Barcoding and morphometry to identify and assess genetic population differentiation and size variability in loliginid squid paralarvae from NE Atlantic (Spain)

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Compliance with Ethical Standards

This study was performed in accordance with existing Spanish guidelines and regulations on animal research (Ley 32/2007, November 7th), and was consequently exempt from an ethics review process. All the authors have revised the manuscript critically for important intellectual content and have approved the final version to be published. The authors declare that the research was conducted in the absence of any relationships that could be construed as a potential conflict of interest. We also declare that the manuscript has not been published previously or split up into several parts, neither the data have been fabricated or manipulated.

Acknowledgments

We are indebted to the captain, crew and technicians of R/V 'Mytilus' (IIM, CSIC Vigo) for their assistance in collecting the zooplankton samples. We are grateful to Mariana Cueto for assisting us with laboratory analyses and Lara García Alves for helping us to sort the paralarvae. This study was supported by the project LARECO (CTM - 2011 - 25929) and CALECO (CTM2015 - 69519 - R) funded by the Spanish Ministry of Economy and Competitiveness. Lorena Olmos-Pérez was supported with a FPI grant funded by the Spanish Ministry of Economy and Competitiveness. We would like to thank the reviewers for their suggestions and comments that improved the quality of the manuscript.

SUMMARY

Accurate species classification is essential to understand complete life-cycles of cephalopods. Identifying freshly caught or fixed loliginid paralarvae to species level with traditional taxonomic guides is challenging. Therefore, the aim of this work was to identify genetically loliginid paralarvae captured in NW Spain (a region where at least three loliginid species are known to coexist) during 2012, 2013 and 2014, and to seek a means to distinguish the species from each other based on their morphometry. Firstly, the barcoding region (COI gene) was amplified to identify each paralarva and to obtain population molecular diversity indices and genetic structure for the different species. Afterwards, discriminant analysis (DA) was used to evaluate the performance of the selected morphometric measurements to distinguish among the species previously identified. Molecular analyses revealed three loliginid species (*Alloteuthis media*, *A. subulata* and *Loligo vulgaris*), with different patterns of molecular diversity. DAs based on body morphometrics correctly categorised 75% of paralarvae to genus (*Loligo* and *Alloteuthis*) and 72% of *Alloteuthis* individuals to species level (*A. media* and *A. subulata*). When statolith measurements were included in the morphometric analysis, successful classification increased to 94% and 82%, respectively. The most useful variables for the discrimination of genus were hatching ring length and head width, while tentacle length helped to differentiate *A. media* from *A. subulata*. These discriminant functions should be tested with more paralarvae from different origins and seasons to account for body shape plasticity, but suggest a promising result to facilitate loliginid paralarvae identification for future research.

KEY WORDS

Squid; early stages; morphometries; population genetics

INTRODUCTION

The Loliginidae is a cephalopod family commercially exploited in Europe. In south Europe, loliginid landings are mainly comprised by *Loligo vulgaris*, and two *Alloteuthis* species (*Alloteuthis media* and *A. subulata*), frequently confused with *Loligo vulgaris* juveniles, because their small size and morphological similarities (UTPB 2005; Hastie et al. 2009, 2013). Due to the importance of loliginid squid for the local economy, their biology has been extensively studied in Atlantic waters of the Iberian Peninsula (Rodhouse et al. 1988; Guerra and Rocha 1994; Moreno 1995; Rocha and Guerra 1999; Moreno et al. 2002; Hastie et al. 2009; González et al. 2010).

Abiotic parameters, especially sea temperature, influence squid growth (Jackson et al. 1997; Hatfield et al. 2001) and thus contribute to intraspecific variability in soft and hard structures (Villanueva 2000; Villanueva et al. 2007). These species present sexual dimorphism, seasonal size differences among cohorts and intraspecific morphometric variability across a large geographic range (Pierce et al. 1994a, 1994b; Villanueva et al. 2003; Moreno et al. 2007, 2012; Van Der Vyver et al. 2016). In *Alloteuthis* species, body shape plasticity, the low utility of morphometric characters traditionally employed in squid taxonomy and indeed, some confusion with the characteristics of each different species (Anderson et al. 2008; Jereb et al. 2015), have hindered their accurate identification. For this reason, some studies have considered *A. media* and *A. subulata* to be the same species (Laptikhovsky et al. 2002) and misidentifications, have probably resulted in erroneous reporting of their occurrence and abundance (Jereb et al. 2015).

