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ORIGINAL PAPER

Evidence of phenotypic plasticity in *Alloteuthis media* (Linnaeus, 1758) from morphological analyses on North Sea specimens and DNA barcoding of the genus *Alloteuthis* Wülker, 1920 across its latitudinal range

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Abstract

Despite being landed in commercial cephalopod fisheries, species of *Alloteuthis* are not yet well defined, with *A. subulata* and *A. media* often confused. DNA barcoding combined with morphometric analyses has begun to clarify the distinction between these two morphologically similar species but has been limited in its geographic coverage to date. Herein, we provide DNA barcodes for 228 specimens collected from Guinea Bissau in the south, up the Atlantic coast, to the Irish shelf and North Sea. Employing species delimitation analyses, and with comparison to the literature, we identified 24 individuals of *A. africana,* 66 individuals of *A. subulata* and 138 individuals of *A. media*. We confirm that *A. media* has the northernmost distribution and is the only species identified by DNA sequencing from the Irish shelf and North Sea. We analysed morphometric measures and indices from 388 individuals from the North Sea, a subset of which ($n = 58$) were barcoded. The most useful traits for identification were tail length as a percentage of dorsal mantle length, and largest club sucker width as a percentage of head width. By comparison to other published data, we determined that *A. media* phenotypes vary substantially across the geographic range of this species. This partly explains the difficulties in morphological identification and suggests regional identification guides may be required in support of fisheries management. Interregional analyses suggest character displacement may occur where species co-exist.

Keywords Molecular identification · Genetic identification · Morphometrics · Fisheries · Character displacement

Introduction

Alloteuthis Wülker, 1920 is a genus of small coastal squids (Order: Myopsida; Family: Loliginidae) comprising three species: *Alloteuthis media* (Linnaeus, 1758), *Alloteuthis subulata* (Lamarck, 1798), and *Alloteuthis africana* Adam, 1950. *Alloteuthis africana* has a more southerly distribution, from Southern Morocco to Namibia, than the other two species (Jereb et al. 2010) so that there is no distribution overlap. Furthermore, *A. africana* is easily distinguished from its congeners by its broader head. The distributions of *A. media* and *A. subulata* are more northerly and overlap in the Mediterranean and North East Atlantic. However, the true range extents of *A. media* and *A. subulata* are unclear due to difficulties in distinguishing these species (Hastie et al. 2015; Lefkaditou et al. 2015) that in part are because morphological differences between the species vary by length

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and sex (Naef 1912, 1923; Grimpe 1925). Both species are likely components of *Loligo* spp. landings throughout their distribution and maybe landed as a secondary target or bycatch in Spain, Portugal and Italy. Even when recognised as *Alloteuthis* spp., they are rarely identified beyond genus level (Hastie et al. 2015) even though landings can be quite substantial: estimated as fluctuating between 55 and 290 tonnes in the Gulf of Cádiz, for example (Lefkaditou et al. 2015).

DNA sequencing studies have clearly established the presence of two genetically distinct species in the Mediterranean and North East Atlantic (Anderson et al. 2008; Lefkaditou et al. 2012; Alujević et al. 2022). Anderson et al. (2008) sampled three locations in the Mediterranean and four in the NE Atlantic (though none were in the North Sea) and sequenced two mitochondrial (COI and 16S rRNA) and one nuclear gene (rhodopsin) for 30 individuals and concluded that *A. media* occurred at all sampled locations, and that *A. subulata* was present only at Mola di Bari in the Adriatic Sea (Mediterranean). Subsequent COI barcoding, with specimens identified due to sequence similarity to those of Anderson et al. (2008), added the Ionian Sea (Lefkaditou et al. 2012), Ría de Vigo (Northwest Spain; Olmos-Pérez et al. 2018) and the Eastern Adriatic (Alujević et al. 2022) as locations where both *A. media* and *A. subulata* are present. A study in the North Sea which included DNA sequencing showed only one species of *Alloteuthis* present there (Gebhardt and Knebelsberger 2015). These North Sea specimens had morphological characteristics of both species, as well as intermediate characteristics. Researchers hesitated to apply a name, but subsequent analysis (Alujević et al. 2022) showed all DNA barcoded specimens to be *A. media*.

There are genuine issues in assigning names to clades. First, the original descriptions are very limited. For *A. media*, the original description translates as 'Sepia with a body flattened and caudate on both sides' (Bello 2019) and the type locality is 'pelago', while for *A. subulata*, the original description translates as 'Loligo with narrow wings coming out of the subulate tail, gladius with three dorsal nerves, subacute on both sides' (Bello 2019) while the type locality was not stated (Lamarck 1798). Each of these descriptions could fit either species. Second, neither type specimen has been located at their respective assumed repositories (Paris Museum and Linnean Society) and are presumed not to exist (Lu et al. 1995; Sweeney and Roper 1998; Anderson et al. 2008). However, detective work on the literature is possible, as demonstrated by Naef (1912), who early on recognised confusion surrounding these species. Several names that are synonyms of *A. media* were in use at that time, including *Loligo marmorae* Vérany, 1839 and *Loligo parva* Froriep, 1806. Naef (1912) reported that the names *marmorae* and *media* were often applied according to tail length, but also recognised that *Loligo parva, L. marmorae* and *L. media* were the same species, and that only mature males of *A. subulata* have long tails. After studying several hundred specimens from Naples and far fewer from elsewhere, Naef concluded that two distinct species of *Alloteuthis* (contemporaneously referred to as *Teuthis*), *A. media* and *A. subulata*, did indeed occur in the Mediterranean, that the most common Mediterranean species was *A. media*, and that this species had strong tentacles and clubs with large suckers, as per the illustration of Rondelet (Rondeletius 1554) that was used by Linnaeus in his description of *A. media*. Grimpe (1925) also concurred that Vérany's *marmorae* and the species depicted by Rondelet's woodcut were the same species. While Naef (1912) did also find some specimens of *A. subulata* in the Mediterranean, most of his *A. subulata* samples came from the Atlantic and North Sea, and he thus referred to *A. subulata* as the northern form ('die nordische Form') of the genus. He described *A. subulata* as having delicate tentacles with small clubs bearing small suckers. These tentacular characteristics were used by Anderson et al. (2008) to apply species names to phylogenetic clades.

Naef (1923) listed additional characters that he felt distinguished *A. media* and *A. subulata*, including differences in tail length, and arrangement of papillae and suckers on the hectocotylus. Anderson et al. (2008) summarised characters listed by Grimpe (1925) based on his own observations, and by Roper et al. (1984) and Nesis (1987), both identification guides which drew on broad literature. Since characters of the hectocotylised arm are less useful as they can only be used to identify mature males, Anderson et al. (2008) focussed on the size and shape of the tentacular stalk and club and its suckers, and the length of the tail.

When the usefulness of these characters in distinguishing genetically identified specimens was tested, it was found that while tail length was not useful, club width and club sucker diameter might be, although the data were confounded by only three *A. subulata* specimens, one of which was missing both tentacles (Anderson et al. 2008). Arm length and tentacular stalk width were most discriminatory in specimens collected from the Eastern Ionian Sea (Lefkaditou et al. 2012), but this study was also confounded by low numbers (only nine *A. subulata* none of which were female). In the Ría de Vigo on the Atlantic coast of northern Spain, tentacle length had the strongest statistical explanatory power in distinguishing paralarvae of *A. media* and *A. subulata* after correcting for paralarval age—with the tentacles of *A. media* being longer (Olmoz-Pérez et al. 2018), reflecting the view of Naef (1923) that long tentacles were indicative of *A. media*. However, in the Eastern Adriatic, indices based on tail length and tentacular club length failed to discriminate species (Alujević et al. 2022).

Each of these studies has progressed, but not solved, our ability to distinguish and correctly identify *Alloteuthis* species. The outstanding questions concern (1) the total range

of each species, which is not known outside the locations sampled for DNA sequence analysis, and (2) whether any of the proposed morphometric characters are effective at discriminating these species throughout their ranges. Certainly, identification problems remain in the North Sea, which is traditionally considered to harbour *A. subulata* (Naef 1912) but apparently harbours *A. media* (Alujević et al. 2022) with individuals displaying a range of morphologies (Gebhardt and Knebelsberger 2015).

