complejo de especies de *E. braziliensis* en la costa de Chile, lo que se suma a la creciente evidencia de la alta abundancia de especies crípticas en invertebrados marinos.

Palabras clave: diferenciación genética, distancia genética, especies crípticas, filogenia, peracáridos marinos.

INTRODUCTION

Cryptic species are species that are difficult to recognize based in morphology (Knowlton 1993, Bickford et al. 2007). While 'sibling species' may refer specifically to cryptic

species that share a common ancestor, the term is usually considered a synonymous of 'cryptic species' (reviewed by Bickford et al. 2007). The abundance of cryptic species discoveries reflects a lack of morphological divergence or an inadequate knowledge of

the morphological differences of divergent groups. Information provided by morphology is limited by our poor understanding of functional morphology, which is strongly influenced by natural selection (Knowlton 1993, Bickford et al. 2007). Moreover, morphological stasis can be promoted by stabilizing or convergent selection under extreme environmental conditions (Bickford et al. 2007). Research to uncover cryptic species complexes can have serious implications for evolutionary biology and for management and conservation plans; for example, the discovery of cryptic species in a particular habitat might reveal underscored levels of diversity and/or endemism upgrading the priority level for conservation for that habitat (reviewed by Bickford et al. 2007). Complexes of sibling species and cryptic species have been widely identified using DNA markers (e.g., Gouws et al. 2004, Moura et al. 2008, Jordaens et al. 2010, Azuma et al. 2011), and most cryptic species are marine invertebrates (Knowlton 1993). Cryptic species have been reported along the geographic range that was assumed for one benthic marine species with low dispersal potential (Held & Wägele 2005, Raupach & Wägele 2006, Remerie et al. 2006, Linse et al. 2007, Raupach et al. 2007, Boissin et al. 2008, Baird et al. 2011, Carr et al. 2011, Doellman et al. 2011). Under a low dispersal scenario, isolation by distance and outbreeding depression are enough to account for a high speciation rate (Hoelzer et al. 2008). Many marine brooders may have an enhanced speciation potential given their small body size and low vagility, resulting in low potential for active dispersal (Teske et al. 2007).

Peracarids are a group of direct developing crustaceans that brood their larvae up to a crawl-away stage. For some peracarids long-distance dispersal may occur through rafting on floating substrata such as detached macroalgae (Thiel & Haye 2006), or via anthropogenic transfer (e.g., Wittmann & Ariani 2009). Other peracarid species, however, live in habitats that are not suitable for alternative dispersal mechanisms, for example, the abundant amount of species that live in soft bottom sandy beaches, cannot survive on floating objects (Thiel & Gutow 2005). Occasional dispersal events between beaches may occur if individuals remain attached to its prey (e.g., live fish) for enough time to complete a passive

transport (Sponer & Lessios 2009). Similarly, marine birds may occasionally transport individuals if they casually remain attached to their plumage or feet, as reported for aquatic non-marine invertebrates (Frisch et al. 2007). These stochastic long-distance dispersal events are likely to be infrequent not allowing for sufficient gene flow to counterbalance the effects of genetic drift. Therefore, the wide range of geographic distribution reported for many soft-bottom peracarids may be the result of undetected species complexes.

The beach dwelling isopod *Excirolana braziliensis* Richardson has a wide geographic range in America and Chile. It has been found in the Pacific Ocean from the Gulf of California in Mexico (Dexter 1976) to Chiloé Island in southern Chile (Jaramillo et al. 2000). In the Atlantic Ocean it has been reported from the Gulf of Mexico (Dexter 1976) to Uruguay (Defeo et al. 1997). For the coast of Panama Lessios & Weinberg (1994) and Sponer & Lessios (2009) reported three divergent lineages in *E. braziliensis*, indicating the presence of a species complex. Along the coast of Chile *E. braziliensis* has been scarcely studied. A highlight is the study of Cardoso & Defeo (2003); they found strong latitudinal variations in reproductive features in populations of *E. braziliensis* from the Pacific and Atlantic Oceans. Even though the authors attributed the variations to phenotypic plasticity, the phenotypic divergence could also be a consequence of genetic differentiation between local populations.

