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New molecular phylogeny of the squids of the family Loliginidae with emphasis on the genus *Doryteuthis* Naef, 1912: Mitochondrial and nuclear sequences indicate the presence of cryptic species in the southern Atlantic Ocean

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ABSTRACT

The family Loliginidae Lesueur, 1821, is currently considered to include seven genera and approximately 50 species of neritic and coastal squids. These commercially important species occur in tropical and temperate coastal waters around the world. The taxonomy of the family has been revised a number of times in recent years, focusing in particular on genera such as *Doryteuthis*, *Sepioteuthis*, *Alloteuthis*, and *Uroteuthis*, which are represented by populations in the New World, Oceania, Europe/Africa, and Asia. However, no detailed phylogenetic analysis is available for the loliginids of the southern Atlantic, in particular the genus *Doryteuthis*. The present molecular study analyzed 81 loliginid taxa from around the world. The partial sequencing of the mitochondrial 16S and Cytochrome Oxidase I genes, and the nuclear rhodopsin gene revealed a number of important patterns, recovering the monophyletic status of the majority of the genera and revealing possible cryptic species in *Doryteuthis plei D. pealei*, *Uroteuthis duvauceli* and *Sepioteuthis lessoniana*.

1. Introduction

The phylogenetic relationships and systematic arrangement of most groups of mollusks are unclear, and taxonomic inferences tend to be ambiguous or hampered by a variety of factors. One of these factors is the considerable variation in the phenotypic characters traditionally used for taxonomic analysis and the lack of reliable morphological data for the definition of specific diagnostic traits. These factors are especially relevant in the case of the cephalopods.

The family Loliginidae Lesueur, 1821, encompasses an ample group of commercially important species of neritic and coastal cephalopods. This family contains seven genera and approximately fifty species that occur in coastal oceanic waters of tropical and temperate regions worldwide (Anderson, 2000b). The taxonomy of the family has been the subject of a number of revisions in recent years. In 1988, the Cephalopod International Advisory Council (CIAC) organized a symposium dedicated to the Loliginidae, at which modifications to the traditional taxonomic scheme were proposed (Vecchione et al., 1998). The family was divided into five genera with four subgenera: *Sepioteuthis* Blainville, 1824; *Lolliguncula* (*Lolliguncula*) Steenstrup, 1881; *Lolliguncula* (*Loliolopsis*) Steenstrup, 1881; *Uroteuthis* (*Uroteuthis*) Rehder, 1945; *Uroteuthis* (*Photololigo*) Rehder, 1945; *Loliolus* (*Loliolus*) Steenstrup, 1856; *Loliolus* (*Nipponololigo*) Steenstrup, 1856; *Loligo* (*Loligo*) Lamarck, 1798, and *Loligo* (*Alloteuthis*) Lamarck, 1798. However, during subsequent years, new cladistic analyses based on morphological (Alexeyev, 1989; Anderson, 1996, 2000a) and molecular techniques (Anderson, 2000a; Brierley et al., 1996) have introduced alternative insights into the taxonomic status of the family.

In a more recent symposium on the systematics of the loliginids (CIAC, 2003), a new classification was proposed, which includes ten genera and nine valid subgenera (Vecchione et al., 2005). However, many species remain undetermined (Table 1).

The phylogenetic status of a number of genera has been tested over the years. These genera include *Doryteuthis*, found in the Americas, *Sepioteuthis* (Caribbean and Oceania), *Alloteuthis* (Europe/Africa), and *Uroteuthis* (Asia). In a phylogeographic analysis of the North American populations of *Doryteuthis* (*Loligo*) *plei* and *Doryteuthis* (*Loligo*) *pealei*, Herke and Foltz (2002) distinguished two populations representing each species within a vast

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Table 1
Current taxonomic classification of the Loliginidae (modified from Jereb and Roper, 2010).

