Distribution and Differentiation Patterns of Sympatric Squids *Alloteuthis media* and *Alloteuthis subulata* (Cephalopoda: Loliginidae) Using Morphological and Molecular Approaches

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Identification of the two sympatric species, *Alloteuthis media* and *Alloteuthis subulata*, has long relied on a set of identifying morphometric parameters and descriptive guidelines. To resolve taxonomic status of *Alloteuthis* in the Eastern Adriatic, we used morphological and molecular approach on a dataset collected during MEDITS expeditions sampling the entire Eastern Adriatic over consecutive summers. Phylogenetic analyses inferred from mitochondrial DNA cytochrome oxidase I (COI) gene sequences confirmed presence of both species in the Eastern Adriatic, with *A. subulata* occurring only in its central and southern parts. Analyses of genetic diversity showed that *A. subulata* samples in the Eastern Adriatic shared a single haplotype while *A. media* showed high haplotype diversity. Comparison of Eastern Adriatic *A. media* samples and populations from other regions showed statistically significant genetic differentiation between the Atlantic haplotypes and each of the Adriatic, Aegean, and Ionian populations. Conversely, *A. subulata* had low genetic diversity with only two haplotypes present across samples collected globally. There was no single morphometric character with strong enough power to discriminate between species, however, when morphological traits were looked as a composite metric rather than in isolation, the majority of individuals were correctly classified into one of three groups (*A. media* males or females and *A. subulata*).

Keywords: *Alloteuthis media*, *Alloteuthis subulata*, Adriatic Sea, morphometry, DNA barcoding

INTRODUCTION

Alloteuthis (Loliginidae) is a genus of slender loliginid squids occurring in the East Atlantic Ocean and Mediterranean Sea. The genus is comprised of three species, *Alloteuthis media*, *Alloteuthis subulata* and *Alloteuthis africana*. These squids are of little fishery importance due to their small body size and are usually caught as bycatch in trawl fisheries (Roper et al., 1984;

Jereb and Roper, 2010). Genus *Alloteuthis* is morphologically easily distinguished from other loliginids due to the characteristic long, sharply pointed "tail" that extends from the mantle (Nesis, 1987) and the characteristic heart-shaped fins (Naef, 1921; Roper et al., 1984). However, morphometric distinction within the genus is less apparent and largely debated (Laptikhovsky et al., 2002; Anderson et al., 2008; Gebhardt and Knebelsberger, 2015).

Alloteuthis africana has almost no geographic overlap with the other two *Alloteuthis* species, as its range is restricted to eastern Atlantic Ocean, extending from southern Angola to southern Morocco (Roper et al., 1984). In addition, *A. africana* is characterized by a larger head width compared to *A. media* and *A. subulata* (Anderson et al., 2008), which allows easier species identification based on sampling location and morphology. However, *A. media* and *A. subulata* have a highly overlapping distribution range in the eastern Atlantic and Mediterranean (Jereb and Roper, 2010). The distribution range of *A. subulata* extends from northwest Africa to the southern coast of Norway and is considered the most prevalent squid species in the North Sea (Zuev and Nesis, 2003). It is more commonly found in deeper waters and is less common in the Mediterranean and the southern part of its range (Zuev and Nesis, 2003). In the Atlantic, *A. media* is considered to occur predominantly between Morocco and Scotland, while only occasionally occurring as far north as the Irish Sea and the southern part of the North Sea, and is considered to be a relatively warm-water species (Zuev and Nesis, 2003). Historically, species identification has relied primarily on two indices: the ratio between the fin length (FL) and dorsal mantle length (DML; but see Anderson et al., 2008), and the ratio between the diameter of the largest tentacular club sucker (CCS) and head width (HW; Grimpe, 1925; Roper et al., 1984; Nesis, 1987). Firstly, the length of slender, elongated tail (i.e. the posterior section of the mantle and fins) exceeds 50% of the total DML in *A. subulata* and *A. africana*, whereas it is shorter and accounts for less than 50% of the DML in *A. media* (Nesis, 1987). Secondly, *A. subulata* and *A. africana* have smaller tentacular club suckers relative to head width (<8%) while the diameter of the largest club sucker in *A. media* is between 9-14% of head width (Naef, 1921; Nesis, 1987). Adults exhibit sexual dimorphism in which females of *A. media* are larger than males, whereas the opposite is true for *A. subulata* and *A. africana*, with males having longer mantle and tail length than females. In addition, mature *A. subulata* and *A. africana* males (DML up to 20 cm) are larger than *A. media* males (DML<14 cm; Fischer, 1987).

Recent efforts to resolve *Alloteuthis* taxonomy and systematics using molecular approaches show that species identification based on established morphological parameters is not accurate and appears to be of low taxonomic value (Anderson et al., 2008; Lefkaditou et al., 2012; Gebhardt and Knebelsberger, 2015). Using the tail length index (TLI; calculated as FL vs DML ratio) as an identifying parameter, Anderson et al. (2008) categorized most of the Atlantic samples as *A. subulata* and most of the Mediterranean samples as *A. media*. However, molecular analyses of mtDNA in the same study showed that

A. subulata occurred only in the southern Adriatic while all Atlantic samples were actually *A. media*. The authors emphasized that TLI is not a reliable parameter for species identification and suggested the diameter of the largest club sucker as the more appropriate morpho-character for *A. media* and *A. subulata* discrimination, albeit the latter is more difficult to measure in the field, especially in smaller individuals. Conversely, Lefkaditou et al. (2012) identified arm length as a variable best suited for successful discrimination between *A. media* and *A. subulata*. Thus, the identification of *Alloteuthis* species remains confusing and the exact species distribution unknown, as the available occurrence data are predominantly based on morphological identification of collected specimens.

To date, molecular studies of the genus *Alloteuthis* have confirmed the occurrence of *A. media* in the Atlantic (Anderson et al., 2008; Olmos-Pérez et al., 2018), English Channel (Anderson et al., 2008), West Mediterranean (Anderson et al., 2008), Ionian Sea (Lefkaditou et al., 2012), Aegean Sea (Lefkaditou et al., 2012) and the Adriatic (Anderson et al., 2008). *Alloteuthis subulata* specimens were recorded only in the southern Adriatic (Anderson et al., 2008), Ionian Sea (Lefkaditou et al., 2012) and the central eastern Atlantic (Olmos-Pérez et al., 2018). Data from previous Mediterranean International Trawl Surveys (MEDITS) indicate the presence of both *A. media* and *A. subulata* in the Eastern Adriatic, with *A. media* present throughout Eastern Adriatic while *A. subulata* is restricted to the southern part (Krstulovic Sifner et al., 2005). Furthermore, Petrić et al. (2014) reported finding 36 *A. media* individuals and no *A. subulata* in a fisheries restricted area (Jabuka Pit) in the central Adriatic. Traditionally, these occurrence data were based on species identification by morphology, which can be misleading. The aim of this study was therefore to use both molecular and morphological data to (i) investigate the co-occurrence and distribution of both Mediterranean *Alloteuthis* species in the Eastern Adriatic, (ii) to assess the level of genetic structure and connectivity of local dems and *Alloteuthis* populations from other geographic regions, and (iii) to evaluate the systematic contribution of morphological characters, as a useful tool for the identification of *A. media* and *A. subulata*.