Given the likelihood of speciation in taxa with low active and passive dispersal potential, the presence of a species complex of *E. braziliensis* on the coast of Panama and the phenotypic variation detected among populations, we hypothesized that there is a species complex in *E. braziliensis* in the Chilean coast. For this, the genetic structure between local populations of *E. braziliensis* along the north-central coast of Chile was analyzed using cytochrome c oxidase subunit I (COI) sequences.

METHODS

Sampling, DNA extraction, PCR and sequencing

Samples were collected from seven localities of the Chilean coast influenced by the Humboldt Current

System (from 18.4° S to 38.2° S, along ~2200 km) (Table 1, Fig. 1A). During low tide, macroinfaunal species were washed out of bulk sand samples with 2 mm mesh size bags. *Excirrolana* species are easily and clearly distinguishable from other macroinfaunal isopods of sandy beaches (Jaramillo et al. 1998). Samples were preserved in 95 % ethanol at -20° C until DNA extraction.

Of the three species of the genus *Excirrolana* present along the Chilean coast, *E. braziliensis* can be easily distinguished from the others (*E. hirsuticauda* and *E. monodi*) by the shape of the cephalon and the telson, as well as by the length of the antennae. In *E. braziliensis* the cephalon width is three times the length, the telson has a rounded apex, antenna 1 reaches half of pereonite 3, and antenna 2 extends until the end of pereonite 5 (Jaramillo 1982). All individuals used in this study had the diagnostic characteristics of *E. braziliensis* (Jaramillo 1982).

Depending on the availability, seven to 22 individuals of *E. braziliensis* per locality were analyzed (Table 1). Whole individuals were used for the DNA extraction; except for individuals larger than 5 mm for which only telson muscle was used. Genomic DNA was extracted with Qiagen's QIAamp DNA mini kit following manufacturer's instructions.

We used the COI gene to detect population genetic structure. This marker has been extensively validated as a good species marker for most animal species, and it is the preferred marker for DNA Barcoding in many animal groups including crustaceans (e.g., Bucklin et al. 2011, Havermans et al. 2011). Sponer & Lessios (2009) also used COI and could easily distinguish the clades that comprise the species complex of *E. braziliensis* in the coast of Panama.

A partial sequence of the COI gene was amplified with PCR using the primers HCO2198 and LCO1490 (Folmer et al. 1994). PCR's were carried out with 1X PCR buffer, 2 mM MgCl₂, 0.4 μM of each primer, 0.2 mM of each dNTPs, 0.03 U μL⁻¹ Taq and 1.5 mg mL⁻¹ Bovine Serum Albumin (BSA), 1 μL of template DNA (~20 ng) and distilled H₂O to complete final volume. The cycling conditions were: initial denaturing of 94 °C for 10 min followed by 35 cycles of 1 min denaturing at 94 °C, 1 min annealing at 51 °C and 2 min extension at 72 °C, and ending with a final extension at 72° C by 13 min. Amplicons were purified by adding 28.8 μL Shrimp Alkaline Phosphatase and 7.2 μL of Exonuclease I to 45 μL PCR product followed by incubation at 37°C for

15 min and 80 °C for 15 min. Purified products were sequenced in both directions with an ABI 3730XL capillary automated sequencer.

CODONCODE Aligner 1.2.0 (CodonCode) was used to inspect chromatographs for ambiguous sites, obtain consensus sequences, and for sequence alignment. Alignment was verified through translation to amino acids. Sequences were submitted to GenBank (Accession Numbers: FJ541198-FJ541243).

Data analyses

Two sequences of *E. hirsuticauda* (Genbank Accession Numbers: FJ532105 and FJ532142) were used as outgroup taxa for phylogenetic analyses. Phylogenetic hypotheses were searched performing Maximum Likelihood Analysis (MLA), and Bayesian Analysis (BA), implemented in PAUP 4.b10 (Swofford 2002) and MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), respectively. The model of DNA evolution that better fit the data according to jModelTest 0.1.1 (Posada 2008) was Hasegawa-Kishino-Yano plus gamma (HKY+G), and was used for MLA and BA. A bootstrap analysis with 10000 replicates was used to obtain support values for the nodes for the MLA using PAUP. In the BA, posterior probability values for the nodes were obtained using 5000000 iterations and sampling every 10 generations. Two simultaneous and independent analyses were run until the standard deviation of split frequencies was below 0.01. The first 25 % of saved trees were discarded as burn-in.