The aims of this study were twofold. First, our intention was to extend the geographic range of available *Alloteuthis* DNA barcodes (Folmer region of mitochondrial COI) to better understand the ranges, including range overlap, of each species. Second, having independently realised that North Sea specimens pertained to *A. media*, we wished to investigate whether morphometric criteria that seemed promising for discerning species in the Mediterranean (Anderson et al. 2008) could be applied to more northerly waters. This information will fill crucial knowledge gaps in our understanding of distribution, improve reliability of identifications, and is essential in respect to fisheries management issues and for our understanding of the ecology of the species (Lishchenko et al. 2021; Oesterwind et al. 2022). In particular, it will help to disentangle the species composition of fisheries landing statistics that are often provided at family level (Loliginidae).

Methods

Sample collection

Between 2018 and 2019, tissue samples from 177 specimens were collected for DNA analyses and preserved in 96–100% ethanol at − 20 °C during six research cruises throughout the North East Atlantic, from Guinea Bissau in the south to the Greater North Sea in the north via a North Atlantic Area INTERREG project 'Cephs and Chefs'. In 2021, a further 397 animals were frozen whole during research cruises in the North Sea in Quarter 1 (Q1) and Quarter 3 (Q3). Morphological measurements were subsequently taken from these 397 whole specimens, while tissue samples from a subset were preserved in ethanol for DNA analyses ($n = 58$; Supplementary Information S1). COI sequence data were available from GenBank for a further 144 *Alloteuthis* specimens. The geographic distribution of specimens for which COI barcodes were available is summarised in Table 1 and Fig. 1. Proxy co-ordinates (based on best available information) were used to plot the GenBank data (Supplementary Information S1) except for samples reported in Gebhardt and Knebelsberger (2015), where exact co-ordinates were available.

Table 1 Numbers of specimens sequenced (excluding seven sequences with ambiguity codes which were discarded from analyses)

ICES ecoregion or other location	Reference or survey code	*A. africana*	*A. subulata*	*A. media*
Baltic Sea	Gebhardt and Knebelsberger 2015	0	0	2
Greater North Sea	This study; DK/DE IBTS 3Q 21	0	0	42
	This study; DE IBTS 1Q 21	0	0	16
	Gebhardt and Knebelsberger 2015	0	0	24
Celtic Seas	This study; IAMS2018	0	1*	0
	This study; IGFS2018	0	0	29
Bay of Biscay and the Iberian Coast	This study; ARSA1119	0	17	50
	This study; DESCARSEL0819	0	48	1
	Anderson et al. 2008	0	0	10
	Olmos Perez et al. 2018	0	3	21
Angola	Anderson et al. 2008	10	0	0
Guinea Bissau	This study; BISSAU1219	24	0	0
Western Mediterranean Sea	Anderson et al. 2008	0	33	2
Ionian Sea and the Central Mediterranean Sea	Lefkaditou et al. 2012	0	1	4
Adriatic Sea	Alujević et al. 2022	0	21	30
	Anderson et al. 2008	0	3	7
Aegean-Levantine Sea	Anderson et al. 2008	0	0	6
		34	94	244

Locations are grouped according to ICES ecoregions (or other location when outside the ICES advisory area). Where sequences were obtained from GenBank, published reference is given. For specimens newly sequenced herein, the field-survey code is provided. Total numbers of specimens sequenced included in analysis are indicated in last row

*Caught in the southern Celtic Sea

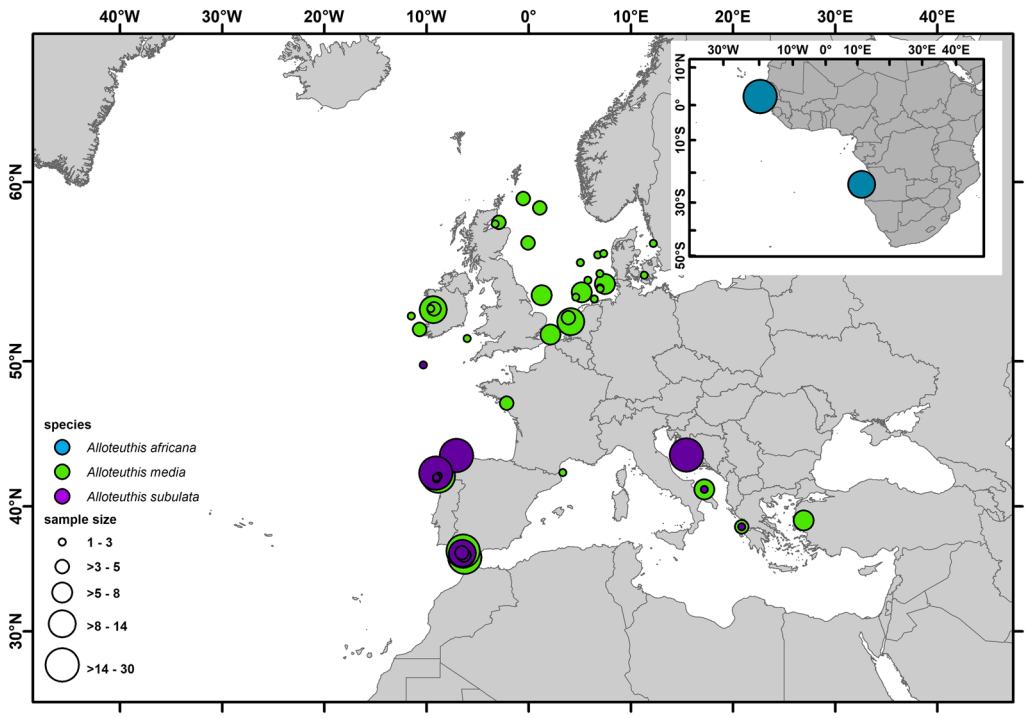

Fig. 1 Geographical distribution of the three *Alloteuthis* species based on 372 barcoded individuals (Table 1). Data collection includes information from our study, Anderson et al. 2008; Lefkaditou et al. 2012; Gebhardt and Knebelsberger 2015; Olmos-Pérez et al. 2018; Alujević et al. 2022

DNA barcoding

DNA was extracted from the mantle tissue of each specimen following the Invitrogen™ PureLink™ Genomic DNA Mini Kit Mammalian Tissue and Mouse/Rat Tail Lysate protocol. DNA was eluted in 100 µL Genomic Elution Buffer and stored at − 20 °C.

DNA barcoding, which targets a 650 bp fragment of the COI gene, was performed using forward primer LCO1490: 5′-ggtcaacaaatcataaagatattgg-3′ and reverse primer HC02198: 5′-taaacttcagggtgaccaaaaaatca-3′ (Folmer et al. 1994) on 235 samples distributed in the Greater North Sea, Celtic Seas, Bay of Biscay and the Iberian Coast, and Guinea Bissau. Each PCR contained 12.5 µL of Thermo Scientific™ DreamTaq Green PCR Master Mix (2X), 0.5 µM of each primer, 2.5 µL of DNA and 9 µL H_20 resulting in final reaction volume of 25 µL. A negative control was also included to ensure contamination did not occur. The PCR conditions were 94 °C for 2 min, 35 cycles of 94 °C for 40 s, 50 °C for 40 s and 72 °C for 90 s, followed by 72 °C for 10 min (Allcock et al. 2007). PCR products were visualised on a 1% (w/v) agarose gel and bands of 650 bp were obtained. PCR products were cleaned using Invitrogen™ PureLink™ PCR Purification Kit according to the manufacturer's instructions. Purified PCR products were standardised to 12 ng/µL using a Biochrom SimpliNano NanoDrop Spectrophotometer in accordance with the DNA sequencing facility specifications. Samples were prepared for sequencing by adding 5 µL of each purified PCR product to 5 µM forward primer LCO1490 resulting in a 10 µL reaction volume. Samples were sent to Eurofins Genomics (Germany) for DNA sequencing on an ABI 3730XL DNA Analyzer.

All electropherograms were checked using 4 Peaks (Nucleobytes™) and aligned in MEGA (Molecular Evolutionary Genetics Analysis) version X (Kumar et al. 2018) together with 144 *Alloteuthis* COI sequences from GenBank (Supplementary Information S1) using the MUSCLE (Edgar 2004) algorithm. The alignment was trimmed such that the maximum number of GenBank samples could be included without introducing regions of ambiguity codes; seven newly generated sequences were removed due to the presence of ambiguity codes in these sequences. The resulting alignment was 445 bp long, aligned by codon, contained no gaps or ambiguities and comprised 372 sequences, 228 of which were newly generated (58 from Greater North Sea, 30 from Celtic Seas, 116 from Bay of Biscay and the Iberian Coast and 24 from Guinea Bissau; Table 1).

