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Phylogeny and Population Genetics of Allotheuthis (Loliginidae) and Discovery of a Cryptic Species

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Introduction

Alloteuthis is a genus of small loliginid squids of minor fisheries importance found in the eastern Atlantic Ocean and the Mediterranean Sea. The three recognized species of *Alloteuthis* are *A. subulata* (Lamarck, 1798) (found from the North Sea to the coast of Morocco and east to the Aegean Sea, but uncommon in the Mediterranean), *A. media* (Linnaeus, 1758) (range largely overlaps with the range of *A. subulata*, but common in the Mediterranean and rare in the Atlantic), and *A. africana* (Adam, 1950) (found along the Atlantic coast of Africa from the southern coast of Spain to Angola) (Nesis, 1987) (Figure 1).

The presence of a "tail"—a slender, often sharply pointed extension of the mantle posterior to the fins—and a small conus on the posterior tip of the gladius are important characteristics that distinguish *Alloteuthis* from other loliginids. The three *Alloteuthis* species can generally be distinguished from one another using several morphological traits (Table 1). *A. africana* has a tail length of 5-6 centimeters (cm) and a total mantle length of up to 20 cm. The length of the fins is more than 50% of the mantle length, and the arm length is 15-20% of the mantle length. *A. africana* also has central tentacular club suckers that are perpendicular to the club axis, with the largest club sucker being 6-8% of the head width. By contrast, *A. media* has a short tail—usually less than 1 cm—and its fin length is always less than 50% of the mantle length. Like *A. africana*, the central club sucker arrangement of *A. media* is perpendicular to the club axis, but the largest club sucker is 9-14% of the head width. *A. subulata* has a long tail (5-6 cm), a fin length that is typically greater than 50% of the mantle length, and the largest club sucker is 6-8% of the head width, as seen in *A. africana*. Unlike *A. africana*, though, the central club suckers of *A. subulata* are arranged obliquely relative to the club axis, and *A. subulata* has an arm length that is 20-25% of the mantle length. Overall, *A. africana* tends to be longer than

A. subulata, due to the presence of the tail. Tail length is measured from the posterior end of the animal to the posterior tips of the fins (by contrast, fin length is measured from the posterior end of the animal to the *anterior* tips of the fins).

A. media and *A. subulata* are typically distinguished from one another by comparing relative fin length. Relative fin length is obtained by dividing the fin length by the mantle length (mantle length is measured from the posterior end of the animal to the anterior tip of the mantle). *A. subulata* has longer fins, relative to body length, than *A. media*. Despite the apparent morphological differences between these two species, Laptikovsky et al. (2002) have suggested that these two species are simply different forms of a single species. To investigate this possibility and to resolve phylogenetic and phylogeographic patterns within *Alloteuthis*, I generated and analyzed sequence data for two mitochondrial gene regions from representatives of each *Alloteuthis* species that were collected from several localities throughout their ranges.

Materials and Methods

Molecular Phylogenetic Analyses

Whole squid specimens were collected from the coasts of Turkey, Italy, Portugal, France (both Mediterranean and Atlantic coasts), Spain, Angola and Mauritania and shipped to SIU in 95% ethanol. DNA was extracted from small tissue samples of 45 animals using DNAzol (Molecular Research Center). Regions of two mitochondrial genes—cytochrome oxidase subunit I (COI) and the ribosomal RNA large subunit (16S)—were amplified via PCR using HotStar Master Mix (QIAGEN) and universal metazoan primers (Folmer et al., 1994; Geller et al., 1997) following manufacturer's protocols (half-reactions). PCR conditions for COI were: 15 min at 95°C; 35 cycles of 94°C for 1 min, 50°C for 1 min, 68°C for 1 min 30 sec; and a final

extension of 7 min at 72°C. PCR conditions for 16S were: 15 min at 95°C; 35 cycles of 94°C for 45 sec, 50°C for 45 sec, 68°C for 1 min; and a final extension of 7 min at 72°C. When reamplification of PCR products was necessary, a "stab gel" method was used, in which the original PCR products were run out on a 1% TAE gel and a needle was used to stab the PCR product band in the gel. The agarose plug captured in the needle (containing some of the PCR product) was then used as a template for another round of PCR. For reamplifications, the PCR cycling parameters were modified: the number of cycles was lowered from 35 to 25 and the final extension temperature was dropped to 68°C for a total of 10 minutes (for both COI and 16S). PCR products were gel-purified using a MinElute gel extraction kit (QIAGEN) following manufacturer's protocols, and the gel-purified PCR product was directly sequenced using BigDye Terminator Ready Reaction Mix (Applied Biosystems) (quarter-reactions). Sequencing reactions were run out on an ABI 377 Prism automated DNA sequencer. Sequences were assembled and edited using Sequencher v4.1 (GeneCodes) and sequences for all individuals were aligned using ClustalX (Thompson et al., 1997), with modifications of the alignment performed manually in Se-Al v. 2.0a11 Carbon (Rambaut, 1996).