The mutational relationships between all haplotypes detected were visualized with a median joining haplotype network generated using NETWORK 4.5 (Bandelt et al. 1999). Ambiguous loops in the network were resolved using the criteria of Crandall & Templeton (1993). The genetic p distance among haplotypes was calculated with PAUP*4.b10. Pairwise F_{ST} values between localities were calculated in ARLEQUIN 3.11 (Excoffier et al. 2005). The significance of the F_{ST} values was evaluated by 10000 permutations.

RESULTS

Sequences of 583 base pairs were obtained for 132 individuals from seven localities of the Chilean coast (Table 1, Fig. 1A). The MLA

TABLE 1
Number of individuals of *Excirrolana braziliensis* analyzed in each locality of the Chilean coast.
Número de individuos de *Excirrolana braziliensis* analizados en cada localidad de la costa de Chile.

Locality	Code	Coordinates	Number of individuals
Arica	Ari	18.4° S-70.3° W	7
Playa Chipana	Chi	21.3° S-70.1° W	20
Península Cangrejo	Pca	24.4° S-70.6° W	22
Caldera	Cad	27.0° S-70.8° W	20
Puerto Aldea	Pal	30.3° S-71.2° W	19
Playa Pangua	Pan	34.5° S-72.0° W	22
Playa Queule	Que	38.2° S-73.6° W	22
Total			132

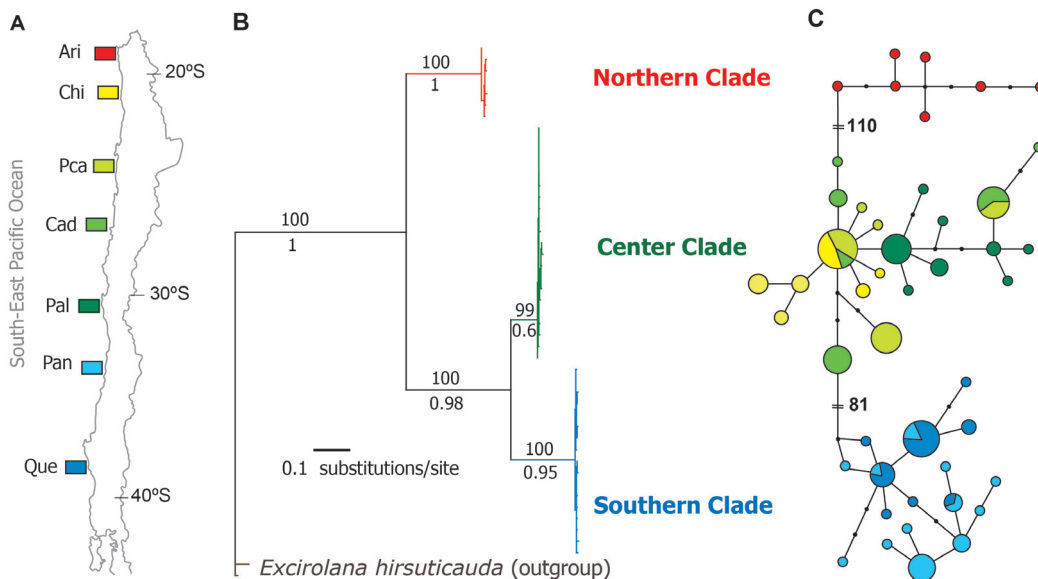


Fig. 1: Partial map of Chile (South-East Pacific coast) showing the localities where *E. braziliensis* were sampled (A). Maximum Likelihood phylogram of 45 COI haplotypes of *E. braziliensis* (B). Median joining haplotype network of *E. braziliensis* (C). In the phylogram, values above the branches correspond to Maximum Likelihood bootstrap and values below to Bayesian posterior probabilities values. Colors in the phylogram and haplotype network represent the different localities shown in the map (A). The symbol || indicates that the line was truncated and the numbers next to show the number of mutational steps between groups of haplotypes.

