*Journal of Biological Research-Thessaloniki* **17**: 169–175, 2012
*J. Biol. Res.-Thessalon.* is available online at http://www.jbr.gr
Indexed in: WoS (Web of Science, ISI Thomson), SCOPUS, CAS (Chemical Abstracts Service) and DOAJ (Directory of Open Access Journals)

# On the occurrence of *Alloteuthis subulata* in the Eastern Ionian Sea and its distinction from the sympatric *Alloteuthis media*

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Received: 13 May 2011 Accepted after revision: 5 October 2011

Despite the efforts made during the last decade, the systematics of the sympatric species *Alloteuthis media* and *Alloteuthis subulata* still remains confused and the distribution of the two species in the Mediterranean Sea doubtful. The nine male specimens identified as *Alloteuthis subulata* in July-August 2008, represent the first finding of this species among samples of the "International Bottom Trawl Survey in the Mediterranean" (MEDITS), which is repeatedly carried out in the Eastern Ionian Sea since 1994. Morphometric data and tissue samples for molecular analysis were collected from these specimens, as well as from male and female specimens assigned to *A. media* from the same and near-by sampling stations. As the longer tale is the most evident characteristic to distinguish at least adult male specimens of the two species, the length of the anterior part of the mantle (from fin's edge to mantle opening) was used as the reference length upon which indices of different body dimensions were calculated. Genetic analyses using mitochondrial DNA cytochrome oxidase I (COI) gene sequences confirmed species allocation for all individuals assigned to *A. subulata*, whereas for *A. media* samples one female and an unsexed juvenile seemed to carry the *A. subulata* COI haplotype. Discriminant function analysis (DFA) of morphometric data suggested that length of arms, tentacles and tentacular clubs, all relatively smaller in *A. subulata*, constitute important variables allowing the distinction of the two species.

**Key words:** *Alloteuthis subulata*, *Alloteuthis media*, taxonomy, discriminant analysis, COI sequences, Mediterranean Sea.

## INTRODUCTION

The squid genus *Alloteuthis* (Cephalopoda: Loliginidae) comprises three nominal species. *Alloteuthis africana* Adam 1950 is distributed along the western coasts of Africa while the other two, *A. media* Linnaeus 1758 and *A. subulata* Lamarck, 1798, occur in the northeastern Atlantic Ocean and the Mediterranean Sea (Roper *et al*., 1984). They can easily be distinguished from the loliginid squids of the genus *Loligo* also occurring in these geographic regions, as well as *from Sepioteuthis lessoniana* that recently invaded the Eastern Mediterranean (Salman, 2002; Lefkaditou *et al*., 2009), due to their small sizes as adults, the relatively narrower mantle ending to its posterior edge in a pointed tail and the heart-shaped fins (Naef, 1921; Roper *et al*., 1984). However the systematic characters permitting the distinction of the two latter species from each other are not yet considered definitive. Both of them present sexual dimorphism in adults, with females growing larger in *A. media* and males developing longer mantle and tail in *A. subulata.*

Mature males *A. subulata*, reaching 20 cm in dorsal mantle length (ML), are larger than mature *A. media* not extending 14 cm in ML (Mangold & Bo-

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letzky, 1987) and are the only ones easily identified due to the particularly longer tail (fin length 72% of mantle length) (Roper *et al*., 1984). The diameter of the largest club suckers, 8.5-14% of the head width in *A. media* but less than 8% of the head width in *A. subulata*, has been considered valuable to distinguish the two species by several authors carrying out morphometric studies in the laboratory (Naef, 1921; Nesis, 1987; Laptikovsky *et al*., 2002; Anderson *et al*., 2008), yet it cannot be easily detected by naked eye or measured in the field, particularly in smaller specimens.

As their identification in the field still remains confused, the exact geographic limits of each of the above species remain unknown. *Alloteuthis subulata* have been recorded twice from the Aegean Sea during the late 1990's (D' Onghia *et al*., 1996; Salman *et al*., 1997), however, morphometric studies on hundreds of *Alloteuthis* specimens collected from the NE Levantine, the Eastern Aegean and the Marmara Sea suggested that the genus *Alloteuthis* is represented by a single taxonomic unit in the Eastern Mediterranean, which should be considered to be *A. media* (Laptikovsky *et al*., 2002, 2005). In the Eastern Ionian Sea *Alloteuthis media* is commonly found on the continental shelf and the upper slope (Katsanevakis *et al*., 2008), while a single record of *A. subulata* has been reported by Kaspiris & Tsiambaos (1986). Despite the systematic trawl surveys carried out in this area since 1994, in the framework of the "International Bottom Trawl Survey" (MEDITS) and other research projects of the Hellenic Centre for Marine Research (HCMR), it was only in July 2008 when a few male *Alloteuthis* sp. specimens with particularly long tails appeared in the catches.