Several phylogenetic analyses were run using individual, combined, and partitioned data sets, with *Afrololigo mercatoris* (the closest known relative of *Alloteuthis*; Anderson, 2000) used as an outgroup. Phylogenetic trees were generated for the COI data and 16S data separately and in combination via maximum parsimony (heuristic search with 100 random addition sequence replicates and TBR branch swapping, holding ten trees at each addition step) (PAUP* 4.0b10; Swofford, 2002) and Bayesian analyses (MrBayes 3.1.2; Ronquist and Huelsenbeck, 2003). Bayesian analyses were performed as follows: 1) COI only, partitioned by codon position (substitution models: 1st position: SYM + I, 2nd: F81, 3rd: HKY; 119,550 generations; 2,392 trees

per run; 1,794 trees post burn-in; 4 runs; 9568 trees total); 2) 16S only (model: HKY; 685,500 generations; 1372 trees per run, 1029 trees post burn-in; 4 runs – 4116 trees total); 3) COI (partitioned by codon position) + 16S (models: COI 1^{st}: SYM + I, 2^{nd}: F81 + I, 3^{rd}: GTR + G; 16S: HKY85 + I + G; 4 runs; 2,852,500). In all partitioned analyses, branch lengths were linked across partitions, but all other model parameters were unlinked. All Bayesian analyses consisted of four independent runs, each consisting of four MCMC chains. Each analysis was set to run for 10 million generations, but the analysis was automatically terminated when a topological convergence diagnostic value (the average standard deviation of tree partition frequency values across all four runs) dropped below 0.01. 25% of the trees from each of the four runs were removed as burn-in and the remaining post burn-in trees were combined to produce a 50% majority-rule consensus tree. Substitution models for Bayesian analysis were chosen using MrDT-ModSel, a modification of DT-ModSel (Minin et al., 2003) developed by F. Anderson. Strength of support for various clades was assessed via parsimony bootstrapping (100 bootstrap pseudoreplicates, each analyzed with ten random addition sequence replicates and TBR branch swapping, holding ten trees at each addition step) (Felsenstein, 1985) and calculation of posterior probabilities. Clade posterior probabilities were drawn directly from the values on the 50% majority-rule consensus tree for each Bayesian analysis; trees sampled after the burn-in phase for each analysis are assumed to be drawn from the tree posterior probability distribution.

Morphometric data collection

Morphometric data was collected from all sequenced specimens of *A. media* and *A. subulata*. These measurements included dorsal mantle length, fin length, head width, maximum club sucker size and sex. All data were collected using calipers and a dissecting microscope.

Fin length as a percentage of mantle length was calculated from these data for each specimen and graphed to assess the validity of using this measurement (and to support or refute Laptikhovsky's hypothesis) as a feature to classify an animal as *A. media* or *A. subulata.*

Hypotheses

If *A. media* and *A. subulata* are distinct, reproductively isolated species, I hypothesized that 1) phylogenetic analysis of two mitochondrial genes would reveal three strongly supported, reciprocally monophyletic groups: one corresponding to *A. africana*, one corresponding to *A. media* and one corresponding to *A. subulata* (with possible phylogeographic breaks within one or both species corresponding to Atlantic and Mediterranean populations of *A. media* and *A. subulata*) and 2) there would be a clear discontinuity between *A. media* and *A. subulata* specimens in the fin length by mantle length plot.

Results

A plot of fin length vs. mantle length for all sequenced specimens does not reveal a clear separation between specimens assigned to *A. media* and *A. subulata* (Figure 2). Originally, these two species were distinguished based largely on the fin length/mantle length comparison: individuals falling above the 50% mark were considered to be *A. subulata*, while those falling below 50% were classified as *A. media*. The morphometric data from the samples collected for this study shows a continuum of measurements and lacks a sharp demarcation between these two "species." A second common measurement used to differentiate all three species is club sucker width as a percentage of head width. If this criterion were used with the samples studied here, all of the specimens would be considered *A. media* (data not shown). However, some specimens

were immature and the measurement method used was rather crude, so club sucker width measurements are probably not accurate, and these measurements cannot be used to assign species here.