Mapa parcial de Chile (Pacífico Sur Este) indicando las localidades donde se recolectaron las muestras de *E. braziliensis* (A). Filograma de Máxima Verosimilitud de los 45 haplotipos de *E. braziliensis* (B). Red de haplotipos de *E. braziliensis* construida con el método "median joining" (C). En el filograma, los valores sobre las ramas corresponden a los valores de iteraciones obtenidos con Máxima Verosimilitud y los valores bajo las ramas a probabilidades posteriores Bayesianas. Los colores en el filograma y en la red de haplotipos representan las diferentes localidades indicadas en el mapa (A). El símbolo || indica que la línea fue truncada y los números al lado de || indican el número de pasos mutacionales entre los grupos de haplotipos.

and BA phylogenetic analyses revealed three highly divergent clades of *E. braziliensis*, each with reciprocal monophyly, that are here on referred to as Northern Clade, Center Clade, and Southern Clade (Fig. 1A and 1B). The phylogenetic hypotheses derived from MLA and BA only differed in the position of the terminal nodes within clades (haplotypes that differed on one nucleotide) with low bootstrap support values.

Consistent with phylogenetic analyses, the arrangement of the 45 haplotypes in the median joining haplotype network shows three distinct haplogroups, each corresponding to the clades detected on the phylogenetic analyses (Fig. 1C). The Northern Clade was found in Arica (18.4° S), the Center Clade from Chipana (21.3° S) to Puerto Aldea (30.3° S), and the Southern Clade in Pangua (34.5° S) and Queule (38.2° S) (Fig. 1A and 1B).

The Northern and Center clades are separated by 110 mutational steps and have 18 % of genetic divergence. The Center and Southern clades are separated by 81 mutational steps and have 14 % of genetic divergence. Northern and Southern clades diverge in 19 %. While the Northern Clade was represented by only one locality, the genetic divergence within the Center clade ranged from 0.45 % to 0.67 % and was 0.66 % for the Southern Clade.

Pairwise F_{ST} values between localities from the three clades were close to 1.0 and highly significant ($P < 0.0001$) (Table 2), indicating almost complete genetic differentiation between clades. Intra-clade divergence was evaluated for the Center and Southern clades, which had more than one location included in the study; both Center and Southern clades had significant intra-clade population-pairwise F_{ST} values ($P < 0.001$), however, genetic

TABLE 2

Pairwise F_{ST} values between localities. Values between localities from the different clades are indicated by "A". All the other correspond to the values between localities within Center and Southern Clades. All the values were significant ($P < 0.001$). Codes as in Table 1.

Valores de F_{ST} entre localidades. Los valores entre localidades de los diferentes clados están indicados por "A". Todos los otros corresponden a los valores entre localidades dentro de los clados Centro y Sur. Todos los valores fueron significativos ($P < 0.001$). Las siglas representan localidades como se muestra en la Tabla 1.

	Ari	Chi	Pca	Cad	Pal	Pan
Chi	0.984 ^A					
Pca	0.975 ^A	0.277				
Cad	0.969 ^A	0.268	0.158			
Pal	0.979 ^A	0.444	0.380	0.328		
Pan	0.974 ^A	0.975 ^A	0.967 ^A	0.963 ^A	0.971 ^A	
Que	0.984 ^A	0.983 ^A	0.976 ^A	0.972 ^A	0.980 ^A	0.460

differentiation between localities within clades was less than half than between clades (Table 2).

DISCUSSION

As hypothesized, COI sequence analyses revealed that the wide range of geographic distribution of the brooder *E. braziliensis* along the north-central Chilean coast is explained by the presence of a species complex. The low dispersal potential of *Excirolana* species, together with their small body size and concomitant low intrinsic dispersal have led to three highly divergent lineages between 18 and 38° S on the South-East Pacific coast under the influence of the Humboldt Current System.