MATERIALS AND METHODS

Sample Collection

Squid samples were collected during the MEDITS trawl survey conducted between May and August for two consecutive years, 2014 (n=32) and 2015 (n=21), covering the whole Eastern Adriatic coast (**Figure 1**). Specimens were caught using the bottom trawl net GOC 73 with 20 mm mesh size at predefined stations. The duration of hauls was set at 30 min at depths less than 200 m and 1 hour at depths greater than 200 m (Bertrand et al., 2002). Samples were frozen on board and transported to the laboratory.
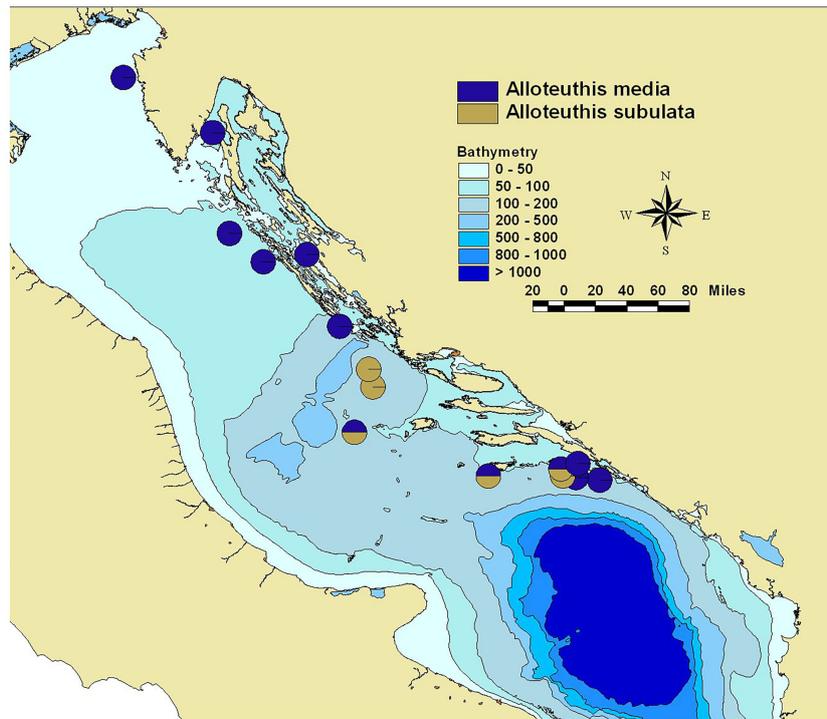
FIGURE 1 | Collection sites in the Eastern Adriatic during MEDITS surveys in 2014 and 2015 for *A. media* (blue) and *A. subulata* (yellow).

Molecular Analyses and Phylogenetic Reconstruction

DNA was extracted from mantle tissue samples previously preserved in absolute ethanol and stored at -20°C, following the modified extraction protocol of Turtinen and Juran (1998). A small amount of each tissue sample was first digested in 180 µL cell lysis buffer (0.2% SDS, 0.01 M TrisBase, 0.01 M EDTA, 0.15 M NaCl) and 4 µL proteinase K (10 mg/mL) and incubated overnight at 55°C on a heat block (BioSan Thermo-Shaker TS-100C, Riga, Latvia). The purity and quantity of DNA preparations were examined using the Genova Nano spectrophotometer (Jenway, Staffordshire, UK). Approximately 650bp long region of cytochrome oxidase subunit I (COI) mitochondrial gene was amplified using universal primers (LCO1490: 5'GGTCAACAAATCATAAAGATATTGG3' and HC02198: 5'TAAACTTCAGGGTGACCAAAAAATCA3'; Folmer et al., 1994). The PCR reaction consisted of 1 U of Taq polymerase (Sigma Aldrich, D1806), 1 X PCR incubation buffer, 0.2 mM dNTP, 3 mM MgCl$_2$, 0.4 µM of each primer, 50 ng of DNA template and distilled water to the final volume (25 µL). Amplification was performed using the MyCycler Thermal Cycler (Bio-Rad, CA, USA) and PCR conditions were as follows: 3 min at 94°C; 5 cycles of 94°C for 30 s, 45°C for 90 s, 72°C for 60 s; 30 cycles of 94°C for 30 s, 50°C for 90 s, 72°C for 30 s; and a final extension of 7 min at 72°C. The success of amplification was visually examined on a 1% agarose gel. PCR products were sent to the sequencing laboratory Macrogen

Europe, where forward sequencing was performed on the ABI 3100 automated sequencer.

Sequence quality was assessed using the Geneious prime software v2020.2.4 (Kearse et al., 2012). Sequences were aligned with the Geneious aligner (Kearse et al., 2012), using default parameters, and uploaded to GenBank with accession numbers MW349463-89 for *A. media* and MW349517-37 for *A. subulata*. All additional COI mtDNA sequences from *A. media* and *A. subulata* that were available on GenBank and for which the sampling location was indicated, were downloaded, as listed in **Table S1**. Analyses were initially performed using only the samples from this study (hereafter, "present dataset"). Subsequently, the *A. media* sequences were combined with those downloaded from GenBank and classified into six broader categories based on sampling location: Adriatic (n=37 of which 30 were sampled in this study and 7 were downloaded from GenBank), Aegean (n=6), Atlantic (n=32), Ionian Sea (n=4), North Sea (n=30) and West Mediterranean (n=2). Similarly, *A. subulata* sequences were grouped into three categories: Adriatic (n=24 of which 21 were sampled in this study and 3 were downloaded from GenBank), Atlantic (n=3) and Ionian Sea (n=1).

The number of haplotypes (NHap), haplotype diversity (Hd), number of polymorphic sites (S), nucleotide diversity (π) and average number of nucleotide differences (k) were calculated using DnaSp 6.12.3.0 (Nei, 1987; Librado and Rozas, 2009) for each species. Haplotype networks were constructed using the

median-joining distance method in PopART 1.7 software (Leigh and Bryant, 2015) while population differentiation index (F_{ST}) was calculated using ARLEQUIN 3.5.2.2 (Excoffier and Lischer, 2010). Statistical significance of F_{ST} indices was adjusted using Bonferroni correction to account for multiple comparisons between populations. Demographic history changes were also examined in ARLEQUIN 3.5.2.2 where Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) indices were calculated, together with simulation of mismatch distribution of DNA pairwise difference using the sudden demographic expansion model (Rogers and Harpending, 1992). Phylogenetic analyses were performed using MrBayes 2.2.4 (Huelsenbeck and Ronquist, 2001). For Bayesian analysis, the best-fit model of sequence evolution was selected with jModelTest 2.1.10 (Posada, 2008) and the phylogenetic tree of the present dataset samples was constructed using the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with a proportion of invariable sites. *Afrololigo mercatolis* (EU668101) was used as the outgroup for phylogenetic analyses.

Morphometric Measurements and Analysis

Samples were defrosted at room temperature and wet body mass (BW) was measured using analytical balance down to ±0.1 mg. Following Roper and Voss (1983), nine morphological traits were measured for each specimen: dorsal mantle length (DML), mantle width (MW), fin length (FL), fin width (FW), head length (HL), head width (HW), tentacle length (TL), tentacular club length (TCL) and length of longest arm (AL). In addition, the tail length index (TLI) was calculated as FL/DML. The TCL/TL ratio was also calculated as two types of tentacular clubs were observed in the samples: longer, wider clubs with larger suckers, typical for *A. media*, and short and narrow clubs with extremely small suckers, typical for *A. subulata*. The diameter of the largest club sucker was not measured as freezing of samples affected the quality of the soft and delicate club sucker tissue. Finally, specimens were sexed and categorized into adults or sub-adults based on the ontogenetic stage, following a modified protocol of Jereb and Ragonese (1995).