Phylogenetic trees resulting from maximum parsimony and Bayesian analysis were congruent. These trees show three distinct, strongly supported clades within *Alloteuthis* (Figures 3-5). However, these groups do not correspond with the three traditionally recognized species. One clade consists of individuals collected from Angola and Mauritania (posterior probability = 1.0, parsimony bootstrap support value = 100). This clade corresponds to the named species *A. africana*. A second, larger clade includes individuals currently recognized as *A. subulata* and *A. media* (posterior probability/parsimony bootstrap support values = 1.0/99), but members of the two species (as assigned by morphological data) do not form two reciprocally monophyletic groups in any analysis (Figures 3-5). Within this *media/subulata* group there are hints of geographical structure—there is some support for a group including all specimens sampled from the east Atlantic and all specimens sampled from the Mediterranean (Figure 5). Finally, a third cryptic clade is revealed that consists of individuals found thus far only in Italian waters (Adriatic Sea) that morphologically resemble *A. media* but are, in fact, genetically distinct from both the *A. media/A. subulata* group and the *A. africana* species (posterior probability/bootstrap support values = 1.0/100).

Discussion

The results of this study suggest that *A. africana* is genetically distinct from both *A. media* and *A. subulata*. Both parsimony bootstrap and Bayesian posterior probabilities are high for this clade. The distinctive morphology of this group—namely the very long tail—and almost

completely disjunct geographic distribution from the other members of *Alloteuthis* (Figure 1) also lends support to this conclusion.

A. media and *A. subulata* were originally thought to be two separate species based on morphological differences, but more thorough morphological comparisons and phylogenetic analysis of their mitochondrial DNA suggest that there is no clear separation between specimens assignable to "*A. media*" and "*A. subulata*". This suggests that these two species may in fact be a single species that simply exhibits morphological variation. Overall, these two "species" do not appear to differ drastically from one another when compared using either morphological or genetic data. The plot of fin length vs. mantle length reveals a continuum of measurements, implying that these animals are showing not genetic differences, but simply a disparity in growth rates. Allometric growth may account for the morphological differences seen in this species (F. Anderson, pers. comm.). It is known that *Alloteuthis* has fins that extend onto the tail; therefore, the length of the fins relative to the body increases very quickly during sexual maturity (Nesis, 1987). The "*A. subulata*" group tends to be larger at maturity and possesses relatively long tails and short tentacles, while "*A. media*" is smaller with relatively short tails and long tentacles. "*A. subulata*" is generally found in the eastern Atlantic, and thus lives in a cooler environment where it may take longer to reach sexual maturity. "*A. media*" tends to be found in the Mediterranean, a shallower and slightly warmer body of water than the eastern Atlantic. Thus, "*A. media*" lives in an environment where it may mature more quickly. This might explain the positive allometric growth of the tail and negative allometric growth of the tentacles. A study by Villanueva *et al* (2003) looked at the length of embryonic development of *Loligo vularis*—a loliginid species with a geographic range similar to that of *Alloteuthis*—from the Eastern Atlantic Ocean and Mediterranean Sea, as a result of the effect of water temperature on

metabolism and growth. They found that the time of egg laying until hatching could last weeks to months, with lower temperatures (like in the Atlantic) corresponding to longer development time and larger hatchlings, while higher temperatures (in the Mediterranean) led to a shorter development time and smaller hatchlings. It is possible that the pattern of embryological development with respect to water temperature seen in *L. vulgaris* could apply to and partly explain the morphological extremes within the *media/subulata* group.

A slight geographic separation is apparent within the *A. media/A. subulata* clade—there is some support for an Atlantic subclade and a Mediterranean subclade. However, the distinction between Atlantic and Mediterranean members of this group is poorly supported at best (Figure 5), and probably does not reflect a difference between two species. If the inferences drawn from the morphological and molecular comparisons described here are correct—i.e., *A. media* and *A. subulata* are actually a single species—the valid name for the "*A. media/A. subulata*" clade is *Alloteuthis media* (Linneaus, 1758), which has priority over *A. subulata* (Lamarck, 1798).

