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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

Table 2
Mitochondrial and nuclear primers used in the present study. The mitochondrial primers (16S rDNA and Cytochrome C subunit I – COI) were obtained from the literature (Palumbi et al., 1991 and Folmer et al., 1994, respectively), while those for the nuclear Rhodopsin gene were designed specifically for the present study.

Primer	Sequence
16S rDNA	
L1987	5′-GCCTCGCCTGTTTACCAAAAAC-3′
H2609	5′-CGGTCTGAACTCAGATCACGT-3′
Cytochrome C Oxidase I (COI)	
LCO 1490	5′-GGTCAAACAAATCATAAAGATATTGG-3′
HCO2198	5′-TAAAATTCAGGGTGACCAAAAAATCA-3′
Rhodopsin	
Invert fwd	5′-ARAAAATGAGCCACAGAAAG-3′
Invert bck	5′-TTSTTGYTGAGCCTGCATCTT-3′

Pharmacia Biotech Inc.). The sequencing reactions were conducting using reagents from the BigDye kit (Applied Biosystems), and the samples were then sequenced in an ABI 3500 automatic sequencer (Applied Biosystems).

2.3. Sequence alignment and statistical analyses

The sequences were aligned using the ClustalW multiple alignment tool in BioEdit v.5.0.6 (Hall, 1999), set at the default parameters (Thompson et al., 1997). Following the automatic alignment, a second file with the sequences of each of the three genes was aligned visually. In the case of 16S mtDNA ambiguous regions were eliminated using Gblocks with default parameters (Talavera and Castresana, 2007). For the phylogenetic analysis individuals genes (16S, COI, and rhodopsin) and all genes combined (16S + COI + rhodopsin) were used.

Prior to the Bayesian analyses, the best-fit model based on Bayesian information criteria (BIC) was chosen for each dataset using a perl script named MRAIC.pl created by John Nylander available in http://www.abc.se/~nylander/mraic/mraic.html (Nylander, 2004). In the case of COI for Bayesian analysis the codon based partition SRD06 model was used (Shapiro et al., 2006).

The PhyML v3.0 program (Guindon et al., 2010) was used to run the Maximum Likelihood (ML) analysis for all data sets individually and combined. A command block containing the evolutionary model based on AIC was chosen by Kakusan v.4 (Tanabe, 2007) for the combined dataset (phyml – run_id whole_AIC_bootstrap – input data.phy – datatype nt – sequential – search BEST – o tlr – bootstrap 1000 – model 012345 – f e – alpha e – nclasses 4). The reliability of the groups formed by the PhyML analysis was checked on the basis of 1000 non-parametric bootstrap pseudoreplicates (Felsenstein, 1985) specified in the command block above.

The Bayesian Inference (BI) of individual genes and multilocus multispecies coalescent Bayesian tree were performed in Beast v. 1.7.5 and in *Beast (Drummond et al., 2012) with model parameters selected by the script MRAIC The MCMC chains were run for at least 200 million generations, and sampled every 20,000 steps. Tracer version 1.5 (utility program distributed with the Beast package) was used to check for convergence on the basis of the effective sampling size (ESS > 200) after a 10% burn-in. According to Drummond et al. (2006), Tracer allows visual inspection of the MCMC chains behavior and estimation of the effective sample size of the parameters. If the effective sample sizes of all continuous parameters are greater than 200 this provides a measure of whether the chain has been run for an adequate length. Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals). The sample of trees was summarized by TreeAnnotator v. 17.5 (utility program distributed with the Beast package) using a 10% burnin.

3. Results

The approximate size of each amplified gene segment and the optimal evolutionary model varied for each dataset and are presented in Table 3.

The phylogenetic trees generated from the sequences of the COI mtDNA and rhodopsin nuclear genes proved to be very effective in recovering the phylogenetic trees. However 16S mtDNA after eliminating ambiguous regions of the alignment proved to be not very useful, this effect is accentuated by the high level of saturation detected (not shown). The Bayesian phylogenetic tree based on mtDNA COI resolved well the most recent relationships, such as the definition of species but not the most of the oldest ones including the monophyletic status at the genus level.