Recent molecular analyses showed genetic differentiation between Atlantic and Mediterranean *A. media* samples, whereas only few specimens from the Adriatic Sea were genetically assigned to *A. subulata* among those sampled in the Mediterranean Sea (Anderson *et al.*, 2008).

The aims of this study were to verify if the haplotype of the long-tailed males caught in the Eastern Ionian was that of *Alloteuthis subulata*, to investigate probable genetic variation among the specimens assigned to *Alloteuthis media*, as well as to test the utility of various morphometric characters, apart from the length of the fins and tail, upon the distinction of the two sympatric species.

## MATERIALS AND METHODS

### *Sampling*

The samples used in the present study were collected during the MEDITS trawl survey carried out in July 2008 in the Eastern Ionian Sea. Hauls were performed during daytime, using a French synthetic bottom trawl net GOC 73 with 20 mm stretched cod-end mesh size, at pre-defined stations distributed in five depth strata: 10-50, 50-100, 100-200, 200-500 and 500-800 m. The total weight and number of individuals were recorded for all species found at each station following the common MEDITS protocol (Anonymous, 1998). Nine long-tailed males assigned to *A. subulata* were collected from five experimental hauls at depths ranging from 36 to 135 m (Fig. 1).

Small pieces of tissue samples were gathered from these specimens for genetic analysis, as well as from one male, seven females and two unsexed specimens assigned to *A. media*, also collected from the same sampling stations. A second sample of *A. media* comprising 14 females and 15 males with respective mantle length ranges 47-76 mm and 35-57 mm, collected from near-by stations was used for morphometric analysis. Samples of both species were kept frozen in order to take detailed morphometric measurements.

### *Molecular analyses*

Total genomic DNA was extracted from eight *A. subulata* and ten *A. media* specimens using standard methods. A region of cytochrome oxidase subunit I (COI) (~650 bp) mitochondrial gene was amplified via PCR using universal primers (LCO1490: GGT CAA CAA ATC ATA AAG ATA TTG G and HCO2198: TAA ACT TCA GGG TGA CCA AAA AAT CA, Folmer *et al*., 1994) and following amplification conditions described in Anderson *et al*. (2008). PCRs were carried out as 25-μL reactions consisting of 0.625 units *Taq*, 0.5 or 0.25 μM each primer, 0.5 μM each dNTP, 2.5 μL of PCR buffer, 50-200 pg of template DNA, and sterile distilled water. Amplification conditions were as follows: 35 cycles of 90s at 94°C, 60s at 50°C and 90s at 72°C. PCR products were purified by ethanol precipitation and single stranded sequencing was performed with primer LCO1490 using the Big-Dye Terminator Cycle sequencing Kit (v. 3.1, Applied Biosystems) on an ABI 3700 automated sequencer following the manufacturer's protocol. Sequences were edited using BioEdit sequence alignment editor (Hall, 1999); additional sequences for *A. subulata* and *A.*