To investigate the discriminatory potential of morphological characteristics between *A. subulata* and *A. media*, we combined principal component analysis (PCA) and quadratic discriminant analysis (QDA). Firstly, we used PCA analyses to examine which morphological variables explained most of the variation in the data. The PCA was performed using the R package 'FactoMineR' (Lê et al., 2008). Visualization of PCA outputs was done using the 'factoextra' R package (Kassambara and Mundt, 2016). Principal components were retained according to the Kaiser-Guttman criterion (eigenvalues >1; Jackson, 1993). Variables with loadings > 0.4 were considered to contribute to a principal component (Tabachnick et al., 2007). Secondly, quadratic discriminant analysis was used as a constrained approach that discriminates multivariate data among *a priori* groups. QDA was run using the R package 'MASS' (Ripley et al., 2013) with the 80/20 split for training and testing data. In addition to multivariate analyses, a two-sample t-test and Wilcoxon rank-sum test was used to specifically examine species differences in TLI and TCL/TL ratio, respectively, as these indices have previously been shown to be important for species discrimination based on morphology. Data were analyzed in R Studio (RStudio Team, 2020) for R (3.5.0).
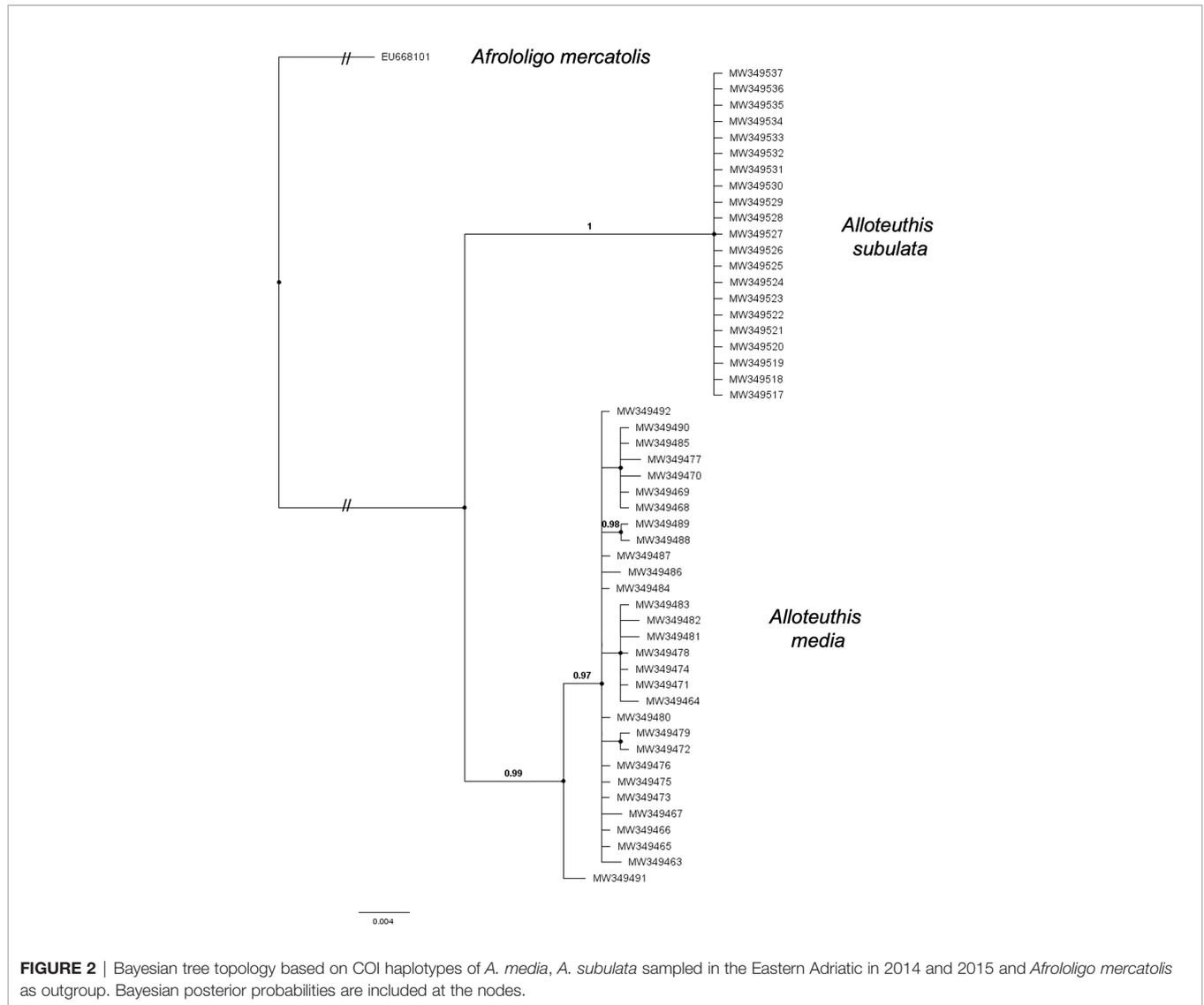
RESULTS

Phylogenetic Inference

Sequences for the cytochrome oxidase I (COI) gene were obtained for 53 individuals, two of which were excluded from further analyses due to poor sequence quality. According to the BLAST search (Altschul et al., 1990) and using ≥97% cut-off for sequence identity, 30 individuals were identified as *A. media* and 21 as *A. subulata*. Phylogenetic reconstruction was later applied to support the species identification results (**Figure 2**). The *A. media* alignment performed on the present dataset included 30 sequences and was 529 bp long. A total of 14 haplotypes were identified in the present *A. media* dataset, indicating high haplotype diversity (Hd=0.89) of populations from the Eastern Adriatic, with nine haplotypes represented by a single individual. Nine individuals shared the most common haplotype while two haplotypes comprised of four individuals each, and two haplotypes of 2 individuals each. Genetic diversity estimates are shown in **Table 1**. The *A. subulata* alignment comprised of 21 sequences and was 522 bp long. However, all of *A. subulata* samples in the present dataset shared the same haplotype with no polymorphic sites present, thus the observed genetic diversity was zero (**Table 1**).

Global *A. media* dataset (i.e., pooled dataset comprising of present dataset plus Genbank sequences) consisted of 111 sequences 384 bp long (**Table 1**). A total of 34 haplotypes were identified, indicating high haplotype diversity (Hd=0.88) with 22 haplotypes represented by a single individual. The majority of individuals shared one of the three most common haplotypes, of which one haplotype represented predominantly samples from East Atlantic and North Sea, second one represented samples from the Adriatic, Ionian and Aegean Sea, while the third haplotype included samples from most regions (**Figure 3**). Moderate and significant global F_{ST} (0.064) was found among the samples of different basins. Pairwise F_{ST} values indicated significant heterogeneity when comparing samples from Atlantic and North Sea with any basin within Mediterranean ($p<0.001$; **Table 2**). The mismatch distribution showed unimodal distribution of pairwise DNA differences, following Rogers and Harpending's model (Rogers and Harpending, 1992) of demographic expansion (SSD = 0.011, $p > 0.05$). Demographic expansion is also evident from the haplotype network star-like shape, with dominant ancestral haplogroups that connect to a network of new haplogroups separated by one or two mutations. Significant negative Tajima's D (-2.09; $p<0.001$) and Fu's F_S (-27.70; $p<0.001$) values indicate an excess of rare haplotypes and presence of recent population expansion (Tajima, 1989; Fu, 1997).