The multispecies coalescent tree (Fig. 1) using rhodopsin nucDNA, 16S mtDNA and COI mtDNA confirmed the monophyly of all genera analyzed (*Sepioteuthis*, *Uroteuthis*, *Loligo*, *and Aloteuthis*) (Fig 1) but *Doryteuthis* appears not to be monophyletic if *Lolliguncula* was not included in the clade (Fig. 1). However, COI alone and the concatenated likelihood analysis of the three genes recover *Doryteuthis* as monophyletic having *Lolliguncula* as sister group (weakly supported) (Figs. 3S and 4S).

In regarding to the intergeneric relationships the multispecies and concatenate tree places *Sepioteuthis* as the most basal genus of the Loliginidae family (Figs. 1 and 3S). However concatenated places *Sepioteuthis australis* as a sister group of all genera. For the remaining genera two groups could be observed (Fig. 1), the first composed by *Uroteuthis*, *Loligo*, *Afrololigo*, and *Aloteuthis* (credibility of 92%) and the second comprised by *Heterololigo* and *Doryteuthis*/*Lolliguncula* (credibility of 85%).

At the species level, the Brazilian specimens identified as *D. plei* were genetically distinct of those from northern and central America (Fig. 2). Furthermore, the multispecies analysis recovered a strongly supported group formed by the two divergent *D. plei* lineages plus *D. surinamensis*. Similarly to the pattern observed in *D. plei*, the Atlantic *D. pealei* specimens were also grouped in two well-supported divergent branches, with North Atlantic specimens being well differentiated from those from the South Atlantic.

In relation to the three *Loligo* species (*L. reynaudii*, *L. vulgaris* and *L. forbesi*) analyzed in the present study *L. vulgaris* and *L. reynaudii* are a sister group significantly supported (Figs. 1 and 2). *Alloteuthis* and *Afrololigo* are sister genera, with good support for the species of each genus.

The multispecies and concatenated tree recovered two monophyletic groups in the genus *Uroteuthis* significantly supported (Fig. 1 and 4S), one formed by *U. chinensis* and *U. Photololigo sp*. and other by *U. sibogae* and *U. duvauceli.* COI tree with more density of taxa corroborate this arrangement but do not clarify the relationships among the additional species analyzed (Fig. 3S).

Sepioteuthis currently contains three species, one of which is found in South America, a second restricted to Australia, and a third found throughout the Indian Ocean and western Pacific.

Table 3
Analytical parameters used in the present study: number of base pairs (bp) and evolutionary model (EM) selected by MRAIC script for individual genes (16S, COI, and rhodopsin) and the combined data sets (16S + COI + Rhod).

Partition	BP	EM (BIC)
16S	412	HKYIG
COI	600	HKYIG
Rhod	653	SYMIG
Concatenated	1665	GTRIG

GBlocks reduced the original 16S mtDNA dataset from 546 to 412 bp after elimination of ambiguous regions of the alignment. The EM for maximum likelihood analysis was chosen by Kakusan script.