Genus	Subgenera	Include species
Loligo		*forbesi*, *reynaudii*, *vulgaris*
Afrololigo		*mercatoris*
Alloteuthis		*subulata*, *media*, *africana*
Doryteuthis	*Doryteuthis*	*plei*, *roperi*
	Amerigo	*gahi*, *ocula*, *opalescens*, *pealei*, *surinamensis*
	Subgenus undiscribed	*sanpaulensis*
Heterololigo		*bleekeri*
Loliolus	*Loliolus*	*affinis*, *hardwickei*
	Nipponololigo	*beka*, *japônica*, *sumatrensis*, *uyii*
Lolliguncula	*Lolliguncula*	*brevis*, *argus*, *panamensis*
	Loliolopsis	*diomedeae*
Pickfordiateuthis		*bayeri*, *pulchella*, *vossi*
Sepioteuthis		*australis*, *lessoniana*, *sepioidea*
Uroteuthis	*Uroteuthis*	*bartschi*
	Aestuariolus	*noctiluca*
	Photololigo	*abulati*, *arábica*, *bengalensis*, *chinensis*, *duvaucelli*, *edulis*, *machelae*, *robsoni*, *sobogae*, *singhalensis*, *vossi*
	Subgenus undiscribed	*Pickfordi*, *reesi*

geographic range. Analyzing allozymes in two *Sepioteuthis* species in Australian waters, Triantafillos (2004) and Triantafillos and Adams (2005) detected the presence of cryptic species in both cases, and concluded that the genus is represented by a species complex in this region.

Similar patterns have arisen in the analysis of other genera. Anderson et al. (2008) reviewed the species of the genus *Alloteuthis*, based on molecular and morphological parameters, and identified a lacuna in the distribution of *A. media* between the eastern Atlantic and the Mediterranean. Sin et al. (2009) found no evidence of the presence of cryptic species in a genetic and morphological analysis of two Asian species of *Uroteuthis*, although two specimens from Australia included in the analysis appeared to represent an as yet-unidentified species.

Despite these recent advances, there has been relatively little research into the phylogenetic relationships of the loliginids of the south Atlantic, based on either molecular (Sales et al., 2011) or morphological inferences. A number of new species were described in the 1970s and 1980s, however (Brakoniecki, 1980, 1984; Cohen, 1976). The most widely studied species is *Doryteuthis gahi*, which has shown to be represented by a single genetic stock throughout its geographic range (Ibáñez et al., 2012; Shaw et al., 1999).

Doryteuthis Naef, 1912 is in fact one of the most important squid genera worldwide, from both ecological and economic perspectives. Jereb and Roper (2010) concluded that eight species occur in the New World, including members of two subgenera – *Doryteuthis* (*Amerigo*) *pealei* LeSuer, 1821; *Doryteuthis* (*Doryteuthis*) *plei* Blainville, 1823; *Doryteuthis* (*Doryteuthis*) *roperi*Cohen, 1976; *Doryteuthis* (*Amerigo*) *ocula*Cohen, 1976; *Doryteuthis* (*Amerigo*) *sanpaulensis*Brakoniecki, 1984; *Doryteuthis* (*Amerigo*) *surinamensis* Voss, 1974; *Doryteuthis* (*Amerigo*) *opalescens* Berry, 1911, and *Doryteuthis* (*Amerigo*) *gahi* Orbigny, 1835.

Cryptic species, which cannot be recognized through morphological criteria, appear to be relatively common in marine invertebrates (Knowlton, 1993; Thorpe et al., 2000). The loliginids are especially problematic due to its considerable morphological variation, which hampers the diagnosis of specimens (Vecchione et al., 2005). This emphasizes the need for the identification of possible cryptic species, in particular in cephalopod populations targeted by commercial fisheries (Triantafillos and Adams, 2005).