The length of the global *A. subulata* alignment included 28 sequences and was 435 bp long. Genetic diversity analysis

FIGURE 2 | Bayesian tree topology based on COI haplotypes of *A. media*, *A. subulata* sampled in the Eastern Adriatic in 2014 and 2015 and *Afrololigo mercatolis* as outgroup. Bayesian posterior probabilities are included at the nodes.

revealed the presence of a single polymorphic site (**Table 1**). Therefore, only two haplotypes were present in the total dataset; 26 samples from the Adriatic, Ionian and Atlantic Seas had the same haplotype while two Atlantic samples shared a different haplotype where a T/C base change occurred. Nucleotide diversity for *A. subulata* across all sampled locations was thus very low (Hd=0.0003) with the average number of nucleotide differences of 0.1376.

Discriminative Power of Morphological Characteristics

Examination of the gonads revealed the presence of 20 female, seven male and three sub-adult *A. media*. Similarly, females formed most of the *A. subulata* sample with 16 individuals, in addition to four males and one sub-adult. Adult individuals were divided into four categories based on sex and species. To reduce

the impact of ontogenetic allometry on study results, sub-adults were excluded from further morphometric analyses (Laptikhovsky et al., 2005). Morphometric variables were tested for correlations using 'car' function in 'caret' R package (Kuhn, 2008), to avoid multicollinearity. Highly correlated morphometric variables (>70%) were excluded and the final reduced dataset comprised of five variables: mantle width (MW), head width (HW), tentacular club length (TCL), TCL/ TL index and TLI index. Principal component analysis of the morphological parameters showed that the first two principal components (PC1 and PC2) were significant and explained 83.7% of the total variance. PC1 showed significant positive loadings (0.62-0.88) for all retained variables, except the TCL/TL index, indicating that individuals with larger TLI also had larger MW, HW and TCL (**Table 3**). Mainly, the first axis separated larger *A. media* females from all other individuals, with *A. media* males and

TABLE 1 | Genetic diversity and neutrality tests for the mitochondrial DNA COI sequences of *Alloteuthis media* and *A. subulata* in the Eastern Adriatic ("Present dataset") and across all sequences available on GenBank ("Global dataset").

| | | | | Polymorphic sites (S) | | Genetic diversity estimates | | | Neutrality tests | |
		Number of individuals	Alignment length (bp)	Haplotypes (H)	Singleton informative sites	Parsimony informative sites	Haplotype diversity (Hd)	Nucleotide diversity (π)	Average number of nucleotide differences (k)	Tajima's D	Fu's FS
Present dataset	*A. media*	30	529	14	11	4	0.8851	0.00327	1.73103	-1.82833*	-9.649*
	A. subulata	21	522	1	0	0	0	0	0	/	/
Global dataset	*A. media*	111	384	34	18	12	0.8830	0.00455	1.73170	-2.0898*	-33.601*
	A. subulata	28	435	2	0	1	0.1376	0.00032	0.1376	-0.74099	-0.380

Statistically significant results for neutrality tests are marked with asterisks.

A. subulata females being characterized by intermediate sizes, and *A. subulata* males at the lower end of the size range (**Figure 4**). The second principal component mostly correlated with the TCL/TL ratio (strong positive loadings of 0.97) and TCL (positive loadings of 0.74). This axis predominantly explained the variation within *A. media* females as this was the only group that showed a substantial level of variation in TCL/TL ratio and TCL compared to other traits (**Figure 4**).

Due to the small number of males sampled for *A. subulata* (n=4), individuals were grouped into three categories for QDA analysis instead of four: *A. media* females, *A. media* males and *A. subulata* (pooled males and females). This is consistent with previous approaches in the literature (Lefkaditou et al., 2012) and was justified by the output of the PCA analysis (**Figure 4**) that showed substantial variation in morphometric characteristics in *A. media* males and females, but generally much less in *A. subulata*. Quadratic discriminant analysis of morphological traits correctly classified 87.5% of individuals to either *A. media* (males or females) or *A. subulata* species, suggesting that the measured morphological traits have relatively high power for accurate species delineation (**Figure S1**). In addition, we found significant variation in the TLI among species ($t(37.2)$ = 4.13, p = 0.0002), the trait historically used for species classification based on morphology. However, there was no significant difference in the TCL/TL ratio between species (Z = -0.35, p = 0.74; **Figure 5**).

DISCUSSION

Sequencing data of the cytochrome oxidase I (COI) unit confirmed the presence of both *Alloteuthis media* and *A. subulata* in the Eastern Adriatic. While this study was limited to the use of a single marker (COI), its efficiency in separating closely related cephalopod species was previously demonstrated, outperforming alternative markers such as 18S rDNA (Gebhardt and Knebelsberger, 2015), 16S and 12S rRNA (Braid et al., 2014). In the north-eastern Adriatic, the genus *Alloteuthis* was exclusively represented by *A. media* (n=19; **Figure 1**). However, both species were found in the central and southern parts of the Eastern Adriatic, suggesting a potential structure of *Alloteuthis* species distribution patterns in the basin. These findings are consistent with previous reports of *A. subulata* in the Adriatic Sea, where three individuals were sampled off the southern Italian coast (Mola di Bari; Anderson et al., 2008) and with the results from MEDITS surveys where this species was reported only in the southern Adriatic (Krstulovic Sifner et al., 2005), albeit latter identification was based only on morphological parameters. Additionally, previous MEDITS surveys reported the occurrence of *A. media* throughout the Adriatic Sea (Krstulovic Sifner et al., 2005; Anderson et al., 2008), further supporting the present findings. The observed difference in the distribution patterns of *A. media* and *A. subulata* in the Eastern Adriatic is not surprising, as *A. subulata* is considered to occur predominantly in deeper waters (Zuev and Nesis, 2003; but see González and Sánchez, 2002), while shallow waters of the
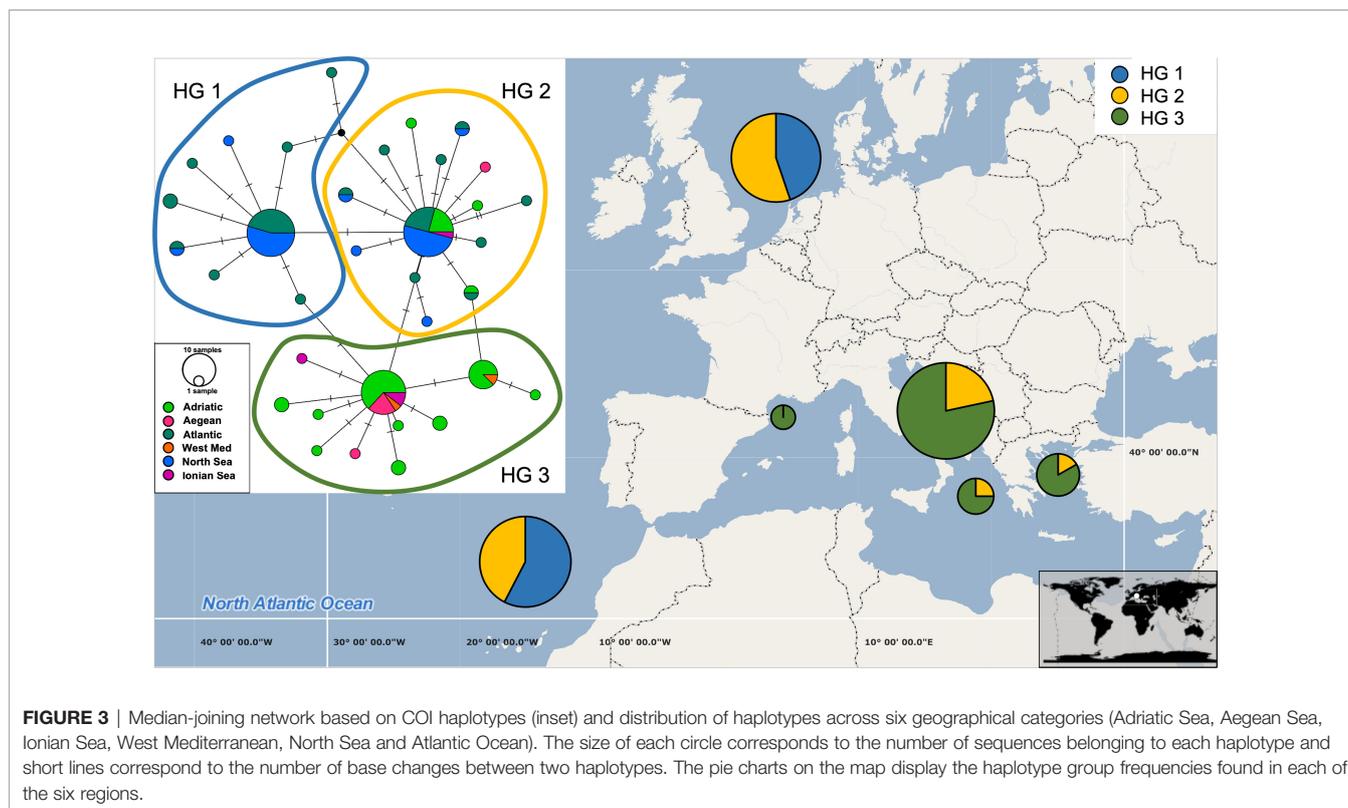
FIGURE 3 | Median-joining network based on COI haplotypes (inset) and distribution of haplotypes across six geographical categories (Adriatic Sea, Aegean Sea, Ionian Sea, West Mediterranean, North Sea and Atlantic Ocean). The size of each circle corresponds to the number of sequences belonging to each haplotype and short lines correspond to the number of base changes between two haplotypes. The pie charts on the map display the haplotype group frequencies found in each of the six regions.