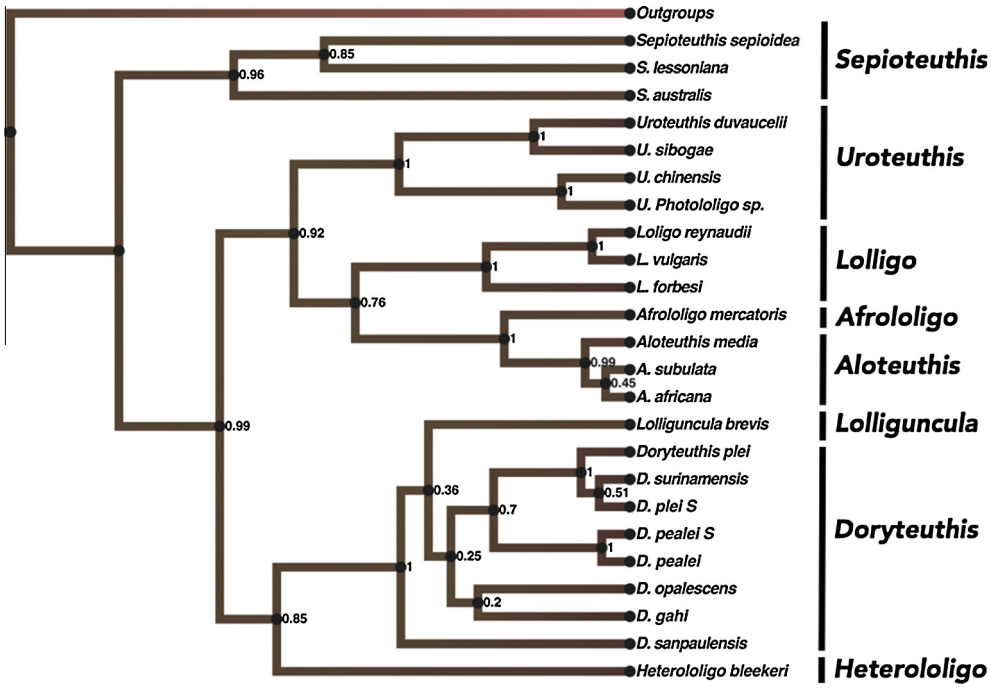

Fig. 1. Multi-species coalescent Bayesian tree obtained for nine genera of Loliginidae based on one nuclear and two mitochondrial genes from 57 individuals. Clade posterior probability in indicated at each node.

The multispecies tree showed a closer relationship between *S. sepiodea* and *S. lessoniana* having *S. australis* as sister group. However the topology generated by COI (Fig. 2) and concatenad (Fig. 3S) did not recover the monophyly of *Sepioteuthis*, but placed *S. australis* very far from the two putative sister species.

4. Discussion

Anderson (2000a) provided the first detailed genetic analysis of the loliginids using sequences of the mtDNA COI and 16S. In a more recent study, Strugnell et al. (2005) combined data from mitochondrial and nuclear DNA sequences, highlighting some contradictions between the phylogenetic reconstruction of mitochondrial and nuclear data and for the arrangements using only data from morphology. The results obtained in the present study for the multispecies coalescent tree using sequences of mitochondrial and nuclear genes are generally consistent with the topology recorded in these earlier studies.

4.1. Sepioteuthis

Curiously, Anderson (2000a) and Strugnell et al. (2005) also reported a paraphyletic arrangement for the genus *Sepioteuthis* using sequences of the mitochondrial data, in contrast with phylogenetic analyses using morphological and nuclear data that confirmed the monophyletic status of this genus.

All three species of *Sepioteuthis* share semi-oval fins which surround the mantle completely, as well as elongated papillae in the row of the dorsal, rather than ventral suckers of the hectocotylus (Anderson, 1996, 2000b). In a more recent molecular phylogenetic study of the modern coleoids, Strugnell et al. (2005) analyzed a set of six genes, three mitochondrial (12S, 16S, COI) and three nuclear (ODH, pax-6, rhodopsin), but they were only able to confirm the monophyly of *Sepioteuthis* in the case of the nuclear genes. This incongruence between mitochondrial and nuclear phylogenies due to the mtDNA limitation was also observed in the present analysis.

Even assigning weights to the different positions of the mitochondrial COI gene it was not possible to recover the monophyly of *Sepioteuthis* in this study. One possible explanation for this is the relatively high evolutionary rate of the mitochondrial genes which is reflected in the relative length of the branches in the mitochondrial in comparison with the nuclear topology and the incomplete lineage sorting (a failure of ancestral gene copies to coalescens copy until earlier than the previous speciation event (Meng and Kubatko, 2009) or even saturation. The genealogical history of individual gene loci may appear misleading or uninformative about the relationship among species or populations due the retention and stochastic sorting of ancestral polymorphism (Maddison and Knowles, 2006).