Traditional morphological identification is even more challenging with cephalopod early life stages, also called paralarvae (*sensu* Young and Harman, 1988), because taxonomic characters like the chromatophore pattern which are useful to differentiate adult wild specimens or laboratory reared paralarvae (Vidal et al. 2010) are usually lost or damaged during trawling and preservation procedures (Sweeney et al. 1992). In fact, preservation of wild paralarvae, usually results in chromatophore bleaching, which prevents successful identification to species level (Vecchione and Lipiński 1995; Martins and Perez. 2006). Number, density and size of suckers have been pointed as promising elements to distinguish ommastrephid squid paralarvae species hatched in laboratory (Fernández-Álvarez et al. 2016), however, they are extremely fragile and easily lost during sampling and conservation, therefore present a low utility to identify wild paralarvae. Therefore, the lack of accurate descriptions to identify paralarvae of certain cephalopod families, including loliginids, has been highlighted as one of the main obstacles to untangling their life histories. Accordingly, in many studies the cephalopod paralarvae have been largely classified to family level (Rocha et al. 1999; González et al. 2005; Moreno et al. 2009; Otero et al. 2016; Roura et al. 2016), thus preventing accurate estimation of relationships between paralarval abundance and oceanographic variability.

Molecular tools have been able to differentiate various cryptic and morphologically similar co-occurring species (Hebert et al. 2003b; Bucklin et al. 2011), where classical taxonomical approaches have failed. In cephalopods, molecular analyses have allowed to distinguish between the two sympatric and frequently confused *Alloteuthis* species (Anderson 2000; Lefkaditou et al. 2012), identify new sepiolid species

(Heij and Goud 2010) and clarify their phylogenetic relationships (Strugnell and Nishiguchi, 2007; Anderson *et al.*, 2008; Groenenberg *et al.*, 2009). Moreover, the analysis of cytochrome oxidase I (COI) gene, has allowed to identify cephalopod paralarvae of several ommastrephid squid species (Gilly *et al.* 2006; Wakabayashi *et al.* 2006; Ramos-Castillejos *et al.* 2010; Staaf *et al.* 2013) and sepiolids (Olmos-Pérez *et al.* 2017).

The COI gene has been widely analysed in marine animals: it has been employed to determine intraspecific molecular variation and genetic differentiation in several marine species (Bucklin 2000; Bucklin *et al.* 2011; Goodall-Copestake *et al.* 2012). Additionally, analysis of COI gene can provide valuable information about population genetic structure, connectivity and exchange of individuals, genetic drift, inbreeding or bottleneck events (Pearse and Crandall 2004; Pineda *et al.* 2007; Hauser and Carvalho 2008; Cowen and Sponaugle 2009) in relationship with oceanic currents. This knowledge is especially essential in cephalopods with planktonic hatchling stages, because ocean currents can facilitate their dispersion. In fact, recent research has shown an inverse relationship between cephalopod size at hatching and developmental planktonic period, with distributional range of adults, and a more restricted distributional range of cephalopods with benthic hatchlings (Villanueva *et al.* 2016).

Morphometric approaches (based on both soft and hard structures) have been successfully employed in several cephalopod species to detect intraspecific seasonal, geographic, sexual and ontogenetic variation (Pierce *et al.* 1994a, b; Borges 1995; Van Der Vyver *et al.* 2016; Braga *et al.* 2017). Morphometric analysis in combination with molecular tools can be especially advantageous to detect differences between sibling species, thus permitting routine discrimination and revealing other morphometric characters that could be more useful for species identification than those previously employed (Anderson *et al.* 2008; Lefkaditou *et al.* 2012; Amor *et al.* 2014).

