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Divergence of cryptic species of *Doryteuthis plei* Blainville, 1823 (Loliginidae, Cephalopoda) in the Western Atlantic Ocean is associated with the formation of the Caribbean Sea

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ABSTRACT

Although recent years have seen an increase in genetic analyses that identify new species of cephalopods and phylogeographic patterns, the loliginid squid of South America remain one of the least studied groups. The suggestion that *Doryteuthis plei* may represent distinct lineages within its extensive distribution along the western Atlantic coasts from Cape Hatteras, USA (36°N) to northern Argentina (35°S) is consistent with significant variation in a number of environmental variables along this range including in both temperature and salinity. In the present study *D. plei* samples were obtained from a large number of localities along the western Atlantic coasts to investigate the distribution of these possible species in a phylogeographic context. Phylogeographic analyses were performed using the mitochondrial Cytochrome Oxidase I gene and nuclear Rhodopsin gene. Divergence times were estimated using Bayesian strict clock dating with calibrations based on fossil records for divergence from the lineage containing *Vampyroteuthis infernalis* (162 mya), the probable origins of the North American loliginids (45 mya), and the European loliginids (20 mya) and fossil statolith from *Doryteuthis opalescens* (3 mya). Our results suggest a deep genetic divergence within *Doryteuthis plei*. The currently described specie consists of two genetically distinct clades (pair-wise genetic divergence of between 7.7 and 9.1%). One clade composed of individuals collected in northwestern Atlantic and Central Caribbean Atlantic waters and the other from southwestern Atlantic waters. The divergence time and sampling locations suggest the speciation process at approximately 16 Mya, which is in full agreement with the middle Miocene orogeny of the Caribbean plate, ending up with the formation of the Lesser Antilles and the adjacent subduction zone, coinciding with a particularly low global sea level, resulting in the practical absence of continental shelves at the area, and therefore an effective geographic barrier for *D. plei*. Furthermore, this study also provides evidence of previously undocumented sub-population structuring in the Gulf of Mexico.

1. Introduction

One of the greatest challenges for marine evolutionary biology is the diagnosis of the processes responsible for genetic differentiation of populations (Pampoulie et al., 2004). Interactions between physical and biological factors such as those between ocean currents, benthic topology and dispersal capacity of different life stages of organisms (gametes, larvae, juveniles, adults) can result

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in different rates and patterns of gene flow (Weersing and Toonen, 2009; Hoffman et al., 2013; Haye et al., 2014; Villamor et al., 2014), and these in turn can greatly influence the evolutionary history of species (Hauser and Carvalho, 2008).

In theory, the presence of a planktonic life phase of marine organisms allows for a high dispersal capacity and can facilitate genetic homogeneity across the species' range (Palumbi, 1994). Evidence linking dispersion potential to reduced genetic differentiation has been found in various species (Avise, 1994). However, there are exceptions where high dispersal capacity does not result in genetic homogeneity (Gerlach et al., 2006; Teske et al., 2007). Considering these factors, invertebrates such as cephalopods that present specific biological characteristics that determine dispersal limits (association to substrates, dispersal time of different life stages, different salinity tolerances) may be important models that can be used to test hypothetical speciation processes. Cephalopods include both sessile species, and species capable of making large scale migrations. They may present internal or external fertilization, direct or indirect development and can be associated to a large range of different substrates (Roper et al., 1984; Nesis, 1987; Vecchione et al., 1998; Haimovici et al., 1994; Boyle and Rodhouse, 2005). Their distributions are often influenced by physio-chemical environmental factors that vary significantly at diverse spatial scales. These include salinity (Hendrix et al., 1981; Vecchione, 1991), ocean currents (Leite et al., 2008; Staaf et al., 2010) and the presence of specific substrates that are used for reproduction (Hanlon and Messenger, 1996; Boyle and Rodhouse, 2005).

Distinct species specific characteristics result in various patterns identified in population genetic studies of cephalopods in recent years. These include genetic homogeneity (Adcock et al., 1999; Shaw et al., 2004), isolation by distance between subpopulations (Pérez-Losada et al., 2002), and reduction in gene flow related to barriers (Shaw et al., 1999; Murphy et al., 2002). The majority of such studies involve species from northeastern Atlantic waters (Shaw et al., 1999; Murphy et al., 2002, Pérez-Losada et al., 2002; Anderson et al., 2008), northwestern and western Atlantic species (Herke and Foltz, 2002; Buresch et al., 2006; Juárez et al., 2010), and, more recently, species from the north and southeastern Pacific (Reichow and Smith, 1999; Staaf et al., 2010; Ibáñez et al., 2011; Sandoval-Castellanos et al., 2010; Ibáñez et al., 2012) and indo-west Pacific (Yeatman and Benzie, 1993; Izuka et al., 1996; Anderson et al., 2011; Cheng et al., 2014). The southwestern Atlantic remains one of the least studied regions (Adcock et al., 1999; Shaw et al., 2004; Moreira et al., 2011).