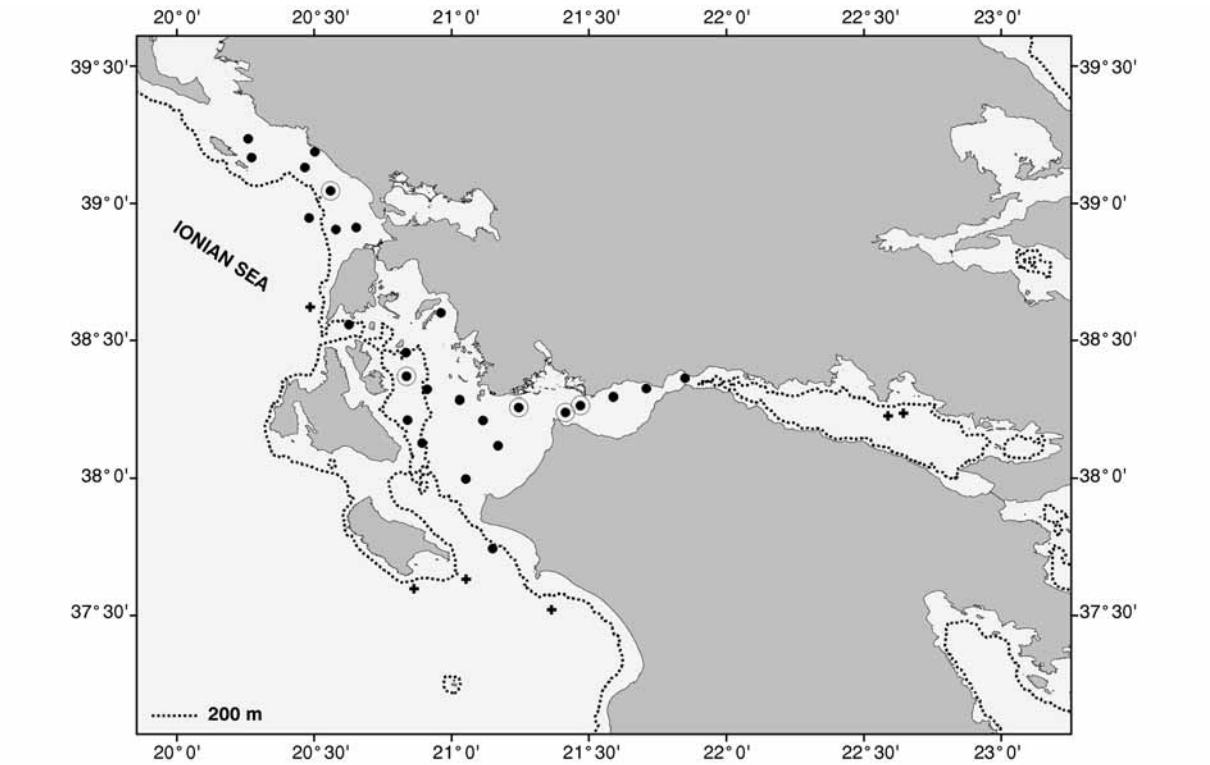


FIG. 1. Grid of sampling stations during the MEDITS-2008 in the Eastern Ionian Sea. Stations where both *A. media* and *A. subulata* were caught are represented with two concentric circles, stations where only *A. media* was fished by one black circle, while stations where neither species of the genus *Alloteuthis* was collected by cross.

TABLE 1. Range, mean and standard deviation for morphometric variables examined in *A. subulata* and *A. media* from the Eastern Ionian Sea (dimension measurements in mm; ML = mantle length; FL = fin length; HL = head length; HW = head width; AL = maximum arm length; TENL = tentacle length; TCL = tentacular club length; TW = tentacular stalk width; MW = mantle width; MLan = ML-FL; I = index)