The third as-yet-unnamed clade (Figures 3-5) may constitute a cryptic species. These specimens are superficially morphologically indistinguishable from *A. media*, but are clearly genetically distinct, and bootstrap support values and posterior probabilities are very high for this grouping. If this is a valid interpretation of the phylogenetic patterns shown in the trees presented here, cryptic speciation within a morphologically homogenous population seems to have occurred. This cryptic species is, at present, only detectable through molecular phylogenetic analysis. The mode of speciation for this group is still in the early stages of speculation. It could be that these particular animals live at a different depth than others found in the same location, and thus in a sense are geographically isolated from populations of the *media/subulata* clade. However, members of both clades were collected in the same trawls (G.

Bello, pers. comm.). The origin of this cryptic species is unclear. It is possible the divergence between the cryptic species and the *A. media/A. subulata* clade occurred during a glacial episode when the Mediterranean Sea may have dried up, leaving only isolated pools of water. This could have been a prime opportunity for allopatric speciation to occur. Further research may reveal more explanations. Whatever the driving force was that caused this event, it seems clear that this cryptic species diverged from the ancestor of the *media/subulata* clade long ago, given the deep evolutionary split seen in the phylogram. Thus far, members of this cryptic species are morphologically indistinguishable from members of the *media/subulata* clade. We are initiating detailed morphological work on the internal anatomy of this cryptic species that may reveal differences from *A. media.*

This data set is an excellent example of two intriguing evolutionary phenomena: 1) a pair of superficially morphologically distinct "species" ("*A. media*" and "*A. subulata*") that are genetically nearly identical, and 2) probable cryptic speciation within an apparently homogeneous population. The results of this study present the first phylogenetic hypothesis for *Alloteuthis* and will serve as the groundwork for reorganizing *Alloteuthis* taxonomy. On a larger scale, this study emphasizes the importance of genetic data in revealing previously hidden biological diversity.

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Figure 1. Range of *Alloteuthis* species in the Eastern Atlantic Ocean and the Mediterranean Sea. A – *A. media*, B – *A. subulata*, C – *A. africana*.