The present analysis further support the hypothesis that *S. lessoniana* is actually a species complex, as indicated by previous genetic and morphological studies. In Okinawa, Japan, local fishermen have traditionally identified three groups based on the external morphology and quality of the meat (Okutani, 1984). Okutani (2005) reported the existence of three different forms: 1-*Sepioteuthis lessoniana sensu stricto*, widely distributed in the western Indo-Pacific; 2-*Sepioteuthis lessoniana* "the Akaika form" found in Japanese waters south of Shikoku and the Nansei-Shoto Islands; and 3-*Sepioteuthis lessoniana* "the Kwaika form" which ranges between the region of Ogasawara and the Nansei-Shoto Islands. Triantafillos and Adams (2005) have also suggested that *S. lessoniana* from northeastern Australia may constitute a species complex. The present study includes specimens from Durban, South Africa, which appear to correspond to the *sensu stricto* form of Okutani (2005), while the rest of the sample is from Indonesia and unknown localities. It was possible to observe two distinct groups in the mitochondrial COI tree (South Africa: Sles1, Sles6, Sles20, Sles21 and Indonesia: Sles2, Sles3, Sles4, Sles5, and SlesA) (Fig 2).

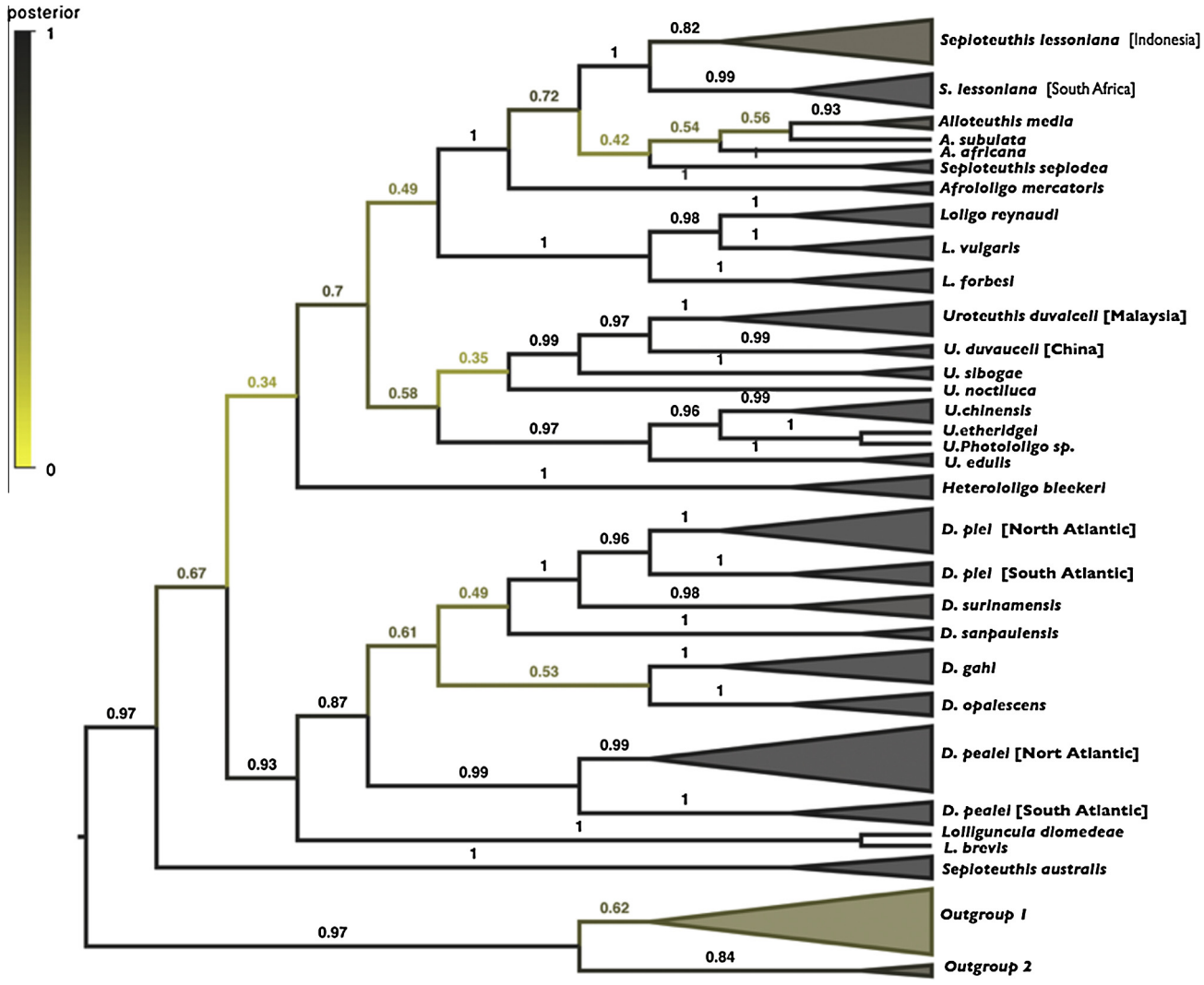

Fig. 2. Bayesian tree based on mitochondrial cytochrome oxidase I (COI) gene from 89 specimens belonging to nine genera of Loliginidae. Clade posterior probability is showed at each branch.

4.2. Uroteuthis

The presence of a pair of photophores on the ventral surface of the ink sac in all the *Uroteuthis* specimens is possibly the most reliable morphological character for the identification of the species of this genus. Until recently, however, *Uroteuthis* was not universally accepted as a valid genus (Jereb and Roper, 2006). With regard to *U. chinensis*, the specimens from Malaysia used in the present study grouped together with specimens from gulf of Thailand reported by Anderson (2000b). This geographic pattern may represent a species complex as proposed by Yeatman and Benzie (1993), although Sin et al. (2009) found no evidence of the presence of cryptic species within a vast area of the geographic range of *U. chinensis*.