In recent years, taxonomic problems in a number of different cephalopod species have been resolved with the support of molecular analyses (Augustyn and Grant, 1988; Brierley et al., 1996; Brierley and Thorpe, 1994; Perez-Losada et al., 2002). These studies have focused on species from all parts of the world, including some from the coast of Brazil (Leite et al., 2008; Levy et al., 1988). However, no detailed phylogenetic analyses are available for the loliginid populations of the southern Atlantic, including those of the genus *Doryteuthis*. The objective of the present study is to verify the possible existence of cryptic species within the squid population identified as *Doryteuthis* on the west coast of the southern Atlantic Ocean, as well as to contribute to the understanding of the phylogenetic relationships among the loliginids in general.

2. Materials and methods

2.1. Samples

Molecular data were collected from 81 specimens belonging to a number of different loliginid taxa (Supplementary data 1): *Doryteuthis* (30 specimens) *Loligo* (9), *Uroteuthis* (17), *Heterololigo* (3), *Alloteuthis* (4), *Sepioteuthis* (14), *Lolliguncula* (2), and *Afrololigo* (2). The outgroup included nine taxa, for which DNA sequences (one sequence for each species) were obtained from GenBank – *Vampyroteuthis infernalis*, *Argonauta nodosa*, *Heteroteuthis hawaiiensis*, *Sepia officinalis*, *Idiosepius notoides*, *Spirula spirula*, *Cranchia scabra*, *Sthenoteuthis oualaniensis*, and *Ommastrephes bartramii*.

Specimens of *Doryteuthis* and *Sepioteuthis* were obtained from two sources in Brazil, where they were either obtained directly from artisanal fishermen or from industrial fisheries. The specimens were identified using specific morphological keys (Jereb and Roper, 2010; Roper et al., 1984). Some of the specimens were fixed in 10% formalin for subsequent morphological analyses.

2.2. Extraction, amplification, and sequencing of the DNA

Three different methods of DNA extraction were used in the present study, depending on the source of the material. Fresh samples were processed using a modified phenol/chloroform method, adapted from Sambrook and Russell, 2001, whereas a Wizard Genomics DNA Purification kit (Madison, WI) or a Quiagen DNEasy kit (Valencia, CA) was used for the specimens obtained from the stomach contents of red snappers (*Lutjanus purpureus*). Additional material stored in ethanol was pre-washed in 600 μl of bi-distilled ultra-pure water in a cooled centrifuge (Sigma Aldrich, 2K15), with two runs of 2 min at 16,000 rpm and then submitted to DNA extraction using Wizard Genomics DNA Purification Kit.

The primers used in the present study were obtained from the literature (Table 2). The PCRs were run in a final volume of 25 μl containing a mixture of 0.5 μl of each primer, 2 μl of $MgCl_2$ (25 mM), 4 μl of the dNTP mixture (1.25 mM), 5.0 μl of 5x buffer (Promega, Madison-WI USA-Tris–HCl and KCl, pH 8.5), 0.2 μl of Taq polymerase (5U/μl, Promega, Madison-WI USA), approximately 100 ng of the total DNA, with ultra-pure water to complete the final volume.

The amplification of the mitochondrial 16S gene was based on the following cycling parameters: 2 min at 94 °C for denaturation, followed by 30 cycles of 30 s at 94 °C, 1 min at 51 °C for annealing, 2 min at 72 °C for extension, and then 7 min at 72 °C for final extension (16S). For COI, the cycle was 2 min at 94 °C for denaturation, followed by 30 cycles of 1 min at 94 °C, 1 min at 45.5 °C for annealing, 2 min at 72 °C for extension, and then 7 min at 72 °C for final extension. For the rhodopsin gene, the parameters were 15 min at 95 °C for denaturation, followed by 35 cycles of 1 min at 94 °C, 1 min at 61 °C for annealing, 1 min and 30 s at 68 °C for extension, and 7 min at 72 °C for final extension. For sequencing, the samples were purified with the ExoSAP-IT enzyme (Amersham