| | *A. subulata* | | | *A. media* – Females | | | *A. media* – Males | | |
|---|---|---|---|---|---|---|---|---|---|
| | Range | Mean | St. Dev. | Range | Mean | St. Dev. | Range | Mean | St. Dev. |
| ML | 69.9-123.0 | 106.2 | 16.2 | 47.0-76.0 | 57.4 | 9.7 | 35.0-57.0 | 44.1 | 6.4 |
| FL | 43.8-77.5 | 65.8 | 10.3 | 18.0-39.0 | 27.6 | 6.1 | 10.0-27.0 | 18.7 | 4.2 |
| HL | 8.1-12.1 | 10.8 | 1.2 | 10.8-14.0 | 12.6 | 1.0 | 9.0-13.5 | 10.7 | 1.4 |
| HW | 8.6-13.2 | 12.0 | 1.4 | 10.9-16.0 | 12.9 | 1.3 | 9.5-13.0 | 11.0 | 1.2 |
| AL | 11.1-20.1 | 16.7 | 3.1 | 22.0-48.0 | 34.9 | 6.9 | 21.0-37.0 | 27.6 | 4.5 |
| TENL | 30.9-46.9 | 42.9 | 4.7 | 65.0-130.0 | 107.5 | 19.9 | 42.0-111.0 | 80.1 | 20.5 |
| TCL | 9.9-11.9 | 11.3 | 0.7 | 12.1-25.1 | 19.9 | 4.1 | 10.2-24.3 | 14.9 | 4.0 |
| TW | 1.3-1.8 | 1.6 | 0.1 | 1.4-3.4 | 2.4 | 0.7 | 0.8-2.9 | 1.6 | 0.5 |
| MW | 10.0-17.7 | 15.5 | 2.5 | 12.0-21.0 | 18.0 | 2.5 | 13.0-22.0 | 15.2 | 2.3 |
| MLan | 26.2-49.4 | 40.4 | 7.2 | 21.0-38.0 | 29.8 | 5.0 | 37.0-50.0 | 42.2 | 4.4 |
| HLI* | 23.1-31.1 | 27.1 | 2.9 | 34.7-52.7 | 43.0 | 6.1 | 37.0-50.0 | 42.2 | 4.4 |
| HWI* | 25.3-35.0 | 30.1 | 4.0 | 34.7-60.5 | 44.2 | 6.9 | 36.7-55.0 | 43.6 | 4.5 |
| ALI* | 25.4-52.3 | 42.1 | 8.3 | 81.5-166.7 | 118.7 | 23.9 | 80.8-130.0 | 109.3 | 15.1 |
| TENLI* | 89.3-122.1 | 107.7 | 12.0 | 240.7-480.0 | 365.8 | 71.9 | 200.0-420.0 | 315.8 | 74.1 |
| TCLI* | 22.1-37.7 | 28.7 | 4.9 | 43.4-84.6 | 67.3 | 13.1 | 39.2-81.0 | 58.4 | 11.6 |
| TWI* | 3.2-5.0 | 4.1 | 0.6 | 5.0-13.1 | 8.2 | 2.2 | 3.2-9.7 | 6.3 | 1.6 |
| MWI* | 30.6-47.1 | 38.6 | 5.0 | 43.2-85.7 | 61.5 | 10.9 | 48.1-73.3 | 60.1 | 6.9 |

* Indices of the body dimensions were calculated as percentages of the MLan

*media* were retrieved from GenBank (Anderson *et al*., 2008), and were used to align the sequences obtained in the present study and finally to assist molecular taxonomic identification.

*Morphometric data and analyses*

Morphometric variables recorded for *A. subulata* and *A. media* included: dorsal mantle length (ML), mantle width (MW), fin length (FL), maximum arm length (AL), tentacle length (TENL), tentacular stalk width (TW), tentacular club length (TCL), head length (HL) and head width (HW), measured to the nearest 0.1 mm.

As the longer tale is valuable only for the distinction of adult male specimens of the two species, ML and FL were excluded from further analyses and the length of the anterior part of the mantle (from fin's edge to mantle opening: MLan = ML-FL) was used as the reference length upon which indices of the different body dimensions were calculated. The data were organized into three groups according to species and sex in *A. media* (Table 1). A stepwise discriminant function analysis (DFA) based on the ability of morphometric character's percentage indices to correctly identify specimens of each group, was carried out using the statistical package SPSS® for Windows™ Professional Statistics™, Release 11. Each morphometric index was tested with an F-statistic test to examine its contribution to the discrimination between the studied groups. The stepwise method was based on the minimization of Wilk's lambda. The significance of each index (either used or excluded by the stepwise DFA) to the group discrimination was shown by ranking the correlation coefficients between this index and the two discriminant functions. The discriminant scores for the two functions were plotted. Finally, the actual cases for sampled specimens were classified according to whether or not they fell within the statistically predicted variability of the group to which they belonged.

## RESULTS

*Molecular identification of A. media and A. subulata specimens*

Sequences for cytochrome oxidase I (COI) gene were successfully obtained for six out of seven male specimens of *A. subulata* and for 10 out of 12 specimens of *A. media*. For *A. subulata*, sequences were from 372 to 600 bp long and were identical to those previously reported for *A. subulata* specimens from the Adriatic Sea (Accession numbers EU668098-668100, Anderson *et al*., 2008); therefore, COI gene sequences confirmed correct species allocation for all individuals morphologically assigned to the species. For the *A. media* samples, the sequences obtained were from 319 to 600 bp long. Interestingly, one female (Am7) and an unsexed juvenile individual (Am10) carried the *A. subulata* COI haplotype mentioned above. The eight remaining *A. media* specimens had '*A. media*'-type haplotypes (Anderson *et al*., 2008). The sequenced part of four individuals (Am1, Am3, Am6 and Am10) was identical and the other four individuals had each one a distinct haplotype (Am2, Am4, Am5 and Am12) with less than 3 differences between them (Am12 *vs* Am2 and Am12 *vs* Am4). When all publicly available *A. media* haplotypes were used with the sequences found in the present study in a combined dataset, less than six mutations were observed between them. Unique nucleotide sequences obtained in this study were submitted to GenBank under accession numbers GU327599-603.