TABLE 2 | Pairwise F_{ST} values showing genetic differentiation between populations of *Alloteuthis media*.

	Adriatic	Aegean	Atlantic	Ionian Sea	North Sea
Aegean	0.002				
Atlantic	0.371*	0.355*			
Ionian Sea	-0.038	-0.091	0.295*		
North Sea	0.422*	0.471*	-0.015	0.420*	
West Med**	-0.206	0.000	0.390	0.000	0.553*

Asterisks indicate statistically significant genetic differentiation between populations.
*significant after Bonferroni correction, p < 0.0083.
**due to the limited number of sequences (n = 2) for West Med group, all pair-wise interactions with the group should not be taken into the consideration.

TABLE 3 | Component loadings of morphological traits observed on two retained orthogonally rotated principal components (PC1 and PC2) from the PCA analysis combining *Alloteuthis media* and *A. subulata* samples from Eastern Adriatic.

Morphological trait	PC1	PC2
MW (g)	0.788	-0.336
HW (mm)	0.882	-0.114
TCL (mm)	0.624	0.741
TCL/TL	-0.007	0.972
TLI	0.874	-0.103
% of variance explained	51.041	32.613
Total variance explained		83.654

Values highlighted in bold indicate variables that were considered to contribute to a principal component (loading of at least 0.4).

northern Adriatic, the most extensive continental shelf of the Mediterranean Sea with an average bottom depth of about 35 m (Trincardi et al., 1994), may not be suitable for this species. However, as the only samples previously identified in the Adriatic using molecular markers were seven *A. media* and three *A. subulata* individuals, all collected from a single location in Mola di Bari, Italy (Anderson et al., 2008), it remains to be ascertained whether the distribution of *A. subulata* is restricted to central and southern parts of the Adriatic basin.

Contrasting intraspecific genetic diversity pattern was observed in these two sympatric species. Based on the available data, *A. subulata* is constituted by a single panmictic unit within the Mediterranean. Namely, lack of haplotype diversity attributed to *A. subulata* in the Eastern Adriatic is consistent with patterns observed on a larger scale, with *A. subulata* occurring only in the eastern part of the Mediterranean, specifically in the southern Adriatic and Ionian Sea (Anderson et al., 2008; Lefkaditou et al., 2012) with whom eastern Adriatic samples share the same haplotype. Molecularly confirmed *A. subulata* samples from the eastern Atlantic (Olmos-Pérez et al., 2018) had a haplotype that differed only in a single base pair from those observed in the eastern Mediterranean. In addition to the general assumption that environmental stability of the deep sea supports low genetic diversity due to niche refinement (Bretsky and Lorenz, 1970), the low evolutionary rate for the coding region, and the loss of diversity following demographic
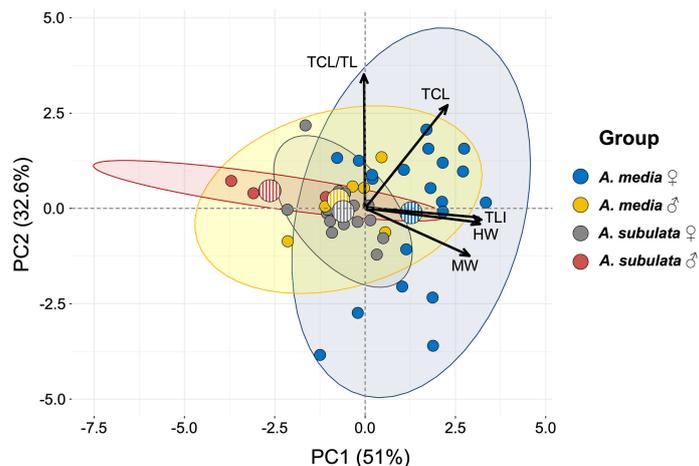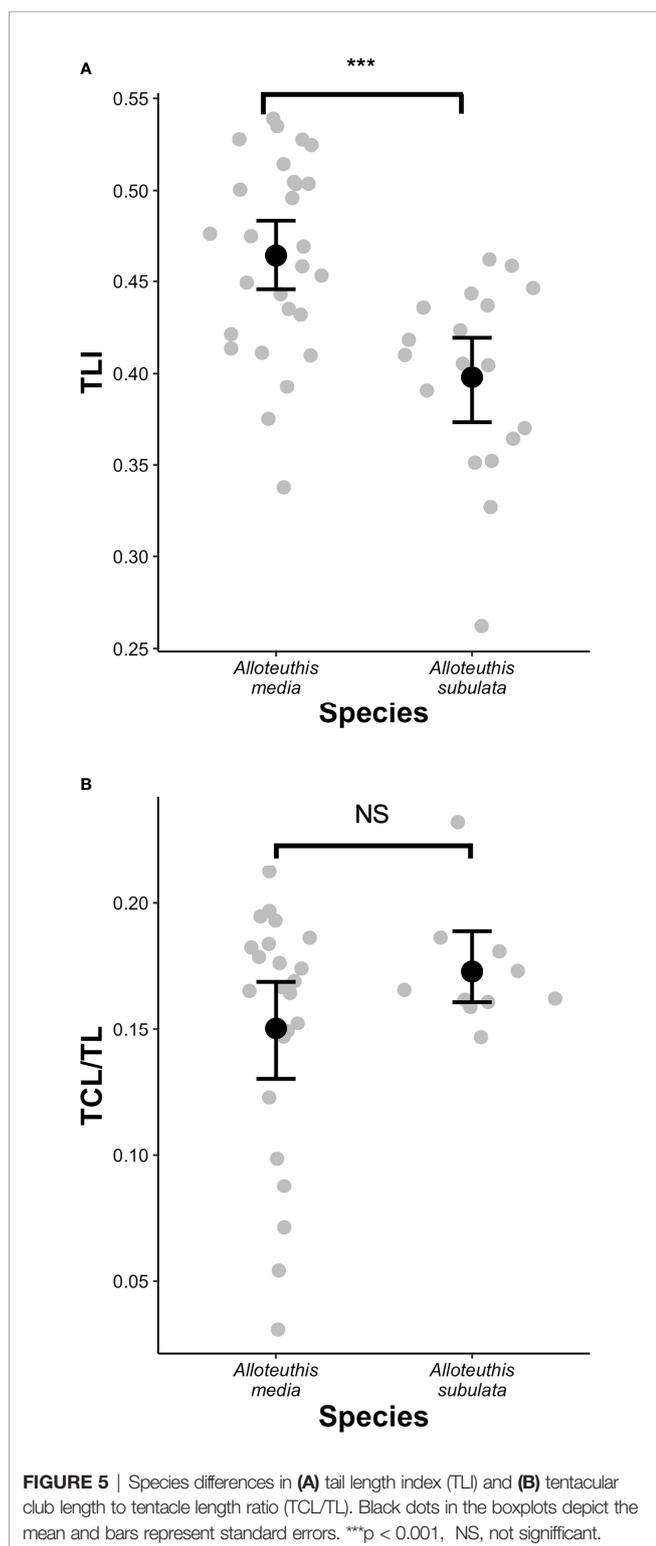
FIGURE 4 | Graphic representation of the PCA analysis combining Eastern Adriatic *Alloteuthis media* and *A. subulata* samples showing the individual placement along the first two principal components based on five retained morphological variables (MW, mantle width; HW, head width; TCL, tentacular club length; TLI, tail length index; TCL/TL, tentacle club length to tentacle length ratio). Group centroids are shown with dashed circles.