Species delimitation

For phylogenetic analysis only, a COI sequence from *Loligo forbesii* (AF075402; Anderson 2000) was added to the

alignment matrix to provide an outgroup, and only unique haplotypes were retained in the matrix. This resulted in a matrix containing 65 unique *Alloteuthis* haplotypes. A maximum likelihood tree was built in IQTree (Nguyen et al. 2015). IQTree called ModelFinder (Kalyaanamoorthy et al. 2017), selected the HKY model (Hasegawa et al. 1985), and applied it with empirical base frequencies and the discrete Gamma model (Yang 1994) with four rate categories. A consensus tree was constructed from 1000 standard bootstraps (Felsenstein 1985).

A species delimitation analysis was performed using ASAP (Assemble Species by Automatic Partitioning (Puillandre et al. 2021), using the 372-sequence alignment matrix. We applied the Kimura (K80) model and default settings. ASAP implements a hierarchical clustering algorithm using pairwise genetic distances and proposes potential species partitions ranked on a scoring system that accounts for the probability of proposed solutions actually having less diversity and the width of the barcode gap. We present the solution with the lowest ASAP score on the maximum likelihood trees, indicating delimited species and the major clades to which they correspond using a colour-blind friendly colour ramp generated in the R (version 4.1.2; R Core Team 2022) package viridis (Garnier et al. 2021). We apply species names which correspond to data and decisions of Anderson et al. (2008). For further details of application of names, see Discussion.

Within-species diversity analysis and genetic structure

Using the 372 sequence, 445 bp alignment (see previous), a haplotype network was created in TCS v1.21 software (Clement et al. 2000) which uses statistical parsimony (Templeton et al. 1992) to detect and connect haplotypes. The threshold of mutational changes was left at the default of 95%, a level which when used with COI tends to split haplotypes into species networks since the 'barcode gap' tends to operate at a similar threshold. The resulting haplotype networks were illustrated using the online tool tcsBU (Santos et al. 2016). A ten-shade colour-blind friendly colour palette was created using the following website https://medialab.github.io/iwanthue. Colours were assigned according to ICES ecoregions. We chose ICES ecoregions because they are more practical from a fisheries management perspective. *Alloteuthis* spp. are landed commercially throughout much of their range and ICES reporting and advice are linked to these ecoregions (ICES 2020). ICES ecoregions are very closely aligned with those of Spalding et al. (2007), with most regions included here mapping directly, except that: ICES Aegean–Levantine Sea maps to two of Spalding et al. ecoregions (Aegean Sea and Levantine Sea); and ICES Ionian Sea and the Central Mediterranean maps to two of Spalding et al. ecoregions (Ionian Sea and Turisian Plateau/Gulf of Sidra). Angola and Guinea Bissau lie outside ICES ecoregions (they map to Angolan and Gulf of Guinea, respectively, in the Spalding et al. scheme) and were colour coded as separate locations.

Species diversity indices were calculated using DnaSP software v5.1 (Librado and Rozas 2009) using separate alignments for each of the three species, constructed by splitting the original 372-sequence alignment according to the results of the species delimitation. DnaSP calculates levels of variation within and between sequences, and also performs some neutrality tests. Haplotype (or gene) diversity shows the probability that two alleles, sampled at random, will differ (Nei 1987). DnaSP implements Nei (1987; Eqs. 8.4 and 8.12 but replacing $2n$ by n) to calculate haplotype (gene) diversity and its variance. Nucleotide diversity Pi (π) is calculated as the average number of nucleotide differences per site between two sequences according to Nei (1987 Eqs. 10.5 or 10.6 and its sampling variance; Eq. 10.7). To test whether mutations are selectively neutral, Fu's *Fs* (Fu 1997) and Tajima's *D* (Tajima 1989) statistic were also calculated, their significance assessed under coalescent simulation. The *Fs* test statistic is used to determine whether there is a greater number of alleles in a sample than predicted by neutral theory, while Tajima's *D* is based on the differences between the number of segregating sites and the average number of nucleotide differences.

Genetic structure of *Alloteuthis media* was assessed by a pairwise F_{ST} test using R packages *adegenet* 2.1.6 (Jombart 2008) and *hierfstat* 0.5–11 (Goudet 2005).

Morphometric traits (North Sea)

The following morphometric traits from Anderson et al. (2008) were measured or calculated: (i) tail length (TL) as % of mantle length (DML); (ii) largest club sucker size (LCS) as % of head width (HW); (iii) maximum DML; (iv) longest arm length (AL) as % DML; and (v) tentacle length (Tentacle L). Note, that Anderson et al. (2008) measured dorsal mantle length (DML) but refer to the measurement simply as 'Mantle Length (ML)'. For clarity, we have specified DML throughout the results and discussion. Similarly, Anderson et al. (2008) refer to arm length (AL) and tail length (TL) but calculated each as a percentage of ML. These indices are identical to our AL/DML and TL/DML, respectively. Finally, 'Maximum ML' of Anderson et al. (2008) is renamed 'Max DML' in our results. For specifics of measurements, see Fig. 2.

For four of the five morphometric traits above, a species assignation was made according to cut-off values for these traits (Table 2; see Anderson et al. 2008). Results of species assignation using morphometric traits for the $n = 58$ barcoded specimens were tabulated separately from

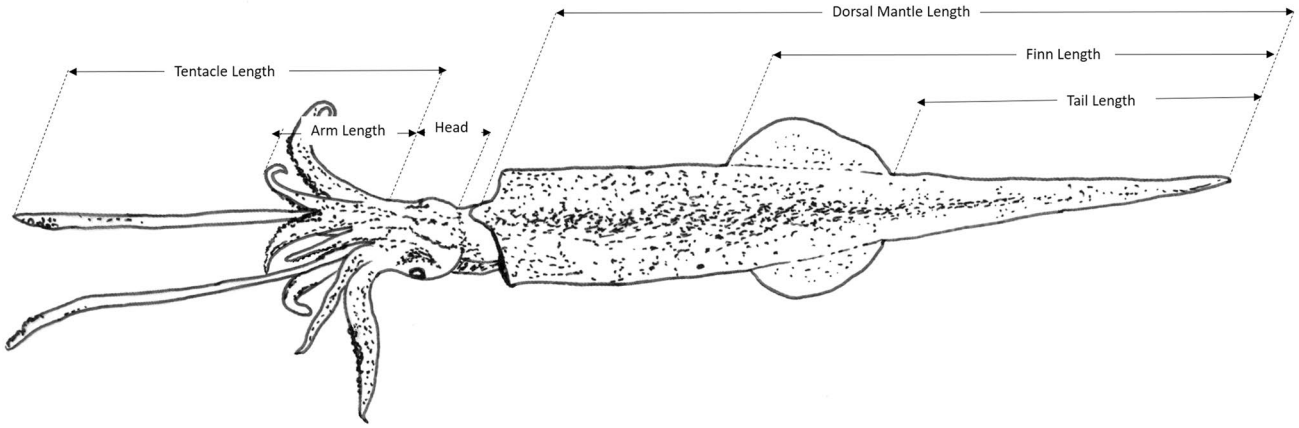

Fig. 2 Measurement dimension details for morphometric traits used in this study

a further $n = 330$ samples which were not barcoded, only measured. All non-barcoded samples came from the same sampling year (2021) and sampling quarters (Q1, Q3) as the barcoded samples. Tentacle length (Tentacle L) was not broken down by species in Anderson et al. (2008), apart from specifying 'long' (in *A. media*) or 'short' (in *A. subulata*), so, although measurements for this trait were included for comparative purposes, species were not assigned to North Sea specimens on this trait.

Further morphometrics were considered after Grimpe (1925) including: fin length (FL) as % ventral mantle length (VML; this trait is reported only to work for males > 3 cm and females > 4 cm so only individuals larger than these values in each sex were included); and ratio of VML: longest AL (although Grimpe (1925) used mean AL rather than longest AL). Because both of these morphometric traits require ventral mantle length (VML) dimensions, VML was measured for a sample ($n = 339$) of non-barcoded individuals and a linear regression was used to convert between DML and VML for the barcoded sample (where VML had not been measured). Species assignations using both FL/VML and AL/VML were examined in both barcoded specimens ($n = 54$) and non-barcoded specimens ($n = 294$, $n = 323$, respectively, for each metric). Species assignation was according to cut-off values for both species (Table 2).