At least three loliginid species inhabit the coasts of NW Spain, but due to the difficulty of identifying loliginid paralarvae to species level, those captured in previous studies in Galician waters had been classified either as Loliginidae (Otero *et al.* 2016; Roura *et al.* 2016) or as *Loligo vulgaris* (Rocha *et al.* 1999; González *et al.* 2005, 2010; Moreno *et al.* 2009) due to its higher abundance and landings as compared to *Alloteuthis media* and *A. subulata*. Furthermore, recent genetic analyses have suggested that *Alloteuthis* species paralarvae are more abundant in Atlantic waters rather than *Loligo vulgaris* as previously expected (Roura 2013). Thus, the aims of this work were: 1) to identify genetically all the loliginid paralarvae captured in the Ría de Vigo from 2012 to 2014 and explore the molecular diversity indices and population structure during this period. 2) to identify morphological measurements that differ among the three loliginid paralarvae species, to evaluate their morphological differences across seasonal changes, and to provide discriminant equations as a tool to distinguish them in the field or laboratory, without the need to perform molecular analysis.

MATERIALS AND METHODS

Sample collection

Zooplankton samples were collected in the Ría de Vigo (NW Spain) onboard RV “Mytilus” in 2012, 2013 and 2014. Ten nocturnal surveys were conducted each year, four in summer (July) and six in early autumn (September and October), corresponding to previously identified periods of maximum paralarval abundance (González et al. 2005; Otero et al. 2016; Roura et al. 2016). Additionally, four 24-hour surveys were conducted, one in summer and other in early autumn in both 2012 and 2013. For each survey, sampling was undertaken along four transects (**Fig. 1a**). Sampling along transects 3, 4 and 5 was cancelled during two nocturnal surveys and the 24-hour survey in autumn 2013, as well as during one nocturnal survey in autumn 2014, due to unfavourable weather conditions. On these occasions, sampling was carried out along additional transects at the North (NM) and South (SM) mouths of the Ría de Vigo, where sea conditions allowed the sampling to take place (**Fig. 1a**). Moreover, two additional surveys were undertaken at the NM and SM sites: one in October 2013 and the second in October 2014. An additional SM survey was undertaken in July 2014, bringing the total number of surveys undertaken over three years to 34 (supplementary material 1).

For each transect, a Multinet® Hydrobios Mammoth with 250 µm mesh size and 1 m² mouth, equipped with two electronic flow meters, was lowered to near the sea floor. The Multinet® was gradually lifted up to the surface at a speed of 2.5 knots. The net thus passed through up to 7 depth layers (105 to 85m, from 85 to 55m, from 55 to 35m, from 35 to 20m, from 20 to 10m, from 10 to 5m, from 5 to 0m; see **Fig. 1b**). Within each layer, the Multinet® filtered 200 m³ and zooplankton captured was stored as discrete samples (one per layer). The zooplankton was fixed on board in 96% ethanol and frozen at -20°C until sorting. Each loliginid paralarva ($N=200$) was separated in the laboratory and preserved individually in 70% ethanol at -20°C.

Genetic identification and molecular analyses

Loliginid paralarvae were dissected under sterilized conditions and DNA from each mantle ($n = 200$) was extracted with a QIAamp DNA Micro Kit (Isolation of genomic DNA from Tissues protocol QIAGEN) following manufacturer’s instructions, with the exception of two steps: Digestion at 56°C was done overnight and the final elution was done in two steps using 15 µl buffer AE in each elution. The universal primers HCO2198 and LCO1490 (Folmer et al. 1994) were employed to amplify the barcoding region. PCR was set up on a total volume of 25µl with 1µL of forward and reverse primers (10µM), 12.5µl Thermo Scientific™ Phusion™ High-Fidelity PCR Master Mix (Thermo Fisher Scientific Inc.) with HF Buffer, 1µl of DNA (40ng/µl) and 9.5µl H₂O. PCR conditions were: initial denaturation at 94°C for 1 min, followed by 39 cycles of denaturation at 94°C for 15 s, annealing at 48°C for 30 s and extension at 72°C for 45 s, with a final elongation at 72 °C for 7 min. Then, 2µl of each PCR product were checked on 1.5% agarose gels. PCR samples were cleaned using USB® ExoSAP-IT® PCR Product Cleanup (Affymetrix, Inc. USA) following the manufacturer’s protocol, and sequenced by Sanger sequencing (Sanger et al. 1977, Stab Vida, Portugal). Out of the total of 200, 14 samples were not successfully sequenced.