collapses during the Messinian Salinity Crisis (MSC) or selective events, could explain the low haplotype diversity of *A. subulata* found in the present work. Similar level of diversity for mtDNA region was reported for *Octopus vulgaris* in the central Mediterranean (Fadhlaoui-Zid et al., 2012). It is still unclear whether *A. subulata* occurs in the rest of the Mediterranean, as research based on molecular markers is sparse resulting in low number of published sequences (n=3, $n_{samples}$=23; Laptikhovsky et al., 2002; Anderson et al., 2008; Lefkaditou et al., 2012), which could also partially explain the low observed genetic diversity in this species. Therefore, any conclusions regarding genetic variation and connectivity along the species distribution range would need to be validated by further research.

In contrast, 34 haplotypes across the *A. media* distribution range have been recorded in the present study. High haplotype diversity and low nucleotide diversity due to changes in a single nucleotide among haplotypes likely indicate sudden demographic expansion from a small initial population throughout recent demographic history. Three major haplotype clusters separated *A. media* individuals: those sampled west of Gibraltar (i.e., Atlantic and North Sea populations) from those sampled east of Gibraltar (i.e., Mediterranean samples; **Figure 3**). An intermediate haplotype cluster was formed with individuals from most sampling sites, but with dominance of the Eastern Atlantic collection (**Figure 3**). Glacial periods throughout history caused geographic isolation between Mediterranean and Atlantic populations, with subsequent genetic divergence observed in many marine invertebrates (Quesada et al., 1995; Pérez-Losada et al., 1999; Ríos et al., 2002). At present, Gibraltar Strait (GS) and the Almería-Oran Front (AOF) represent oceanographic discontinuities caused by the difference in salinity between the colder less saline Atlantic

waters that enter through the GS into the Mediterranean (warmer higher density waters). The presence of three haplotype clusters and the significant genetic differentiation between Atlantic and Adriatic, Aegean and Ionian populations found in this study suggest that the GS and/or the AOF might represent a geographic barrier that reduces gene flow between Atlantic and Mediterranean populations of *A. media*. Furthermore, despite the pelagic larval and adult stage, gene flow could be restricted by strong marine currents that occur at the narrow and shallow passage of the GS and along the Almería-Oran line (Pascual et al., 2017).

Although it has been commonly used as a species specific parameter, several recent studies have found TLI as potentially unreliable morphometric trait for the identification of *Alloteuthis* species (Laptikhovsky et al., 2002; Pilsits, 2007; Anderson et al., 2008; Lefkaditou et al., 2012). While our results indicate that the TLI index differs between *A. media* and *A. subulata* with the TLI being significantly greater in *A. media*, this also contradicts the guidelines for morphological species identification which indicate the TLI<0.5 in *A. media* (TL is less than 50% of DML) and >0.5 in *A. subulata* (Nesis, 1987). With the mean TLI of 0.46 in *A. media* and 0.40 in *A. subulata*, our results suggest that the TLI threshold of 0.5 is not appropriate for discriminating between these two species. In addition, TLI values of adults of each species included in this study vary considerably (**Figure 5**), indicating that the ratio of FL to DML should not be used for species differentiation. In addition, research conducted from 1990 until 2002 across the Aegean, Mediterranean and East Atlantic have shown that relative fin length increases with body length (Laptikhovsky et al., 2005), thus the smallest *Alloteuthis* specimens are likely to be identified as *A. media* while the largest get attributed to *A. subulata*, following the traditionally accepted species assignment based on the 0.5 TLI

FIGURE 5 | Species differences in **(A)** tail length index (TLI) and **(B)** tentacular club length to tentacle length ratio (TCL/TL). Black dots in the boxplots depict the mean and bars represent standard errors. ***p < 0.001, NS, not signiffcant.

While Anderson et al. (2008) indicated that the ratio of central club sucker to head width could be a good discriminatory parameter of the two species, Pilsits (2007) showed that the CCS/HW index is not reliable for species identification and reported lack of consensus between molecular and morphological assignment based on this index. In the present study, the CCS/HW index was not examined due to the delicate nature of club suckers severely affected by defrosting process, especially in individuals with smaller TCL. However, a simple visual inspection of these squids revealed that two types of tentacular clubs were observed in our sample (**Figure S1**), differing substantially in length and width of the tentacular club. Analyses of the TCL/TL index revealed that all *A. subulata* samples had long tentacular clubs (15-23% of TL) while a wide range of sizes was observed in *A. media* (3-21% of TL). Some *A. media* had extremely short and narrow tentacular clubs (**Figure 5**), which is usually considered a distinguishing feature of *A. subulata*. In addition to the higher observed variation in size and shape of tentacular clubs in *A. media* and the lack of significant differences in the TCL/TL index between the two species, the use of TCL or TCL/TL index alone as identifying parameter is rendered difficult since these structures can be easily damaged or often missing in frozen samples.

Given the lack of reliable indices for species identification, this study focused on examining a full set of morphological variables to try to determine if species can be classified into separate categories based on a combination of morphological parameters. Among the morphological parameters examined, mantle width, head width, TCL, TCL/TL index and TLI were selected as variables that best described interspecies variation. Overall, individuals with larger TLI also had larger MW, HW and TCL. Females of *A. media* were generally larger than male conspecifics, and *A. subulata* males and females, that were at the smaller end of the size spectrum. However, a wide variation in morphometric parameters was observed in females of *A. media*, mainly reflected by TCL/TL index and TCL. Nevertheless, when morphological traits were considered as a composite metric rather than in isolation, most individuals were correctly classified into one of the three groups (*A. media* males or females, and *A. subulata*). These results suggest that individual morphometric parameters are not accurate enough for species identification, but each of the groups studied is characterized by a specific combination of values of the morphological traits. To date, it is not clear what drives the observed variation in morphology between species, as well as within *A. media* females, where observed variation in morphology largely exceeds observed variation in *A. subulata* and *A. media* males. This morphological variability may explain why the search for a morphological key to species identification has not yielded conclusive results in the case of *A. subulata* and *A. media*.