Finally, North Sea samples (this study) were compared with samples taken in the eastern Ionian Sea (Lefkaditou et al. 2012). The morphometric traits for this comparison were: HLI = head length index; HWI = head width index; ALI = maximum arm length index; TENLI = tentacle length index; TCLI = tentacular club length index; TWI = tentacular stalk width index; MWI = mantle width index. In each case, indices were calculated relative to MLan which was ML-FL (mantle length minus fin length) (Table 2). All North Sea specimens (barcoded and non-barcoded) were assigned a sex and maturity stage according to the criteria outlined in ICES (2010).

Results

COI sequences for 228 specimens are deposited in GenBank with accession numbers OP831327-OP831496 (Supplementary information S2).

Species delimitation

IQTree produced a Maximum Likelihood Tree (Fig. 3) with three clearly distinguishable clades. ASAP identified each of these clades as a separate species (Fig. 3), which could be assigned to named species (*A. subulata, A. media, A. africana*) based on sequences previously published by Anderson et al. (2008) included in the species blocks (Supplementary Information S3). Our dataset comprised 7 haplotypes and 34 individuals of *A. africana,* 5 haplotypes and 94 individuals of *A. subulata* and 53 haplotypes and 244 individuals of *A. media.*

Within-species diversity analysis

Statistical parsimony analysis produced three unconnected haplotype networks which corresponded to each of the species delimited by ASAP (Figs. 3, 4). There were 65 haplotypes in total: no haplotypes were shared between species, and the number of haplotypes per species was as described above. *Alloteuthis africana* was captured among samples from Guinea Bissau and Angola only; the same haplotype was dominant at both geographic locations. *Alloteuthis subulata* was captured among samples from the Bay of Biscay and the Iberian Coast, Celtic Seas (southern), the Adriatic Sea and the Ionian Sea and the Central Mediterranean Sea;

Table 2 Species' cut-off values for morphometric indices according to different studies

Trait	Species	range	Mean ± SD	Reference
TL as % DML	*A. subulata*	> 50		Anderson et al. 2008
	A. media	< 50		
LCS as % HW	*A. subulata*	6–8		Anderson et al. 2008
	A. media	9–14		
Max DML	*A. subulata*	20 cm (M)		Anderson et al. 2008
		12 cm (F)		
	A. media	12 cm		
AL as % DML	*A. subulata*	20–25		Anderson et al. 2008
	A. media	No value[1]		
Tentacle L	*A. subulata*	Short		Anderson et al. 2008
	A. media	Long		
FL/VML	*A. subulata*	52–70%	61%	Grimpe 1925
	A. media	45–52%	48%	
VML: longest AL[2]	*A. subulata*	≥ 3		Grimpe 1925[3]
	A. media	~ 2½		
HLI	*A. subulata*	23.1–31.1 (M)	27.1 ± 2.9 (M)	Lefkaditou et al. 2012
	A. media	37.0–50.0 (M)	42.2 ± 4.4 (M)	
		34.7–52.7 (F)	43.0 ± 6.1 (F)	
HWI	*A. subulata*	25.3–35.0 (M)	30.1 ± 4.0 (M)	Lefkaditou et al. 2012
	A. media	36.7–55.0 (M)	43.6 ± 4.5 (M)	
		34.7–60.5 (F)	44.2 ± 6.9 (F)	
ALI	*A. subulata*	25.4–52.3 (M)	42.1 ± 8.3 (M)	Lefkaditou et al. 2012
	A. media	80.8–130.0 (M)	109.3 ± 15.1 (M)	
		81.5–166.7 (F)	118.7 ± 23.9 (F)	
TENLI	*A. subulata*	89.3–122.1 (M)	107.7 ± 12.0 (M)	Lefkaditou et al. 2012
	A. media	200.0–420.0 (M)	315.8 ± 74.1 (M)	
		240.7–480.0 (F)	365.8 ± 71.9 (F)	
TCLI	*A. subulata*	22.1–37.7 (M)	28.7 ± 4.9 (M)	Lefkaditou et al. 2012
	A. media	39.2–81.0 (M)	58.4 ± 11.6 (M)	
		43.4–84.6 (F)	67.3 ± 13.1 (F)	
TWI	*A. subulata*	3.2–5.0 (M)	4.1 ± 0.6 (M)	Lefkaditou et al. 2012
	A. media	3.2–9.7 (M)	6.3 ± 1.6 (M)	
		5.0–13.1 (F)	8.2 ± 2.2 (F)	
MWI	*A. subulata*	30.6–47.1 (M)	38.6 ± 5.0 (M)	Lefkaditou et al. 2012
	A. media	48.1–73.3 (M)	60.1 ± 6.9 (M)	
		43.2–85.7 (F)	61.5 ± 10.9 (F)	
Tentacle L	*A. subulata*	3.09–4.69 cm (M)	4.29 ± 0.47 cm (M)	Lefkaditou et al. 2012
	A. media	4.20–11.10 cm (M)	8.01 ± 2.05 cm (M)	
		6.50–13.00 cm (F)	10.75 ± 1.99 cm (F)	
AL as % DML[4]	*A. subulata*		15.7 (M)	Lefkaditou et al. 2012
	A. media		62.6 (M)	
			60.8 (F)	

Indices were calculated in each case relative to MLan which was ML-FL (mantle length minus fin length)

TL tail length, *DML* dorsal mantle length, *LCS* largest club sucker size, *HW* head width, *AL* longest arm length, *FL* fin length, *VML* ventral mantle length, *HLI* head length index, *HWI* head width index, *ALI* maximum arm length index, *TENLI* tentacle length index, *TCLI* tentacular club length index, *TWI* tentacular stalk width index, *MWI* mantle width index, *M* males, *F* females. Measurements from Lefkaditou et al. (2012) are converted from mm to cm

[1]no data for *A. media* are given in Anderson et al. (2008); we have assumed values > ~25% are *A. media*

[2]applies only to males with VML > 3 cm and females with VML > 4 cm

[3]Grimpe (1925) used VML: *mean* AL. We used VML: *longest* AL as the latter is faster to measure

[4]calculated based on mean measurements in Table 1 of Lefkaditou et al. (2012)