The genetic divergence among clades of *E. braziliensis* ranged from 14 % to 19 %, while the divergence within clades was very low (< 0.67 %). Comparisons of species of the family Cirolanidae revealed a sequence divergence from 13.6 % to 14.7 % (Wetzer 2001). Other studies comparing species within a genus of peracarids have led to similar or slightly higher values. For the genus *Idotea*, Wares (2001) determined a genetic divergence of 17-25 %; Markow & Pfeiler (2010) reported divergences from 14.9 % to 30.3 % among species of *Ligia*, including terrestrial and marine-terrestrial species; for the amphipod genus *Eusirus* interclade distances fell between 4.6 to 10.9 % (Baird et al. 2011); and Baratti et al. (2005) suggested that

Sphaeroma terebrans was a complex of cryptic species with genetic distances of 15 to 18 %. The high values of genetic divergence among clades detected within *E. braziliensis* were close to the divergence of the COI gene generally reported between species. When genetic divergence between clades exceeds the typical values for congeners it is reasonable to infer there are sibling/cryptic species. Therefore, the detected levels of genetic divergence between clades whose individuals have the diagnostic features of *E. braziliensis* suggest that there is a complex of cryptic species under the formal taxon *E. braziliensis* on the Chilean coast, as had been shown for the coasts of Panama (Lessios & Weinberg 1994, Sponer & Lessios 2009). So far, few studies have identified marine cryptic species on the Chilean coast. Using morphological, developmental and genetics features, Véliz et al. (2003) found three cryptic species of *Crepidula* from northern Chile. Later, Tellier et al. (2009) identified two divergent lineages of the kelp *Lessonia nigrescens* at 30° S that likely evolved through parapatric speciation. Recently, Montecinos et al. (2012) detected three highly divergent clades along the Chilean coast in the red alga *Mazzaella laminarioides*, which likely correspond to cryptic species. Further studies are needed to reveal the possible existence of other cryptic species on the Chilean coast.

The results of this study provide insights into a former study of *E. braziliensis* performed by Cardoso & Defeo (2003). They found that

individuals from two distant local populations (at 23 and 39° S) of *E. braziliensis* had differences in their breeding and recruitment seasons, fecundity, and size of mature females and juveniles. According to our results, individuals from these two locations correspond to different clades, suggesting that the differences in reproductive features most likely reflect differences between two species and not within species phenotypic plasticity. Additional morphological analyses should aid in determining intra and inter-clade morphological variability of the *E. braziliensis* species complex on the coast of Chile. Lessios & Weinberg (1994) found that body length, body width, eye diameter and length of antennule were important characters to delimit species of the *E. braziliensis* complex in Panama and should also assist in morphological delimitation of *E. braziliensis* species on the Chilean coast. It is necessary to integrate and combine different sources of evidence, e.g. morphology, reproductive characteristics, and phylogenetics, to evaluate the taxonomic status of marine species biodiversity (Castro-Longoria et al. 2003, Padial et al. 2009). Target taxa should be those with low intrinsic and extrinsic dispersal potential that have a large geographic range reported, as is the case of *E. braziliensis* and other beach dwelling peracarids.

Within the detected lineages of *E. braziliensis* there was a high and significant genetic differentiation. Similarly, other marine taxa with low dispersal potential show high and significant genetic differentiation, such as the Atlantic clade of the mysid *Mesopodopsis slabberi* (Remerie et al. 2006), and Mediterranean clades of the brooding brittle star, *Amphipholis squamata* (Boissin et al. 2008). High levels of genetic differentiation within clades or species seem to be a common pattern in marine brooders, and may reflect an ongoing process of speciation.

In conclusion, *E. braziliensis* from the Chilean coast shows a similar genetic pattern to that detected in Panama for this species, for others isopods worldwide, and for few marine taxa from the Chilean coast. There are highly divergent clades that most likely correspond to a complex of cryptic species. Our study supports growing evidence of the high abundance of cryptic species within marine invertebrate taxa. Further studies in other

geographic areas where *E. braziliensis* is found (e.g., between Panama and Chile) are required to determine the number of cryptic species that make up the complex and how are they distributed in the Atlantic and Pacific coast.

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