While early research suggested that *A. subulata* is widespread in the Atlantic, and *A. media* is more common in the Mediterranean, molecular analyses conducted in the last decade largely contradict these conclusions. Judging from the frequent errors in morphological species assignment noted in

cut-off (Nesis, 1987). Similarly, in this study larger individuals had a higher TLI, regardless of species. However, *A. media* individuals (especially females) were larger than *A. subulata* (**Table 4** and **Figure 4**).

TABLE 4 | Range, mean and standard deviation for morphometric variables measured in adult *A. media* and *A. subulata* in the Eastern Adriatic.

	A. media			*A. subulata*		
	Range	Mean	St. Dev.	Range	Mean	St. Dev.
BW (g)	1.98-13.71	6.59	2.95	1.36-4.87	3.05	1.03
DML (mm)	34.49-90.39	63.86	14.77	32.91-57.45	43.76	7.13
MW (mm)	12.64-19.89	16.58	2.19	11.99-17.31	14.98	1.25
FL (mm)	11.65-48.51	30.29	9.95	8.63-24.21	17.48	4.40
FW (mm)	14.40-35.03	24.24	5.19	13.62-25.12	19.41	3.32
HL (mm)	7.59-16.12	13.32	2.04	8.25-13.72	10.90	1.24
HW (mm)	10.09-16.02	13.23	1.60	9.63-14.36	12.03	1.41
TL (mm)	67.48-118.84	96.01	15.48	44.50-83.75	66.39	10.53
TCL (mm)	3.06-23.28	14.50	5.04	8.29-14.45	10.64	1.52
AL (mm)	8.05-34.80	26.77	5.31	10.38-20.77	16.02	2.99

BW, body weight; DML, dorsal mantle length; MW, mantle width; FL, fin length; FW, fin width; HL, head length; HW, head width; TL, tentacle length; TCL, tentacular club length; AL, arm length.

studies using molecular data (Anderson et al., 2008; Lefkaditou et al., 2012), it is possible that the geographic distribution of these species is widely misinterpreted and it is unclear whether *A. subulata* is common in the Atlantic as originally assumed. Furthermore, assumptions about habitat preferences are largely based on occurrence data obtained from morphological identification of species, which raises the question of whether *A. media* is a warm-water species and *A. subulata* prefers deeper, colder waters, although this has been widely accepted in the literature (Zuev and Nesis, 2003). Therefore, to understand the global distribution patterns and ecology of *A. media* and *A. subulate*, a detailed review is needed that prioritizes molecular data to delineate the species and attempt to provide context for the observed morphological variability.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because squid samples were collected during the MEDITS trawl survey (fishing landings).

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic Local Alignment Search Tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Anderson, F. E., Pilsits, A., Clutts, S., Laptikhovsky, V., Bello, G., Balguerías, E., et al. (2008). Systematics of Alloteuthis (Cephalopoda: Loliginidae) Based on Molecular and Morphometric Data. *J. Exp. Mar. Bio. Ecol.* 364, 99–109. doi: 10.1016/j.jembe.2008.07.026

Bertrand, J. A., de Sola, L. G., Papaconstantinou, C., Relini, G., and Souplet, A. (2002). The General Specifications of the MEDITS Surveys. *Sci. Mar.* 66, 9–17. doi: 10.3989/scimar.2002.66s29

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Braid, H. E., McBride, P. D., and Bolstad, K. S. R. (2014). Molecular Phylogenetic Analysis of the Squid Family Mastigoteuthidae (Mollusca, Cephalopoda) Based on Three Mitochondrial Genes. *Hydrobiologia* 725, 145–164. doi: 10.1007/s10750-013-1775-3

Bretsky, P. W., and Lorenz, D. M. (1970). An Essay on Genetic-Adaptive Strategies and Mass Extinctions. *Geol. Soc Am. Bull.* 81, 2449–2456. doi: 10.1130/0016-7606(1970)81[2449:AEOGSA]2.0.CO;2

Excoffier, L., and Lischer, H. E. L. (2010). Arlequin Suite Ver 3.5: A New Series of Programs to Perform Population Genetics Analyses Under Linux and Windows. *Mol. Ecol. Resour.* 10, 564–567. doi: 10.1111/j.1755-0998.2010.02847.x

Fadhlaoui-Zid, K., Knittweis, L., Aurelle, D., Nafkha, C., Ezzeddine, S., Fiorentino, F., et al. (2012). Genetic Structure of Octopus Vulgaris (Cephalopoda, Octopodidae) in the Central Mediterranean Sea Inferred From the Mitochondrial COIII Gene. *C. R. Biol.* 335, 625–636. doi: 10.1016/j.crvi.2012.10.004

Fischer, W. (1987). Fiches FAO D'identification Des Especes Pour Les Besoins De La Peche.(Rev 1). Mediterranee Et Mer Noire. Zone De Peche 37. *Vertébrés* 2, 761–1530.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA Primers for Amplification of Mitochondrial Cytochrome C Oxidase Subunit I From Diverse Metazoan Invertebrates. *Mol. Mar. Biol. Biotechnol.* 3, 294–299. doi: 10.1071/ZO9660275

Fu, Y.-X. (1997). Statistical Tests of Neutrality of Mutations Against Population Growth, Hitchhiking and Background Selection. *Genetics* 147, 915–925. doi: 10.1093/genetics/147.2.915

Gebhardt, K., and Knebelsberger, T. (2015). Identification of Cephalopod Species From the North and Baltic Seas Using Morphology, COI and 18S rDNA Sequences. *Helgol. Mar. Res.* 69, 259–271. doi: 10.1007/s10152-015-0434-7

González, M., and Sánchez, P. (2002). Cephalopod Assemblages Caught by Trawling Along the Iberian Peninsula Mediterranean Coast. *Sci. Mar.* 66, 199–208. doi: 10.3989/scimar.2002.66s2199

Grimpe, G. (1925). Zur Kenntnis Der Cephalopodenfauna Der Nordsee. Wissenschaftliche Meeresuntersuchungen. Neue Folge. Abteilung Helgoland, Vol. 16, Part 1, Number 3. *Ad. Littmann. Oldenbg.* 34.

Hasegawa, M., Kishino, H., and Yano, T. (1985). Dating of the Human-Ape Splitting by a Molecular Clock of Mitochondrial DNA. *J. Mol. Evol.* 22, 160–174. doi: 10.1007/BF02101694

Huelsenbeck, J. P., and Ronquist, F. (2001). MRBAYES: Bayesian Inference of Phylogenetic Trees. *Bioinformatics* 17, 754–755. doi: 10.1093/bioinformatics/17.8.754

Jackson, D. A. (1993). Stopping Rules in Principal Components Analysis: A Comparison of Heuristical and Statistical Approaches. *Ecology* 74, 2204–2214. doi: 10.2307/1939574

Jereb, P., and Ragonese, S. (1995). An Outline of the Biology of the Squid Illex Coindetii in the Sicilian Channel (Central Mediterranean). *J. Mar. Biol. Assoc. United Kingdom* 75, 373–390. doi: 10.1017/S0025315400018245

Jereb, P., and Roper, C. F. E. (2010). "Cephalopods of the World-an Annotated and Illustrated Catalogue of Cephalopod Species Known to Date," in *Myopsid and Oegopsid Squids*, vol. 2. (Washington, D.C: FAO). doi: 10.7312/nei-92038