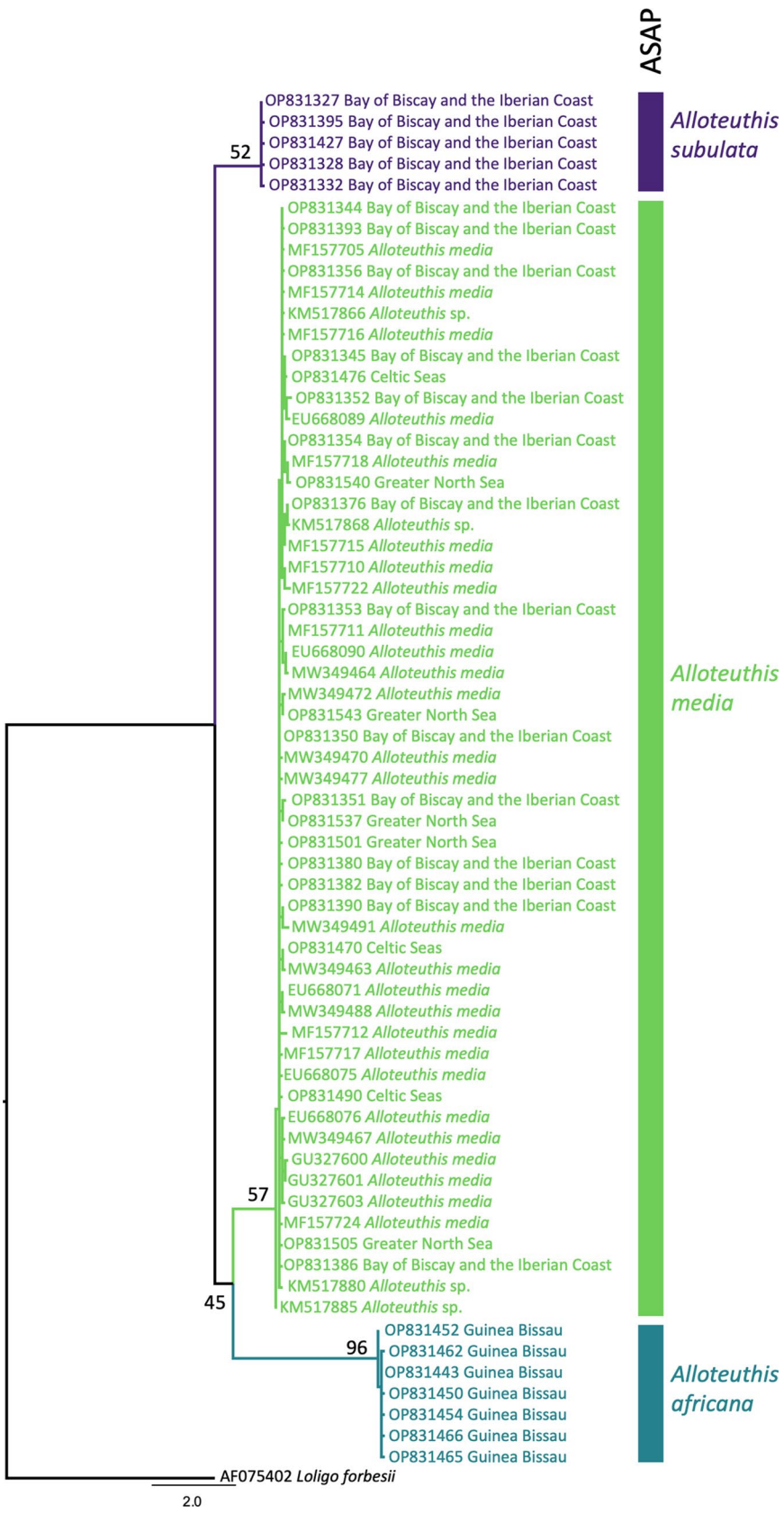

Fig. 3 Maximum likelihood of 65 *COI* haplotypes of *Alloteuthis*, rooted on *Loligo forbesii*. The matrix of haplotypes was generated in RAxML from the 372-sequence alignment. RAxML (Stamatakis 2014) assigns one sequence of each haplotype to a new alignment file; thus, the included sequences are an eclectic selection, but they represent all 65 haplotypes of the three species. Species delimitation based on ASAP (Puillandre et al. 2021)

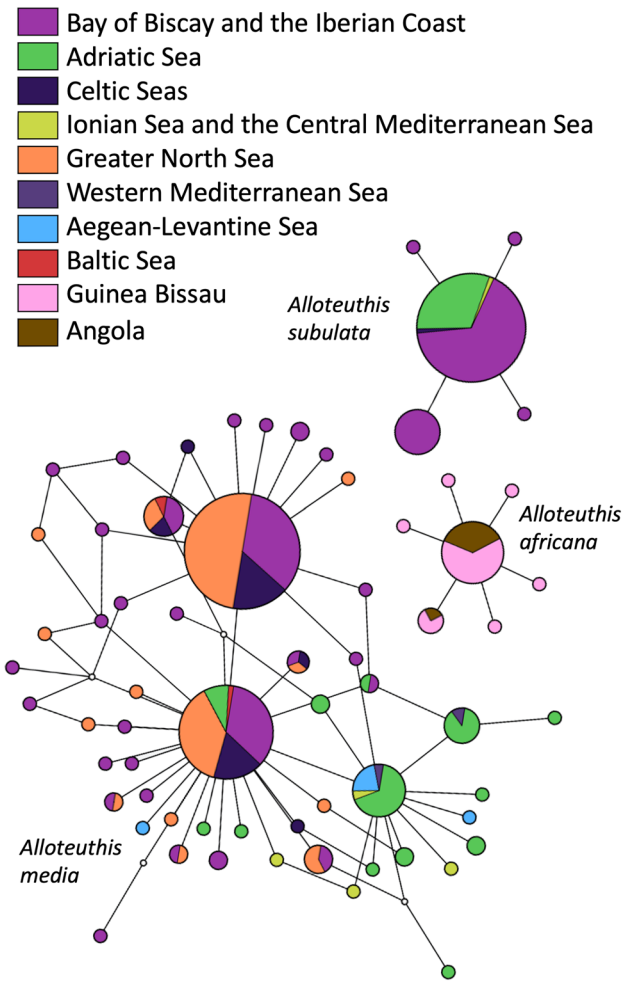

Fig. 4 Haplotype network created using TCS 1.21 (Clement et al. 2000) and illustrated using tcsBU (Santos et al. 2016)

again, one single haplotype was dominant at all geographic locations. *Alloteuthis media*, which had the greatest haplotype diversity ($n = 53$), also had the greatest geographic range and was captured among samples from Bay of Biscay and the Iberian coast, Celtic Sea, Greater North Sea, Adriatic, West Mediterranean, Aegean-Levantine Sea, Baltic Sea and the Ionian Sea and the central Mediterranean Sea. Two haplotypes dominate *Alloteuthis media* samples from the Greater North Sea, Bay of Biscay and the Iberian Coast and Celtic Seas (Fig. 4). Two other *A. media* haplotypes are prevalent among Adriatic Sea samples: one consisted of the Aegean–Levantine Sea, the Ionian Sea and the Central Mediterranean Sea, and the Western Mediterranean Sea; the other consisted of samples from the Western Mediterranean Sea and the Adriatic Sea. Although there is geographic structure in the *A. media* network, there is no clear separation of locations. Many of the 53 *A. media* haplotypes ($n = 43$) were singletons.

All measures of within species diversity were lower in *A. africana* and *A. subulata* (Table 3) but both the number of samples and the number of sampling sites of these two species were also smaller than in *A. media* (Tables 1 and 3). The 244 *Alloteuthis media* samples were collected from a wide geographic area, from Greater North Sea to the Iberian Peninsula across to the Aegean Sea (Fig. 1).

Alloteuthis africana and *A. media* had significantly negative Tajima *D* values. Fu's *Fs* was also significantly negative for *A. africana* and *A. media* indicating an excess number of alleles (Table 3).

Pairwise F_{ST} values for *Alloteuthis media* showed a marked differentiation between samples from the Mediterranean and samples from the Atlantic (Fig. 5).

Morphometric traits (North Sea)

All individuals barcoded from the North Sea were *Alloteuthis media* (see above). Morphometric indices concerning tail length, club sucker size, max DML and arm length, as averaged across all North Sea barcoded specimens returned mean values indicative of *Alloteuthis media* (Table 4). We also considered morphometric traits at the level of individuals. In every barcoded North Sea individual, TL as % DML

Table 3 Summary statistics for genetic diversity indices based on sequence alignments of 445 bp

Species	No. of specimens	No. Haplotypes (nH)	Polymorphic sites		Nucleotide diversity (π)	Haplotype diversity (Hd)	Neutrality tests	
			Singleton informative sites	Parsimony informative sites			Fu's Fs	Tajima's D
A. africana	34	7	5	1	0.00114 ± 0.01037	0.455 ± 0.102	– 5.257***	– 1.810*
A. subulata	94	5	3	1	0.00068 ± 0.00014	0.295 ± 0.056	– 2.930	– 1.223
A. media	244	53	22	18	0.00333 ± 0.00017	0.807 ± 0.019	– 77.547***	– 2.243**

Nucleotide and haplotype diversity reported as mean ± standard deviation. Significance of neutrality tests indicated as:

*$P = < 0.05$

**$P = < 0.01$

***$P = < 0.001$

was less than 50% (< 50% is *A. media*; > 50% is *A. subulata*), i.e. this trait accurately assigned every individual as *A. media*. In all but one of the individuals, LCS as % HW was greater than 9. Previous authors suggested 6–8% indicated *A. subulata* and 9–14% indicated *A. media* (Table 2). In fact, we found values ranged as high as 22, but, if we take > 9% as indicative of *A. media*, then this trait accurately assigned the species *A. media* to all but one individual. For the trait AL as % DML (where *A. subulata* falls between 20 and 25%), individuals were mostly correctly assigned as *A. media*. However, for 5 out of 56 individuals, this index was < 25 and thus incorrectly indicated that these individuals could be *A. subulata* (Supplementary Information S4). The five exceptions were sampled in quarter 3 and comprised a mix of males and females.