*Distinction of Alloteuthis species and sexes by morphometric indices*

According to the stepwise method in DFA two indices were kept in analysis, the arm length (AL) and the tentacular stalk width (TW). All the remaining excluded indices appeared to carry information similar to AL, which contributed higher to the first discriminant function (Table 2). This first discriminant

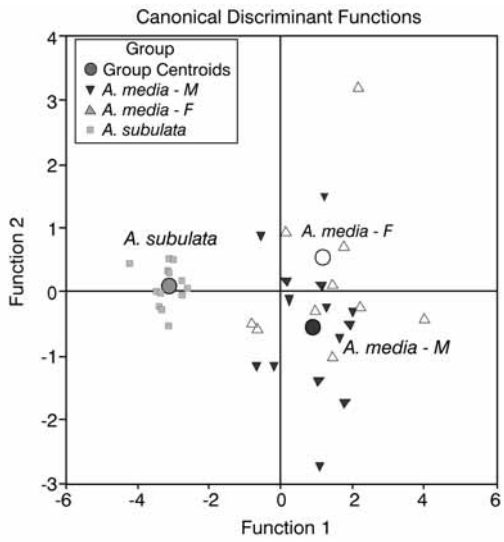


FIG. 2. Graphic presentation of the values of the two discriminant scores for each case and the group centroids.

TABLE 2. Correlation coefficients between each of the examined variables and the discriminant functions produced

| | Function | |
|---|---|---|
| | 1 | 2 |
| AL | 0.976* | 0.216 |
| TENL[a] | 0.713* | 0.332 |
| HW[a] | 0.696* | 0.044 |
| TCL[a] | 0.694 | 0.524 |
| HL[a] | 0.626* | 0.012 |
| MW[a] | 0.469 | 0.176 |
| TW | 0.460 | 0.888* |

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables ordered by absolute size of correlation within function.

* Largest absolute correlation between each variable and any discriminant function

[a] This variable not used in the analysis

function perfectly distinguished *A. subulata* from both male and female *A. media* (Fig. 2). The TW index contributed higher to the second discriminant function, which separated best the two sexes of *A. media*. Male specimen of *A. media* appeared to be more easily discriminated than female ones according to the classification results in Table 3 (correct classification percentages of 80% and 71.4% for males and females, respectively). Overall the DFA classification performance achieved 81.6% for the studied cases.

## DISCUSSION

### *Validating and assigning Alloteuthis specimens to species using molecular markers*

Sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene were successfully used to correctly assign *Alloteuthis* specimens from the Eastern Ionian Sea. In the case of *A. subulata*, all six specimens genetically analyzed carried the haplotype previously reported for three specimens sampled in the geographically close region of Bari in the Adriatic Sea (Anderson *et al*., 2008). Regarding *A. media* samples, two out of ten specimens analyzed had the *A. subulata* haplotype found above and the other eight had haplotypes similar to those reported for the species in Anderson *et al*. (2008). In the latter study, *A. media* samples could be assigned to Mediterranean and Atlantic groups based on more than 1,690 bp sequences obtained from three genes, two mitochondrial (COI and 16S) and one nuclear (rhodopsin gene); in the present study, however, the use of approximately 600 bp sequences of only one mitochondrial gene (COI) does not permit this subtle allocation to any particular group. Overall, specimens may simply be identified as belonging to the *A. media* or *A. subulata* species.

### *Evaluation of morphometric indices as diagnostic characters of Alloteuthis species*

Among the indices examined in the present study, ratios of arm length, tentacle length, tentacular club length, head length and width to the length of the mantle's anterior part, being relatively smaller in *A. subulata* than in *A. media*, have proven significant for classification of the examined specimens in each species. Furthermore, those morphometric characters relevant to arm, tentacle and club length might be quite easily compared even by naked eye while sorting in the field. The length of the anterior dorsal part of the mantle (from fin's edge to mantle opening) could be defined as a new standard measurement useful for *Alloteuthis* taxonomy, as well as for any other squid species which develop a long tail as adults.