Loliginid squid are demersal, usually occupying coastal regions and limited to the continental shelf (to around 200 m depth), owing to a dependence on specific marine substrates for egg mass deposition, a characteristic considered to be important for delimiting the distribution of these species (Boyle and Rodhouse, 2005). Of the more economically and ecologically important species, *Doryteuthis plei* Blainville, 1823 is notable because of the presence of fisheries directed at this species in temperate regions of the distribution range (Jereb et al., 2010). Along with *Lolliguncula brevis* Steenstrup, 1881, this species displays the greatest geographic range for cephalopods along the western Atlantic coasts from Cape Hatteras, USA (36°N) to northern Argentina (35°S) (Jereb et al., 2010).

Phylogeographic inferences for populations of *D. plei* in the Gulf of Mexico and north-western Atlantic were made using the mitochondrial cytochrome oxidase I gene (COI), indicating the presence of sub-populations in these regions (Herke and Foltz, 2002). Furthermore, the potential for cryptic species in the genus *Doryteuthis* (*D. plei* and *D. pealei*) was suggested using mitochondrial and nuclear markers for small sample numbers from few sampling locations (Sales et al., 2013a). The present study uses both mitochondrial and nuclear markers to infer phylogeographic relationships of *D. plei* across its range to (1) determine whether *D. plei* is a single species presenting isolation by distance but with gene flow across its range or whether two, or more, cryptic species exist and (2) determine the distributional limits of any cryptic species identified and assess the potential for further sub-populations in the western Atlantic.

2. Material and methods

2.1. Sampling and laboratory methods

A total of 169 specimens were collected for the present study from various localities between Campeche in Mexico (19°13′N, 91°02′W), to southern Santa Catarina state in Brazil (27°38′S, 48°13′W) (Figs. 1 and 2, Supplementary data 1). A set of reference individuals were fixed in 10% formalin and deposited in the zoological collection of Oceanographic Museum at Federal University of Rio Grande (FURG). Additionally, 23 sequences from Herke and Foltz (2002) and three sequences from Sales et al. (2013a), were downloaded from Genbank and included in the final dataset (Supplementary data 1), resulting in a COI population analysis dataset containing sequences representing 195 individuals of *D. plei* covering the great majority of the distribution of the species (Jereb et al., 2010). All new specimens were identified using the taxonomic keys of Roper et al. (1984) and Jereb et al. (2010). Procedures for preservation of tissues, DNA extraction, amplification by PCR and cycle sequencing are the same as in Sales et al. (2013a, 2014).

In addition to the new COI *D. plei* haplotypes, COI sequences were downloaded to produce a second dataset for analyses of divergence time including sequences for: *Loligo vulgaris* (Lamarck, 1798), *Loligo forbesi* (Steenstrup, 1856), *Loligo reynaudii* (Orbigny, 1839–1841), *Doryteuthis opalescens* (Berry, 1911), *Lolliguncula brevis* Blainville, 1823, *Lolliguncula diomedeae* Steenstrup, 1881, *Sepioteuthis sepioidea* Blainville, 1824, *Ommastrephes bartramii* Lesueur, 1821; *Stenoteuthis oalaniensis* Lesson, 1880 and *Vampyroteuthis infernalis* Chun, 1903 (Supplementary data 1). New rhodopsin haplotypes for *D. plei* were also obtained for various samples using amplification by PCR and cycle sequencing as in Sales et al. (2013a, 2014) (Supplementary data 1).

2.2. Molecular diversity analyses, genetic structuring and demographic history

Sequences were aligned using ClustalW (Thompson et al., 1997), implemented in BioEdit v.7.0.4 (Hall, 1999). Visual inspection was made after every alignment to correct possible errors in the automatic alignment process. The genetic diversity indices: Haplotype number (Hap); haplotype diversity (*H*); and nucleotide diversity (π) (Nei, 1987), were estimated for each sampling locality using Arlequin v. 3.11 (Excoffier et al., 2005).

The program Geneland (Guillot et al., 2005) implemented in R, was used to estimate similarity of populations across the sampling area, using multiple runs to infer the number of population groups (clusters) utilizing the COI + Rhodopsin database concatenated following these parameters: 5,000,000 Monte Carlo Markov Chain (MCMC) iterations, sampling every 5000 iterations, and discard of the first 10% of samples as a "burn-in" phase. The best run was selected using the greatest mean posterior probability using the *post processing* function, and the "Map of population membership" and "Map of probability of population membership" produced based on populations identified under Hardy-Weinberg equilibrium with linkage equilibrium between loci (HWLE).

To confirm the clusters obtained in Geneland analyses a hierarchical analysis of molecular variance (AMOVA, Excoffier et al.,