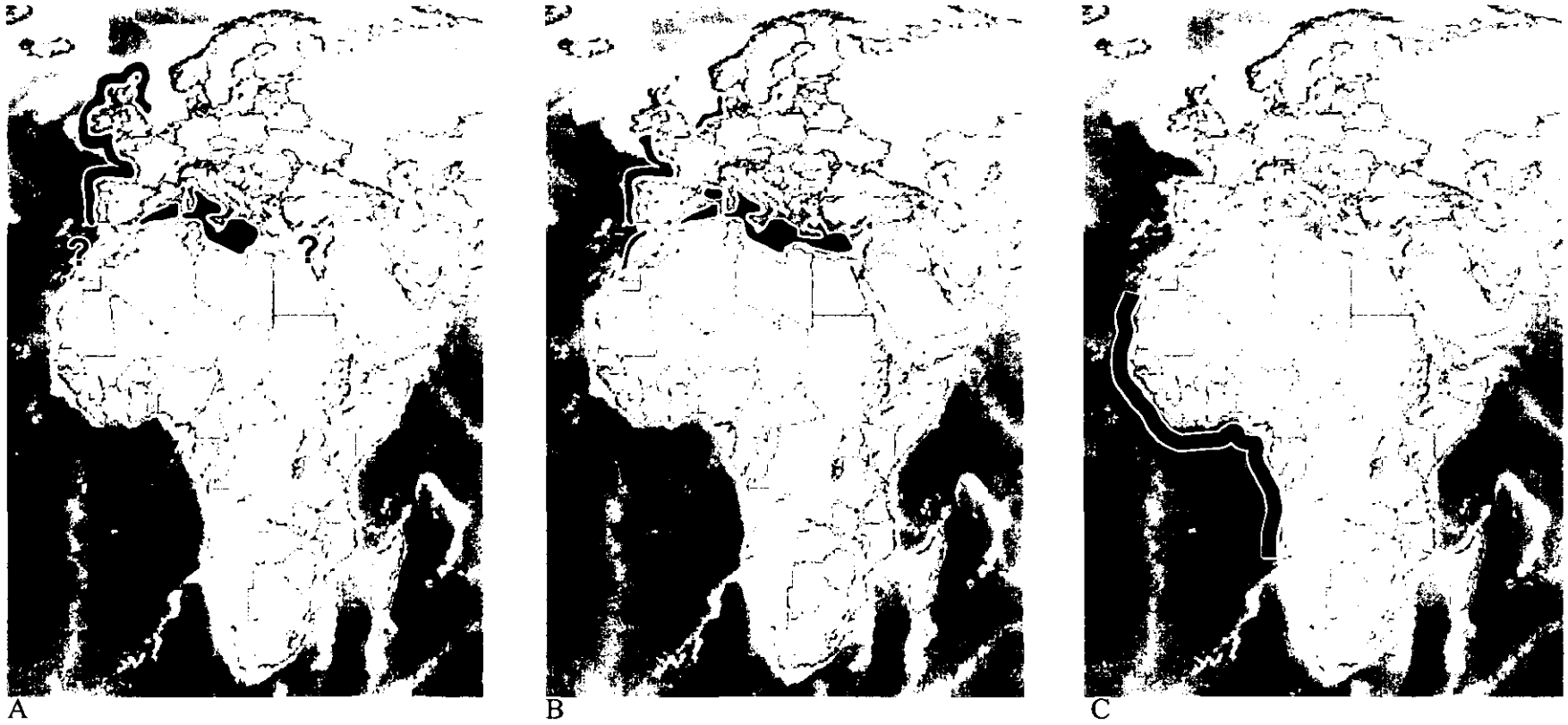


Figure 2. Plot of relative fin length for specimens of *A. media* and *A. subulata*.