Nevertheless, since no female *A. subulata* were examined and in neither species the whole size range

TABLE 3. Classification results of the stepwise discriminant function analysis showing the number and percentage of specimens classified in each group. 81.6% of the original grouped cases were correctly classified

| | | Predicted Group Membership | | | |
|---|---|---|---|---|---|
| | GROUP | *A. subulata* | *A. media* – F | *A. media* – M | Total |
| Count | *A. subulata* | 9 | 0 | 0 | 9 |
| | *A. media* – F | 0 | 10 | 4 | 14 |
| | *A. media* – M | 0 | 3 | 12 | 15 |
| % | *A. subulata* | 100.0 | 0.0 | 0.0 | 100.0 |
| | *A. media* – F | 0.0 | 71.4 | 28.6 | 100.0 |
| | *A. media* – M | 0.0 | 20.0 | 80.0 | 100.0 |

was sampled, the results of the present study should be considered with caution. In future, a larger sample, covering the whole size range for both species and sexes, from different geographic areas, should be subjected to both genetic and morphometric analyses, in order to confirm the usefulness of the above morphometric variables in the distinction of specimens of the species *A. subulata* and *A. media*.

*Geographical range and abundance of Alloteuthis subulata in the Mediterranean Sea*

The long-tailed males of *A. subulata* were described for the first time in the Mediterranean Sea by Jatta in 1896 (as cited in Naef, 1921), who called them "Loligo media". Naef (1921), who continued the work of Jatta on the cephalopod fauna of the Bay of Naples, established the currently used scientific names *A. media* and *A. subulata* following the rules of nomenclature determined at international congresses and described in detail the two sympatric species and their distinctive characteristics, based on several specimen of both sexes. As mentioned for the Bay of Naples (Naef, 1921), *A. subulata* is not so rare but either can be considered common in the Western Mediterranean. As it is evidenced by faunistic studies in the Adriatic Sea (Gamulin-Brida & Ilijanic, 1972; Bello, 1990; Ungaro *et al*., 1999; Krstulovic-Sifner *et al*., 2005), the Western Ionian Sea (Panetta, 1974; Maiorano *et al*., 1999), the Catalan and Alboran Seas (Mangold-Wirtz, 1963; Sanchez *et al*., 1998; Gonzalez & Sanchez, 2002), *A. subulata* is caught mainly on upper shelf, in much lower numbers than *A. media*, constituting an indicator species for cephalopod assemblages only along the Iberian shelf (Gonzalez & Sanchez, 2002). In the regions of Mediterranean east of 20° E, the species is much rarer. The earliest document including species of the genus *Alloteuthis* concerned Syrian waters, where two loliginid squid species with a pointed tail not exceeding 10 cm in ML, "*Loligo officinalis*" and "*Loligo subulata*", were mentioned and the latter was reported as much more abundant (Gruvel, 1931). As there is not in the text of Gruvel (1931) any further description of these species and their distinctive characters, it is probable that the name "*Loligo subulata*" corresponded to *A. media*, as it has been noted for other cases by Naef (1921). Next to this reference, two females were recorded from the waters of Israel (Ruby & Knudsen, 1972), while the occurrence of *A. subulata* in the Sea of Marmara was mentioned in the review on squid biology and fishing by Zuev & Nesis (1971). In the Eastern Ionian and the Aegean Sea, a few individuals of *A. subulata* had been reported from trawl catches in the early 1980's (Kaspiris & Tsiambaos, 1986) and early 1990's (D' Onghia *et al*., 1996; Salman *et al*., 1997) respectively, but the species was never reported before 2008 among catches of the MEDITS trawl surveys, which cover most of trawlable fishing grounds in these areas on an annual basis, since 1994. Recent morphometric and genetic analyses performed for different *Alloteuthis* morphotypes from the Aegean Sea, suggested that they should be considered as *A. media* (Laptikovsky *et al*., 2002; Anderson *et al*., 2008). Similar studies, failed in confirming *A. subulata* identity among examined specimens from the Western Mediterranean and the north-eastern Atlantic, but have verified the presence of *A. subulata* in the Adriatic Sea (Anderson *et al*., 2008), which according to our results is also confirmed in the Eastern Ionian Sea. Certainly, further morphometric and molecular analyses of specimen macroscopically identified as *A. media* and *A subulata*, by different scientists along the Mediterranean shelf, are needed to clarify the identity of appearing morphotypes.

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