For the non-barcoded samples from the North Sea ($n = 330$), morphometric indices of Anderson et al. (2008) concerning tail length, max DML and arm length, as

Fig. 5 Heat map of pairwise F_{ST} values for *A. media* among eight sampling locations based on SNPs extracted from *COI* sequence data. Colour ramp indicates marked differentiation between Mediterranean and Atlantic samples

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1) Baltic Sea							
(2) Greater North Sea	0.026						
(3) Celtic Seas	-0.046	-0.016					
(4) Bay of Biscay and the Iberian Coast	-0.052	-0.008	0.015				
(5) Western Mediterranean Sea	0.4	0.641	0.643	0.57			
(6) Ionian Sea and the Central Mediterreanean Sea	0.456	0.512	0.522	0.438	0.111		
(7) Adriatic Sea	0.457	0.543	0.527	0.491	-0.167	-0.073	
(8) Aegean-Levantine Sea	0.618	0.564	0.582	0.496	0.226	-0.236	-0.022

Table 4 Variation in morphometric traits in barcoded samples ($n = 58$; all identified by COI barcode as *A. media*)

Trait	Females		Males	
	Values	Designation	Values	Designation
TL as % DML	27.3 ± 5.3 ($n = 26$)	*A. media*	25.1 ± 6.9 ($n = 27$)	*A. media*
LCS as % HW	14.2 ± 3.2 ($n = 25$)	*A. media*	14.9 ± 2.7 ($n = 23$)	*A. media*
Max DML (cm)	10.0 ($n = 27$)	*A. media*	10.9 ($n = 29$)	*A. media*
AL as % DML	29.7 ± 4.5 ($n = 27$)	*A. media*[§]	30.3 ± 4.3 ($n = 27$)	*A. media*[§]
Tentacle L (cm)	6.47 ± 1.1 ($n = 27$)		6.58 ± 1.1 ($n = 25$)	

Two barcoded samples were too small and immature to determine their sex and were not measured. Values represent mean ± sd of all samples, with sample size in brackets. Designation of species by trait is provided for each trait and sex separately according to comparison of mean against species cut-off values in Table 2

TL tail length, *DML* dorsal mantle length, *LCS* largest club sucker size, *HW* head width, *AL* longest arm length. Measurements are given in cm

[§]No data for *A. media* are given in Anderson et al. (2008); however, the measurements shown here rule out either *A. subulata* or *A. africana*; we have assumed values > ~25% are *A. media*

Table 5 Variation in morphometric traits in samples not barcoded ($n = 330$; see individual traits for precise sample sizes as this varied by trait)

Trait	Females		Males	
	Values	Designation	Values	Designation
TL as % DML	23.5 ± 9.7 ($n = 156$)	*A. media*	27.2 ± 8.2 ($n = 150$)	*A. media*
LCS as % HW	not measured		not measured	
Max DML (cm)	10.9 ($n = 162$)	*A. media*	15.2 ($n = 168$)	*A. media*
AL as % DML	39.3 ± 7.6 ($n = 161$)	*A. media*[§]	38.2 ± 7.0 ($n = 168$)	*A. media*[§]
Tentacle L (cm)	7.36 ± 1.4 ($n = 141$)		6.95 ± 1.1 ($n = 136$)	

Values represent mean ± sd of all samples, with sample size in brackets. Designation of species by trait is provided for each trait and sex separately according to comparison of mean against species cut-off values in Table 2

TL tail length, *DML* dorsal mantle length, *LCS* largest club sucker size, *HW* head width, *AL* longest arm length. Measurements are given in cm

[§]No data for *A. media* are given in Anderson et al. (2008); however, the measurements shown here rule out either *A. subulata* or *A. africana*; we have assumed values > ~25% are *A. media*

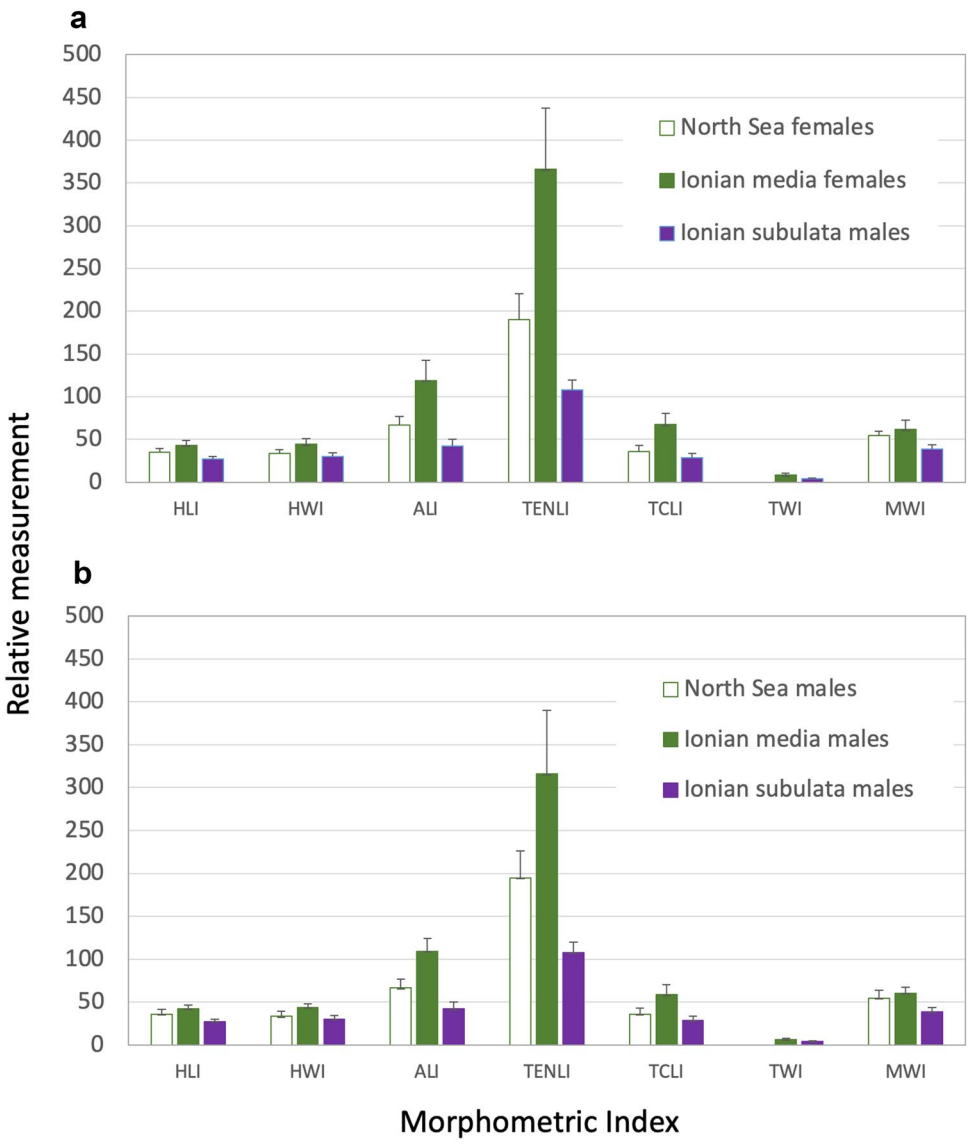

Fig. 6 Comparison of North Sea samples (barcoded samples from the present study, $n = 56$) with morphometric traits from the east Ionian Sea from Lefkaditou et al. (2012) for **a**. females and **b**. males. *A. subulata* were all males and are presented in both **a**. and **b**. The morphometric traits were: HLI = head length index; *HWI* head width index, *ALI* maximum arm length index, *TENLI* tentacle length index, *TCLI* tentacular club length index, *TWI* tentacular stalk width index, *MWI* mantle width index. Indices were calculated in each case relative to MLan which was ML-FL (mantle length minus fin length)

averaged across all non-barcoded North Sea specimens returned mean values indicative of *Alloteuthis media* (Table 5). We again considered morphometric traits at the level of individuals. On the trait TL as % DML (< 50% is *A. media*; > 50% is *A. subulata*), all were assigned as *A. media*. On the trait AL as % DML (*A. subulata* falls between 20 and 25%), the same 330 individuals were mostly assigned to *A. media*, but there were 6 out of 330 exceptions, which were assigned as *A. subulata* on this trait. These exceptions were sampled in quarter 3 and comprised a mix of males and females (Supplementary Information S5).

There was a statistically significant linear relationship between DML and VML (VML = 0.9458 DML—2.2591, $R^2 = 0.9883$). The Grimpe (1925) morphometric traits gave contradictory findings in the present study. VML: AL ratio assigned a sizeable minority of barcoded samples and a majority of non-barcoded individuals as *A. media*, whereas FL/VML assigned a majority of individuals from both datasets as *A. subulata* (Supplementary Information S5). Thus, these morphometric traits were not considered useful for making species assignations as they contradicted one another and did not agree strongly with species assignation from DNA barcodes.