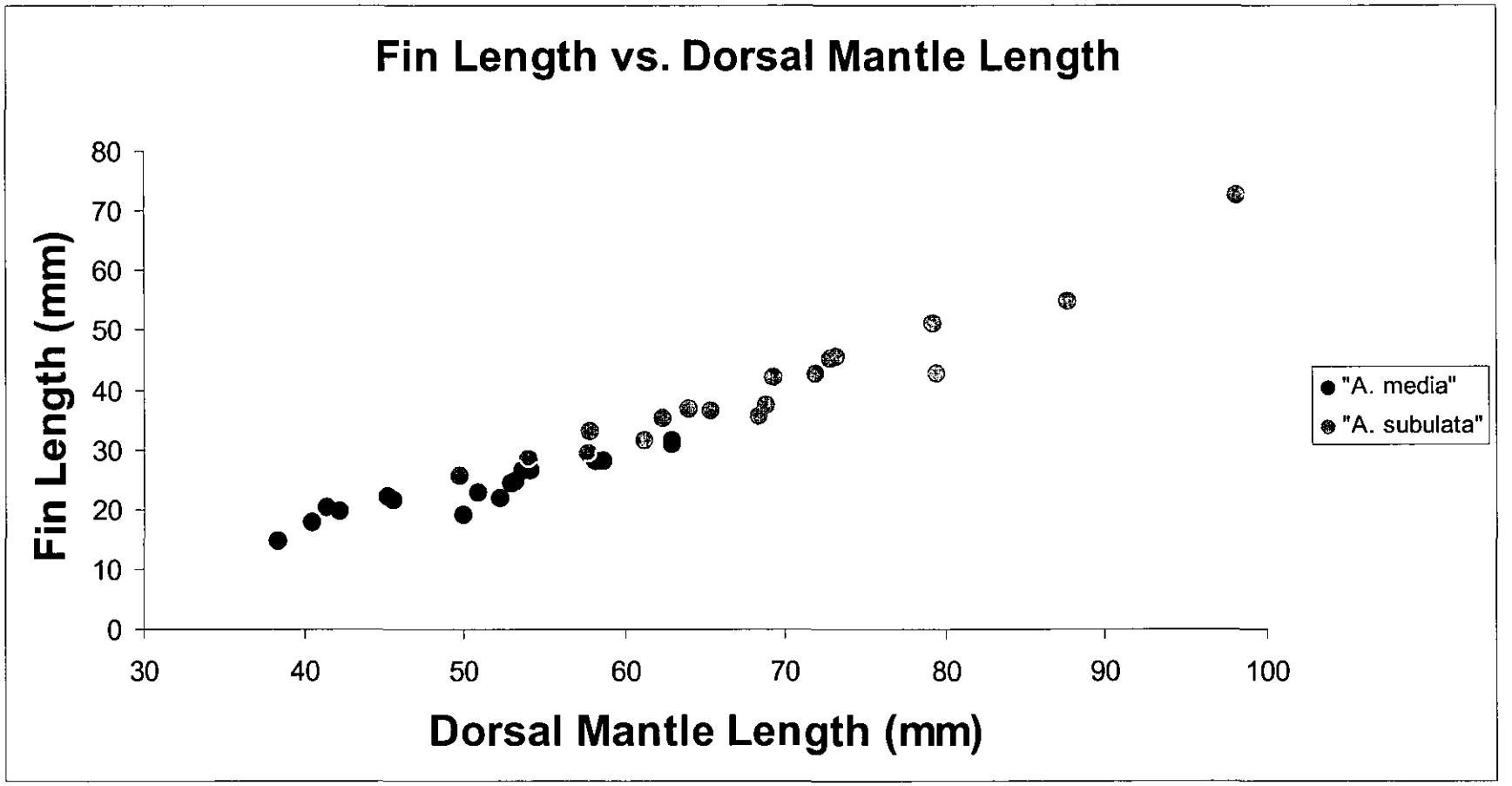


Figure 3. Phylogram of COI data partitioned by codon position.

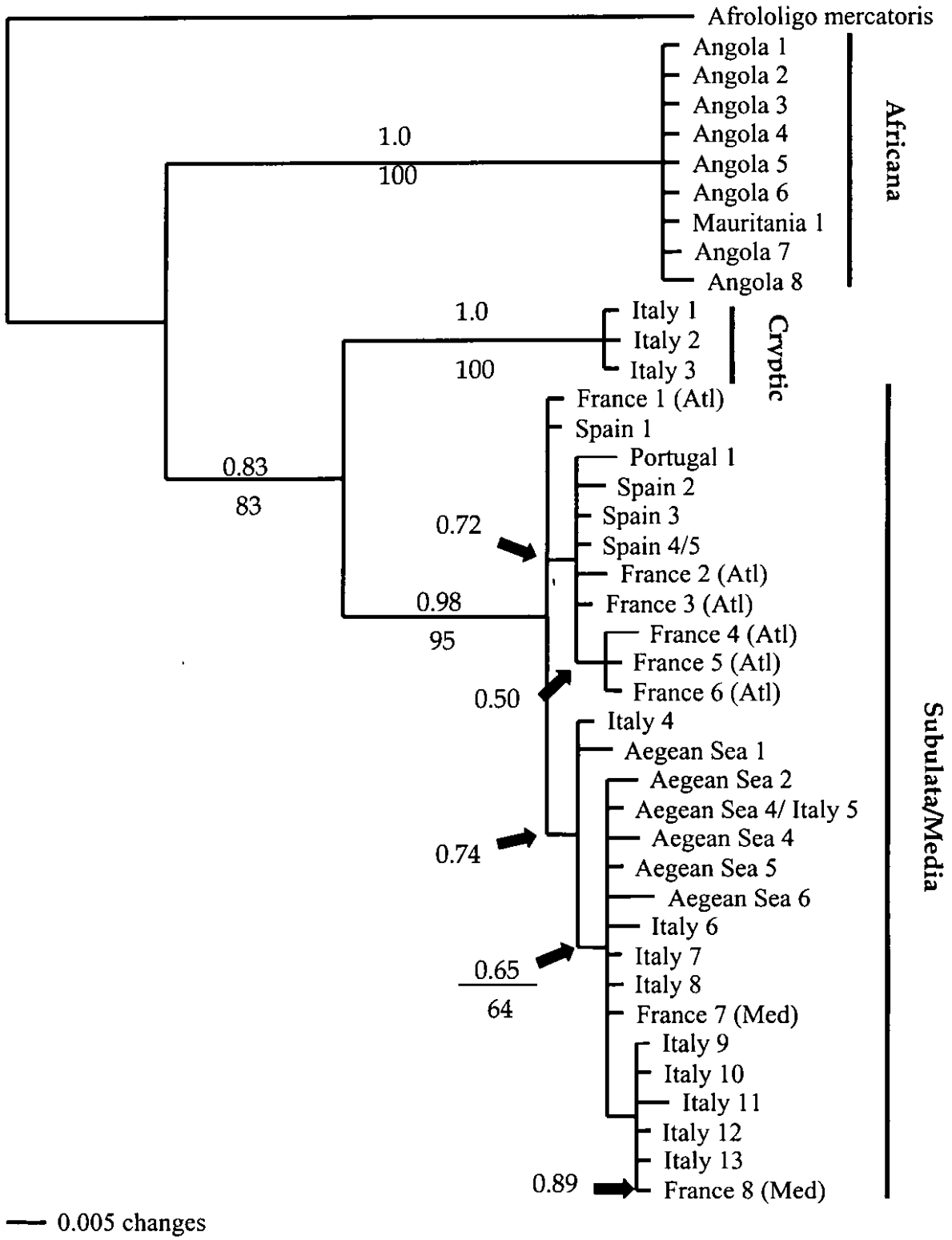


Figure 4. Phylogram of 16S data.