Another form that may contain cryptic species, *U. duvauceli*, presents considerable morphological and genetic polymorphism (Sin et al., 2009) within a relatively ample geographic range. The possible presence of cryptic species is further reinforced by the results of the present study. When the sequences obtained here (Malaysia) are compared with those available in GenBank, derived from specimens collected in China, two well-supported geographic clades were found (Fig. 2). Different forms of *U. duvauceli* are recognized by commercial fisheries, including a relatively slender form and a more robust one from the eastern Pacific (Okutani, 2005), and two others – one large and the other smaller – from the Gulf of Aden and the Arabian Sea (Nesis, 1982/1987). The results of the present study support the necessity of further morphological and genetic studies of specimens collected over a wider area for a more conclusive understanding of the variability found in *U. duvauceli*.

4.3. Doryteuthis

The New World *Loligo* species were recently allocated to *Doryteuthis*, based primarily on the paraphyletic position of *Loligo*, in which the New World, European, and Asian specimens were arranged in distinct geographic groups (Anderson, 2000a,b; Vecchione et al., 2005). Up until now, however, no diagnostic morphological trait has been identified for a reliable distinction of these genera (Jereb and Roper, 2010).

In the present study, only two of the recognized species (*D. roperi* and *D. ocula*) were not included in the analysis, given the lack of specimens or GenBank sequences. Even so, the evidence was clear on the separation of the specimens of *D. plei* from the northern and southern Atlantic, with quite high genetic distances (data not shown) of 1.9% (16S rDNA), 7.7% (COI), and 0.6% (rhodopsin). There were also observed clear differences between North and South American *D. pealei*, with genetic distances of 1.5% (16S rDNA), 4.9% (COI), and 1.1% (rhodopsin) (data not shown).

The present study included specimens of *D. plei* of southern Atlantic, from the northernmost Brazilian state of Amapá to the southern state of Santa Catarina. All these specimens diverged significantly from those collected in the North Atlantic (data not shown). *A priori*, the morphology of the South American specimens is consistent with that of *D. plei*, including the longitudinal lines in the anterior ventrolateral region of the adult males (personal observation). A similar degree of consistency was found in the morphology of the *D. pealei* specimens from the southern and northern Atlantic.