Kassambara, A., and Mundt, F. (2016). Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. *R Packag. version* 1, 76. https://cran.r-project.org/web/packages/factoextra/index.html.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: An Integrated and Extendable Desktop Software Platform for the Organization and Analysis of Sequence Data. *Bioinformatics* 28, 1647–1649. doi: 10.1093/bioinformatics/bts199

Krstulovic Sifner, S., Lefkaditou, E., Ungaro, N., Ceriola, L., Osmani, K., Kavadas, S., et al. (2005). Composition and Distribution of the Cephalopod Fauna in the Eastern Adriatic and Eastern Ionian Sea. *Isr. J. Zool.* 51, 315–330. doi: 10.1560/4LT4-K01W-C9GF-7YK3

Kuhn, M. (2008). Building Predictive Models in R Using the Caret Package. *J. Stat. Softw* 28, 1–26. doi: 10.18637/jss.v028.i05

Laptikhovsky, V., Salman, A., and MoustahfId, H. (2005). Morphological Changes at Maturation and Systematics in the Squid Genus Alloteuthis. *Phuket Mar. Biol. Cent. Res. Bull.* 66, 187–193.

Laptikhovsky, V., Salman, A., Önsoy, B., and Katagan, T. (2002). Systematic Position and Reproduction of Squid of the Genus Alloteuthis (Cephalopoda: Loliginidae) in the Eastern Mediterranean. *Mar. Biol. Assoc. United Kingdom. J. Mar. Biol. Assoc. United Kingdom* 82, 983. doi: 10.1017/S0025315402006483

Lefkaditou, E., Tsigenopoulos, C. S., Alidromiti, C., and Haralabous, J. (2012). On the Occurrence of Alloteuthis Subulata in the Eastern Ionian Sea and its Distinction From the Sympatric Alloteuthis Media. *J. Biol. Res.* 17, 169.

Leigh, J. W., and Bryant, D. (2015). Popart: Full-Feature Software for Haplotype Network Construction. *Methods Ecol. Evol.* 6, 1110–1116. doi: 10.1111/2041-210X.12410

Lê, S., Josse, J., and Husson, F. (2008). FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Software* 25, 1–18. doi: 10.18637/jss.v025.i01

Librado, P., and Rozas, J. (2009). DnaSP V5: A Software for Comprehensive Analysis of DNA Polymorphism Data. *Bioinformatics* 25, 1451–1452. doi: 10.1093/bioinformatics/btp187

Naef, A. (1921). *Fauna E Flora Del Golfo Di Napoli, Monograph No. 35, Cephalopoda.* (Berlin: R. Friedländer & Sohn)

Nei, M. (1987). *Molecular Evolutionary Genetics* (New York City, NY: Columbia University Press).

Nesis, K. N. (1987). *Cephalopods of the World: Squids, Cuttlefishes, Octopuses, and Allies.* (Neptune, NJ: TFH Publications, Inc).

Olmos-Pérez, L., Pierce, G. J., Roura, Á., and González, Á.F. (2018). Barcoding and Morphometry to Identify and Assess Genetic Population Differentiation and Size Variability in Loliginid Squid Paralarvae From NE Atlantic (Spain). *Mar. Biol.* 165, 1–18. doi: 10.1007/s00227-018-3387-y

Pascual, M., Rives, B., Schunter, C., and Macpherson, E. (2017). Impact of Life History Traits on Gene Flow: A Multispecies Systematic Review Across Oceanographic Barriers in the Mediterranean Sea. *PloS One* 12, e0176419. doi: 10.1371/journal.pone.0176419

Pérez-Losada, M., Guerra, Á., and Sanjuan, A. (1999). Allozyme Differentiation in the Cuttlefish Sepia Officinalis (Mollusca: Cephalopoda) From the NE Atlantic and Mediterranean. *Heredity (Edinb).* 83, 280–289. doi: 10.1038/sj.hdy.6885520

Petrić, M., Šifner, S. K., Ferri, J., Škeljo, F., Brčić, J., and KržELj, M. (2014). Cephalopods in the Trawl Catches in the Jabuka Pit Wider Area. *Proceedings of the 49th Croatian & 9th International Symposium on Agriculture.* Marić, M., and Lončarić, Z. (eds) (Faculty of Agriculture, University of Josip Juraj Strossmayer in Osijek), 504–508, in Croatian.

Pilsits, A. (2007). Phylogeny and Population Genetics of Allotheuthis (Loliginidae) and Discovery of a Cryptic Species. *Honor. Theses* 327.

Posada, D. (2008). Jmodeltest: Phylogenetic Model Averaging. *Mol. Biol. Evol.* 25, 1253–1256. doi: 10.1093/molbev/msn083

Quesada, H., Beynon, C. M., and Skibinski, D. O. (1995). A Mitochondrial DNA Discontinuity in the Mussel Mytilus Galloprovincialis Lmk: Pleistocene Vicariance Biogeography and Secondary Intergradation. *Mol. Biol. Evol.* 12, 521–524. doi: 10.1093/oxfordjournals.molbev.a040227

Ríos, C., Sanz, S., Saavedra, C., and Pena, J. B. (2002). Allozyme Variation in Populations of Scallops, Pecten Jacobaeus (L.) and P. Maximus (L.)(Bivalvia: Pectinidae), Across the Almeria–Oran Front. *J. Exp. Mar. Bio. Ecol.* 267, 223–244. doi: 10.1016/S0022-0981(01)00371-9

Ripley, B., Venables, B., Bates, D. M., Hornik, K., Gebhardt, A., Firth, D., et al. (2013). Package 'Mass.'. *Cran r* 538, 113–120.

Rogers, A. R., and Harpending, H. (1992). Population Growth Makes Waves in the Distribution of Pairwise Genetic Differences. *Mol. Biol. Evol.* 9, 552–569. doi: 10.1093/oxfordjournals.molbev.a040727

Roper, C. F. E., Sweeney, M. J., and Nauen, C. (1984). *Cephalopods of the World. An Annotated and Illustrated Catalogue of Species of Interest to Fisheries.* (Washington, D.C: National Museum of Natural History Smithsonian Institution)

Roper, C. F. E., and Voss, G. L. (1983). Guidelines for Taxonomic Descriptions of Cephalopod Species. *Biol. Resour. Potential Cephalopods. Mem. Natl. Museum Victoria* 44, 48–63. doi: 10.24199/j.mmv.1983.44.03

RStudio Team (2020). RStudio: Integrated Development for R. RStudio. Boston, MA: PBC. Availabale at: http://www.rstudio.com/.

Tabachnick, B. G., Fidell, L. S., and Ullman, J. B. (2007). *Using Multivariate Statistics* (Pearson Boston, MA: Pearson Education, Inc).

Tajima, F. (1989). Statistical Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. *Genetics* 123, 585–595. doi: 10.1093/genetics/123.3.585

Trincardi, F., Correggiari, A., and Roveri, M. (1994). Late Quaternary Transgressive Erosion and Deposition in a Modern Epicontinental Shelf: The Adriatic Semienclosed Basin. *Geo-mar Lett.* 14, 41–51. doi: 10.1007/BF01204470

Turtinen, L. W., and Juran, B. D. (1998). Protein Salting-Out Method Applied to Genomic DNA Isolation From Fish Whole Blood. *Biotechniques* 24, 238–239. doi: 10.2144/98242bm14

Zuev, G. V., and Nesis, K. N. (2003). *Squid (Biology and Fishing)* (Washington, D.C: Smithsonian Institution Libraries).