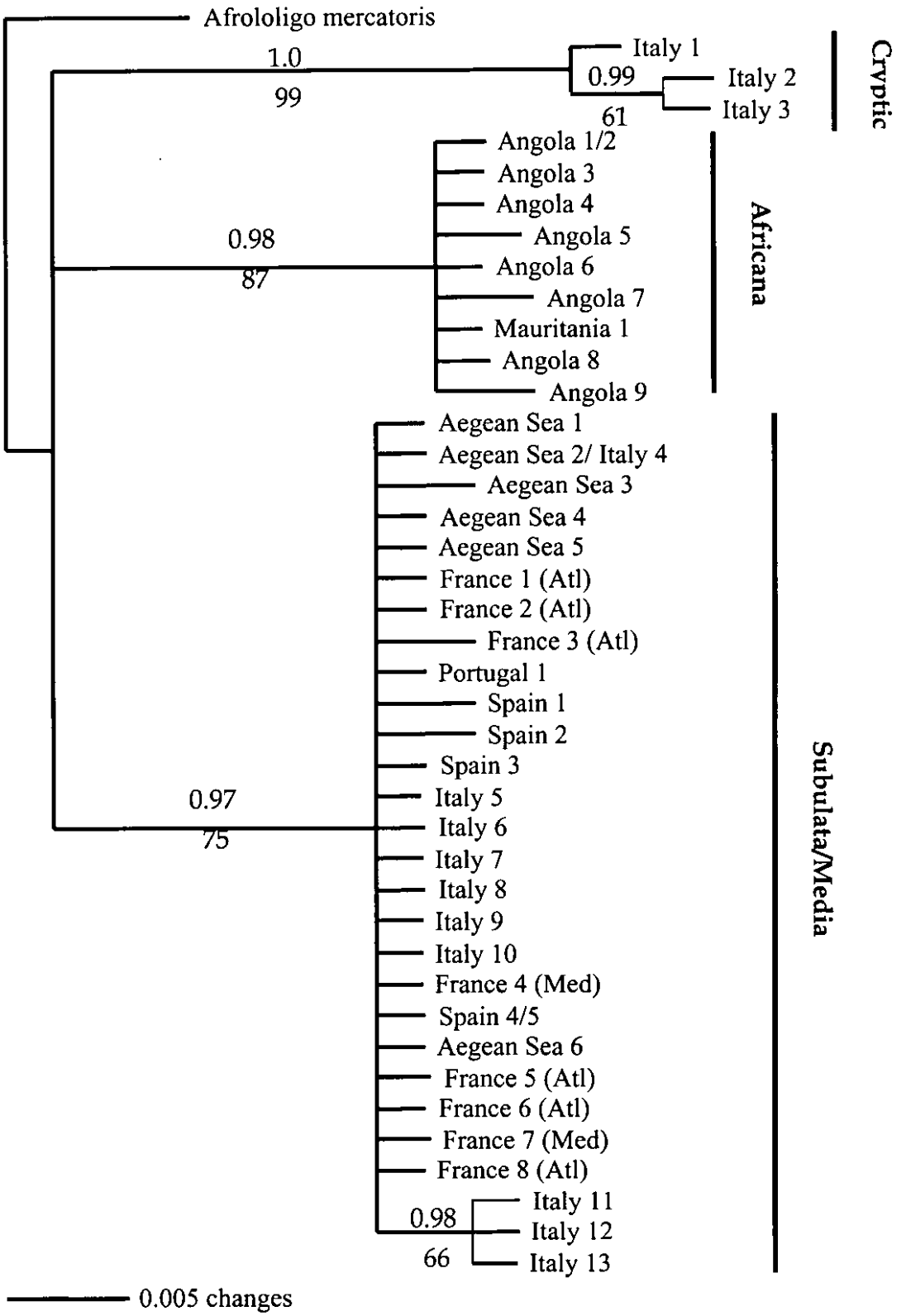
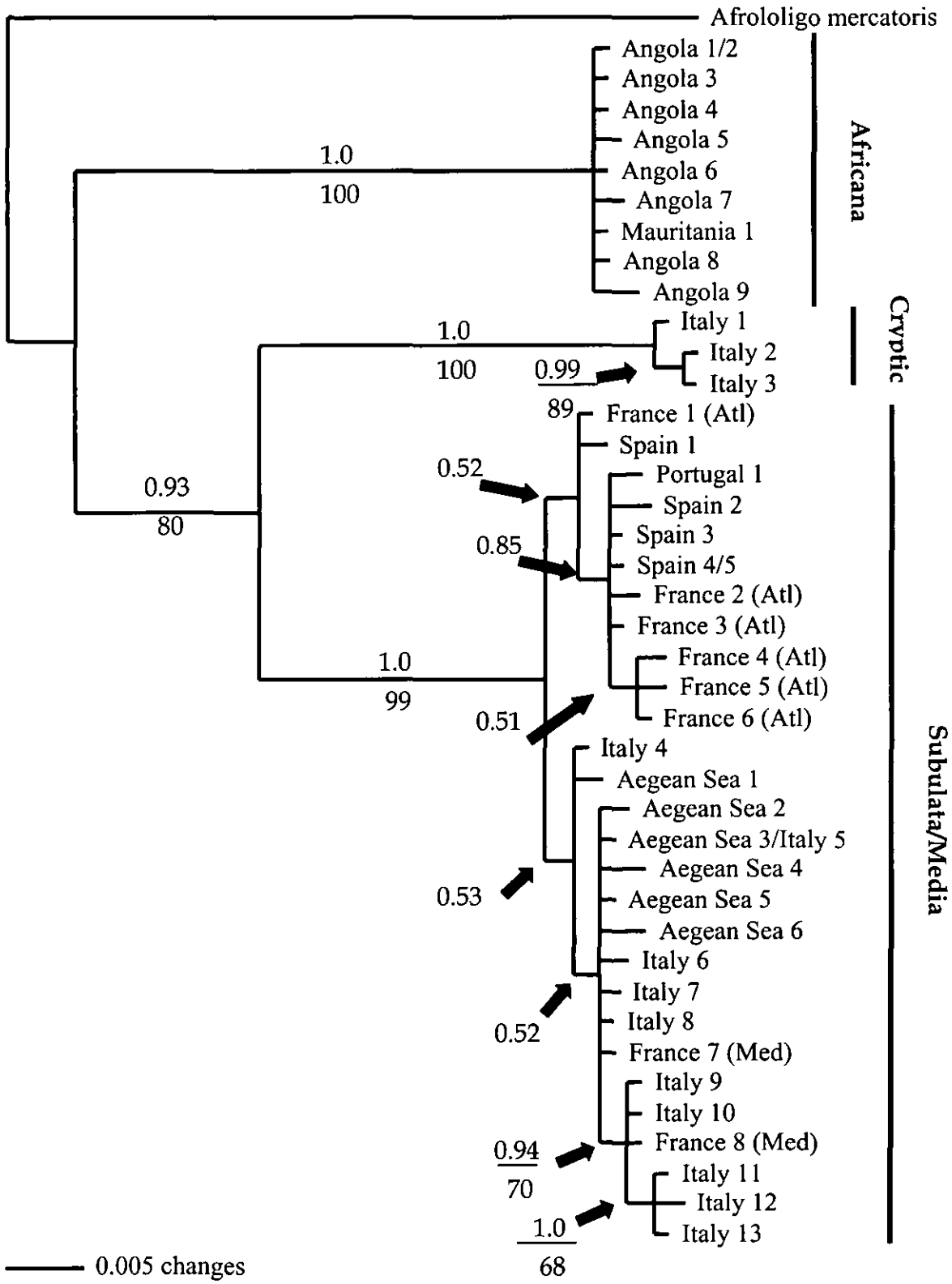


Figure 5. Phylogram of combined COI data (partitioned by codon position) and 16S data.



	Tail length	**Fin length**	**Central club sucker angle relative to club axis**	**Largest club sucker size**	**Arm length**
A. Africana	long (5-6 cm)	>50% ML	perpendicular	6-8% HW	15-20% ML
A. media	short (<1 cm)	<50% ML	perpendicular	9-14% HW	
A. subulata	long (5-6 cm)	>50% ML	oblique	6-8% HW	20-25% ML

Table 1. A summary of the morphological measurements traditionally used to distinguish species of *Alloteuthis*.