Herke and Foltz (2002) analyzed COI sequences and RFLPs in *D. plei* and *D. pealei* from the central and northern Atlantic, and identified "genetic breaks" within the distribution of each species. In *D. plei*, the break appears to coincide with the discharge of the Mississippi River, which separates the populations from the northwestern Gulf of Mexico and the northwestern Atlantic. In *D. pealei*, this break was located between the northern Gulf of Mexico and the Atlantic.

Given the lack of significant oceanic barriers within the geographic ranges of the two species, the principal factor determining the genetic differences between the specimens from the northern and southern Atlantic may be the discharge of freshwater from the mouth of the Amazon River in northern Brazil. This may constitute an effective barrier to the dispersal of cephalopods, given that the majority of species, except *L. brevis*, are typically stenohaline (Vecchione, 1991).

4.4. Doryteuthis/Lolliguncula

The grouping of *Lolliguncula brevis* within the *Doryteuthis* clade in the multispecies coalescent analysis including the rhodopsin sequences is very intriguing. The genus *Lolliguncula* was validated on the basis of the morphological differentiation of *L. brevis* from the *Loligo* species recognized at the time, related to the shape of the body and fins, and the distribution pattern of the spermatophores, in addition to the fact that the members of this genus are characterized and differentiated from other loliginids by the long cement body present in their spermatophores (Vecchione et al., 1998). An additional feature of the species is its euryhaline behavior, which enables it to tolerate much lower levels of salinity than other loliginids (Vecchione, 1991). The present study provided some support for the inclusion of *Lolliguncula brevis* into the *Doryteuthis* clade, but considering the contrasting results between mitochondrial and nuclear genes, this issue deserves an additional more dense genomic analysis.

4.5. Doryteuthis/Heterololigo

The sister relationship of *Doryteuthis* and *Heterololigo* recovered in the multispecies analysis including nuclear and mitochondrial markers, although with lower support, is in agreement with the previous mitochondrial topology of Anderson (2000b). According to this author, the ancestor of *Heterololigo* and *Doryteuthis/Lolliguncula* was a more muscular form that gave origin to a less muscular lineage that can tolerate much lower salinity, and a more muscular swimmer lineage that can live in more saline environment (*Doryteuthis*). Perhaps the Glaciations have influenced the appearance of less migratory species able to tolerate much lower levels of salinity. The estimation of divergence time between all those genera can shed new light in the cladogenesis of Western Atlantic species of *Heterololigo*. *Lolliguncula* and *Doryteuthis*.

4.6. Cryptic species

The phylogenetic reconstructions presented here also indicate the possible presence of more cryptic species in other loliginids, such as *U. duvauceli* and *S. lessoniana*, although more specimens or sequences will be required in order to investigate this phenomenon more conclusively. In the specific case of *Doryteuthis*, the lack of recent studies of *D. roperi* and, in particular, *D. ocula* brings the validity of these species in skepticism, since no one individual from this two species was sampled in the present study, as well as no other genetic or morphological inferences were made about these two species since their description. Further inferences on the loliginid fauna in the Caribbean region as well as in the central and southern Atlantic will be required for the genetic validation of these two species.

5. Conclusions

This study clearly demonstrated the monophyly of the majority of genera in the Loliginidae family, with the exception of *Doryteuthis* that should include *Lolliguncula*. The data also suggest the presence of cryptic species mainly within the American genus *Doryteuthis* where the specimens of *D. plei* and *D. pealei* species from South Atlantic are genetically distinct from specimens that occur in the Northern Hemisphere of the Atlantic of each species, respectively. The phylogenetic reconstructions presented in this study also suggest the possible presence of more cryptic species within other loliginid species like *U. duvauceli* and *S. lessoniana*. Based on the patterns observed in the present study, all loliginids with a relatively ample geographic distribution should be subject to more systematic genetic and morphological analyses in order to establish the possible occurrence of cryptic species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.03.027.

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