Comparison of samples barcoded as *A. media* from the North Sea (present study) and the east Ionian Sea (Lefkaditou et al. 2012) on a range of traits showed that the morphometry of North Sea specimens conformed to the traits of neither *A. media* nor *A. subulata* from the east Ionian Sea, but were intermediary between the two; however, there was a general trend for the North Sea samples to be closer in size to *A. media* from the east Ionian Sea than to *A. subulata* for several metrics including HLI, HWI and MWI, in both males and females (Fig. 6). Furthermore, the east Ionian Sea samples had mean tentacle lengths which were longer in *A.*

media than in *A. subulata* (Table 2) and tentacle lengths for the North Sea were closer in size to *A. media* from the east Ionian Sea than to *A. subulata* (Tables 4 and 5). Tentacle length measurements of *A. subulata* were not available from the North Sea (as we did not find this species there), but there is overlap in this trait in *A. media* and *A. subulata* from the eastern Ionian Sea, particularly in males (Table 2). Finally, in the east Ionian Sea, the trait AL as % DML was much higher in *A. media* than in *A. subulata*, with values of 62.6% in males and 60.8% in females (Lefkaditou et al. 2012; Table 2). This trait is also elevated in *A. media* in the North Sea (assuming values > ~25% are *A. media*; Table 2) but the values are lower, from 30 to 40%, than in the Ionian Sea (Table 4–5).

Discussion

Species delimitation analyses including newly sequenced specimens and all available *Alloteuthis* sequences from GenBank clearly indicated that three species of *Alloteuthis* occur in the North East Atlantic and Mediterranean Sea (Fig. 3). Comparison with material sequenced by Anderson et al. (2008) allowed us to place names on clades, and this revealed that the North Sea, Baltic Sea and Irish Shelf waters were exclusively occupied by *A. media* for the periods and quarters sampled (Q1 and Q3 of 2021 in the North Sea, Q1 and Q4 of 2018 on the Irish Shelf by us, and Q4 of 2011 in the Baltic Sea, and Q4 of 2010, Q1 and Q3 of 2011, and Q1 of 2012 in the North Sea by Gebhardt and Knebelsberger 2015; Fig. 1, Supplementary Information S1). Anderson placed names on his clades with reference to the historical literature, assigning the name *A. media* to a clade whose members had central tentacular club suckers more than 9% head width, essentially following Naef (1923). The type material for *Loligo subulata* Lamarck, 1798 and *Sepia media* Linnaeus, 1758 is lost, but Naef (1923) considered *A. media* to be the species with larger tentacular clubs, following the illustration by Rondelet (Rondeletius 1554), which Linnaeus used to illustrate *Sepia media* in his Systema Naturae (Bello 2019). The nomenclature could be stabilised by the deposition of neotypes from type localities ('Mediterranean' for *A. subulata* and 'pelago' for *A. media*) but we advocate very strongly that material should be DNA barcoded prior to deposition such that deposited specimens concur with the body of evidence that has been building since Anderson et al. (2008) first attempted to resolve questions relating to *Alloteuthis* with molecular techniques.

When considering only those specimens that have been identified by DNA barcoding across all studies (Fig. 1), *A. media* has a considerably more extensive distribution than *A. subulata. Alloteuthis media* appears to be more genetically diverse than either *A. subulata* or *A. africana* (Fig. 4, Table 3), and this may not simply reflect the increased sampling. The large and negative value of Fu's *Fs*, the significantly negative Tajima's *D*, and the large number of haplotypes, many of which were singletons could all indicate a species that has undergone recent population expansion. The diversity indices reported for *A. media* herein are consistent with those in other recent studies (e.g. Olmos-Pérez et al. 2018; Alujević et al. 2022).

We found genetic structure within *A. media*, with differentiation between Atlantic and Mediterranean samples (Figs. 4, 5) as observed by previous studies (Anderson et al. 2008; Lefkaditou et al. 2012; Alujević et al. 2022), and in other cephalopods e.g. *Loligo forbesii* (Göpel et al. 2022) and *Sepia officinalis* (Pérez-Losada et al. 2007). Although some mixing apparently takes place, or has at least done so in the past, barriers to gene exchange between the Atlantic and Mediterranean populations could lead to morphological differentiation between samples from these two locations.

Historically, *A. media* has been considered 'the relatively warmer water species' (Anderson et al. 2008), which only occasionally inhabits cooler Irish Shelf and North Sea water (Zuev and Nesis 2003), whereas *A. subulata* has been assumed to range as far north as southern Norway (Anderson et al. 2008). For this reason, the North Sea and Irish Shelf were previously assumed to mainly comprise *A. subulata*, which is also the species specified in the online database of trawl surveys hosted by ICES (DATRAS; https://www.ices.dk/data/data-portals/Pages/DATRAS.aspx). It now appears that this is incorrect. *Alloteuthis media* occupies the relatively northern distribution including the North Sea and Irish shelf, and is also found in the southern Celtic Sea, western English Channel and in the Mediterranean (Fig. 1). These results explain the 'surprising' lack of *A. subulata* in the east Atlantic referred to by Anderson et al. (2008), at least in northern waters, but also confirms via barcoding that *A. subulata* is indeed present in more southern Atlantic waters. Our study barcoded *A. subulata* specimens from the southern Celtic Sea to the Iberian Coast, Olmos-Pérez et al. (2018) barcoded *A. subulata* from Vigo, while previous barcoding studies confirmed *A. subulata* in the Mediterranean (Anderson et al. 2008; Lefkaditou et al. 2012; Alujević et al. 2022). Naef (1912) reported 60 specimens of *A. subulata* from the English Channel (Plymouth), but also 1 from Liverpool (Irish Sea), 5 from the Netherlands (southern North Sea), and 1 from Bergen (northern North Sea); he specified delicate tentacles with small suckers, which are indeed indicative of *A. subulata* as currently understood. The present study barcoded samples from three seasons (Q1, Q3, Q4). Three unusually large samples in the unbarcoded dataset could potentially be *A. subulata* (Supplementary Information S5). These three largest specimens (Max DML 12.4, 13.7, 15.2 cm) had the three smallest values for AL as a % of DML (21.0–24.1), the three largest values for VML:

longest AL (3.9–4.5) and were the only specimens assigned *A. subulata* on these three metrics which we found most reliable. They additionally had the largest values for TL as % of DML (46.0–48.7) although they were slightly below the suggested *A. subulata* threshold of 50. Thus, absence of *A. subulata* from the Celtic Seas/North Sea should be verified by additional future DNA barcoding, and specifically for Q2. Given the regard in which his work is held, it seems unlikely that Naef was mistaken in his northern records of *A. subulata* but, if the current distribution of *A. subulata* is confirmed as more southern, the alternate hypothesis is that the distribution has shifted over time.

What are good morphometric traits for distinguishing *A. media* in the North Sea? This is difficult to answer as we did not find any *A. subulata* in the North Sea (or Celtic Seas, apart from one specimen far south in the Celtic Seas) based on the barcoding results. However, we found some traits which were consistent for barcoded *A. media*, and for a larger set of individuals ($n = 330$) that were not barcoded but which were measured so as to represent as much morphological variation as possible in the North Sea region. These consistent traits had previously been suggested by Naef (1923) and Anderson et al. (2008). Largest club sucker size (LCS as % HW), where values of 9–14% are consistent with *A. media*, and tentacular club width were the most promising distinguishing traits according to Anderson et al. (2008). In the present study, we also found that LCS as % HW was highly consistent with values greater than 9% (often exceeding the 14% upper limit supposed previously) corresponding to *A. media* (we did not measure club width). However, as tentacles are often missing, additional traits that can be used for reliable identification are required.

In the North Sea, at least two additional traits suggested by Anderson et al. (2008) were consistent with barcodes in the present study: TL/DML (*A. media* < 50%) and Max DML (*A. media* = 12 cm), although these traits may not work for comparisons between Atlantic and Mediterranean populations (see below). The trait AL/DML (also called AL as % DML), which in *A. subulata* = 20–25% (Table 2), also has potential for identification. Mean values for AL/DML for North Sea specimens were higher than *A. subulata* cut-off values, with values of 29.7–39.3% in females and 30.3–38.2% in males, showing that our barcoded *A. media* had relatively much longer arms than *A. subulata*. Interestingly, Grimpe (1925) also observed that "*A subulata* is always relatively more short-headed and short-armed than *A. media*", showing that this feature has been recommended several times in the past. Nevertheless, the AL/DML trait also gave rise to 11/386 individuals which did not agree with cut-off values for *A. media*. However, 5 of the 11 exceptions were ≥ 24% (Supplementary Information S4, S5), i.e. very close to the > 25% needed to be considered *A. media*. Thus, the cut-off for *A. media* on this trait may simply need to be decreased slightly, at least for North Sea specimens. In fact, Anderson et al. (2008) did not sample morphology in the North Sea and also did not provide any values for *A. media* on this trait, only giving cut-off values for *A. subulata* and *A. africana*. And since the values obtained in the present study did not correspond to either *A. subulata* or *A. africana*, it is by a process of elimination that we assume that individuals of > ~ 25% are *A. media*. It is hoped that providing details for each one of the *A. media* individuals which did not match their barcode in the present study, along with a large sample of other measurements from the North Sea (Supplementary Information S4, S5), will aid a more precise definition using this trait in future in the North Sea and elsewhere.

Given the similarity of the two species, it may also be possible for hybrids to form in regions of geographic overlap. It is theoretically possible that the five samples barcoded as *A. media* but which had phenotypic characters of *A. subulata* (specifically AL/DML < 25%) are hybrids. DNA barcoding with mitochondrial COI would likely not detect hybrids because of the maternal inheritance of the mitochondrial genome. Anderson et al. (2008) found that the positions of three *Alloteuthis* specimens differed between mitochondrial (COI and 16S) and nuclear (rhodopsin) trees. While two of those could potentially be explained by hybridization, only incomplete lineage sorting satisfactorily explained all three. Nonetheless, the possibility of hybridization remains an interesting avenue for further explanation.

Because barcoded individuals of *A. subulata* were found in the southern Celtic Sea and the western English Channel (albeit few individuals) in the present study, it is not unreasonable to assume that *A. subulata* has been recorded in the North Sea in the past (for example by Naef, and see comments above). This is difficult to assess from older literature because some traits suggested as reliable identifiers for North Sea specimens (Grimpe 1925), specifically FL/VML and VML: AL ratio (for the longest arm), were not found to be useful traits in the present study. FL/VML and VML: AL ratio gave mutually contradictory species assignments in the North Sea, although, of the two, VML: AL was the more congruent with barcodes. Inconsistency in VML: AL is a little surprising because this trait is likely to be highly correlated with the AL/DML metric that was reasonably useful in the present study (see above). However, the misassignment rate of VML: AL was much higher in the barcoded samples which used a regression to predict VML from DML. It maybe that this trait is more accurate when VML is measured directly. Relative fin length, on the other hand, was dismissed as not useful by Naef (1923) and, like many potential traits, this varies with maturity, making it difficult to apply across the entire population (Laptikhovsky et al. 2002). Tail length (TL) also increases at maturity and is made more difficult by the fact that maturity occurs over a range of sizes (Laptikhovsky et al. 2005). Therefore, in

addition to the fact that TL presents issues for Mediterranean versus Atlantic comparisons (as shown in Fig. 4 of Anderson et al. 2008), the maturity of individuals ought to be considered for this trait. Unsurprisingly, therefore, neither relative FL nor TL plotted against DML clearly separated *A. media* and *A. subulata* across their range (Anderson et al. 2008) and only the 'most extreme' examples can give reliable identification on these traits according to Naef (1923). Nevertheless, we found that one of these, TL as % of DML was consistent with *A. media* cut-off values (*A. media* is < 50% on this trait) in North Sea specimens. This may be because our morphological measurements were restricted to the North Sea, where *A. media* is more easily distinguished as regional variations (phenotypic plasticity) do not confuse matters. More importantly, however, a variety of maturities were sampled for barcoded (stage 0, 2a, 2b, 3a, 3b with $n = 2$, 11, 11, 24, 8, specimens respectively, maturity was not determined for two specimens) and non-barcoded (stage 0, 1, 2a, 2b, 3a had $n = 9$, 116, 50, 82, 81) specimens in the present study; hence, the widest possible extent of morphological variation associated with maturity should have been represented by the *A. media* morphology results presented here. Another related metric, Max DML (species cut-off *A. media* = 12 cm), was also highly consistent with barcodes in the North Sea, including specimens across a range of maturities.

Finally, consideration of *A. media* variability across a wider geographic range was possible by examining a series of morphometric indices (HLI, HWI, ALI, TENLI, TCLI, TWI, MWI) and comparing these with individuals from the east Ionian Sea (Lefkaditou et al. 2012). When we applied these traits, the North Sea individuals (all barcoded as *A. media*) matched neither species; however, they were closer to *A. media* on several traits than to *A. subulata*. For instance, while the North Sea samples were generally mid-way between Ionian Sea *A. media* (largest) and *A. subulata* (smallest) for each index-based trait of Lefkaditou et al. (2012) (Fig. 6), the North Sea samples were much closer in size to *A. media* than *A. subulata* for HLI, HWI and MWI in both males and females (Fig. 6, Table 2), so these indices may be useful distinguishing characters across the range, especially with region-specific cut-off values. An example of this was AL as % DML, which was much higher in the east Ionian Sea with values of 62.6% in males and 60.8% in females (Table 2), and also high in the North Sea, though to a lesser degree (equivalent values of 30–40%). This trait, thus, may be useful in other parts of the range. Alujević et al. (2022) found that tail length index (equivalent to TL/DML in the present study) was a useful trait for distinguishing species in the eastern Adriatic Sea but the species' cut-offs required a change from the usual > 50% in *A. subulata* and < 50% = *A. media* (Table 2) to > 40% in *A. subulata* and < 46% in *A. media* (Alujević et al. 2022). Regarding intraspecific variability more generally, Naef (1923) remarked that *A. media* possesses the 'more homogeneous appearance' of the two species. Indeed, Naef (1923) discovered two 'varieties' of *A. subulata*; an Atlantic and a Mediterranean form, which supports our suggestion to define separate species cut-off values for both regions. As the full range of these species is established, *Alloteuthis* species may provide an interesting case study of character displacement. Certainly, there is evidence that traits of *A. media* are more marked in the Ionian Sea, where the species occurs sympatrically with *A. subulata* than in the North Sea where *A. media* is apparently the only species present (Fig. 6). Such a phenomenon would explain the historical difficulty in establishing criteria for identification. In general, there are very few specimens for which both barcode and morphological measurements exist, particularly in *A. subulata*. Further sampling, especially of *A. subulata*, is, therefore, a prerequisite for advancing identification techniques to take regional variation into account.

Overall, the morphometric results illustrate (1) that *A. media* phenotypes do vary substantially across the range, so that even for useful identification features, cut-offs for each species will vary between regions, and (2) that our findings were broadly consistent with the results of both Lefkaditou et al. (2012) and Anderson et al. (2008), with differences only in the absolute trait cut-off values because of phenotypic plasticity. The morphometric traits which were most consistent with barcoding in the North Sea in the present study were: TL as % DML (*A. media* = < 50%), LCS as % HW (*A. media* = 9–22%) and Max DML (*A. media* = 12 cm). A third finding was that when the full distribution of each species is considered, the ranges of the values of some morphometric measurements and indices overlap, even where there is no local overlap, and thus regional adjustments to cut-off values may be helpful; specifically, the cut-off for *A. media* using AL as % DML (*A. media* = > ~ 25%) needs to be adapted in the North Sea, as some individuals here were slightly below the cut-off and thus misassigned. While consideration of intraspecific differences in sex, maturity (Naef 1923) and length (Grimpe 1925) is likely to influence morphometric trait selection, traits of North Sea specimens were consistent across the range of maturity sampled in the present study. Overall, we suggest that region-specific morphological identification guides are necessary to take phenotypic plasticity into account. These may be more user-friendly than analyses based on composite or multivariate morphometric data (Olmos-Pérez et al. 2018; Alujević et al. 2022) and should be explored further.

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Author contributions Conceptualization: ALA, AMP, DO. Sample collection: ES, DO, DH, EA, LB, MP, IS, JV. Morphometric data collection: DH, DO. Morphometric data analysis: AMP. DNA sequencing: ES. Molecular analyses: ES, ALA. Writing—original draft preparation: AMP, ES, DO, ALA. Writing—reviewing and editing: ES, AMP, DO, DH, EA, LB, MP, IS, JV, ALA.

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Data availability All morphometric data generated or analysed during this study are included in this published article and its supplementary information files. Genetic sequence data are available from GenBank, https://www.ncbi.nlm.nih.gov/genbank/

Declarations

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval Samples were collected as bycatch either during government statutory monitoring surveys or during ICES coordinated international fishing trawl surveys with all applicable permits in place.

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