The sequences obtained ($n = 186$) were aligned in MEGA 6.0 (Tamura et al. 2013) and compared using the BLAST algorithm to GenBank database (Altschul 2014), using the following GenBank as reference

sequences: *Alloteuthis media*, EU668085; *Alloteuthis subulata* EU668098; *Loligo vulgaris* KF369142. Sequences that presented blurry chromatograms were excluded from subsequent analysis. The remaining sequences ($n=162$) were translated using the invertebrate mitochondrial coding gene with EXPASY translate tool (<http://www.expasy.org/>) to detect pseudogenes with stop codons (Song et al. 2008). The final database included 162 sequences and 505 bp. All these sequences were collapsed into haplotypes using FaBOX (<http://users-birc.au.dk/biopv/php/fabox/>) and haplotypes were aligned using MUSCLE (Edgar 2004) default options in MEGA 6.0 (Tamura et al. 2013).

Evolutionary analyses were conducted in MEGA6.0 (Tamura et al. 2013). The analysis involved 34 haplotype nucleotide sequences and 505 nucleotide positions obtained from our samples, and 3 species as outgroups: *Loligo forbesii* (KM517925.1, Gebhardt and Knebelsberger 2015), *Loligo reynaudi* (AF075406.1, Anderson 2000) and *Doryteuthis pealeii* (KF854065.1, Sales et al. 2013).

We employed MEGA 7.0 with default settings of the Model Selection based on the Maximum Likelihood (ML) statistical method to estimate best-fitting substitution DNA models. Afterwards, the best model was selected based on the lowest Akaike Information Criterion (AIC) scores.

The evolutionary history was inferred using a Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (16 categories (+G, parameter = 2.4580)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 61.49% sites). All positions containing gaps and missing data were eliminated. Phylogenetic confidence in the tree was assessed with 1000 bootstrap replicates (Felsenstein 1985).

The following molecular diversity parameters were calculated with DNAsp (Librado and Rozas 2009) separately for each species for each year and for the three years: variable sites (S), nucleotide diversity (π), haplotype diversity (Hd) and total number of mutations (Eta). The reference values of nucleotide and haplotype diversity were those of Goodall-Copestake et al. (2012). To test neutrality of mutations, Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997), were calculated in Arlequin 3.0 (Excoffier et al. 2005) for each of the three years to assess demographic changes and detect patterns of natural selection. F_{ST} pairwise genetic distances and their significance were also calculated in Arlequin 3.0 (Excoffier et al. 2005) as a measure of genetic differentiation among paralarvae captured in different transects and years (Michalakis and Excoffier 1996). Finally, a median joining haplotype network was built for each species (Bandelt et al. 1999) using Nexus software (www.fluxus-engineering.com).

Morphometric measurements

Morphometric measurements were taken for every loliginid paralarva to the nearest 0.05 mm using a LEICA M205C stereomicroscope and LEICA Application Suite (LAS) image analysis system. Mantle length (DML),

total length (TL), head width (HW) and length of right Arms 1 and 2 (A1, A2) were measured dorsally. Tentacles were measured along the ventral and lateral surfaces (TeL1, TeL2) (**Fig. 2**) and the average of both measurements was used to minimise error (**Fig. 2**).

Statoliths were removed with fine dissecting needles under a LEICA M205C and mounted on a microscope slide with CrystalbondTM, with the anterior concave side uppermost. Measurements (to 0.05 mm) were taken when possible on all statoliths according to Clarke (1978): statolith length (SL) from the end of the dorsal dome to the tip of the rostrum and statolith width (SW) across the widest part of the dorsal dome (**Fig. 3**). Statoliths were photographed under a LEICA DM4M microscope at $\times 400$ magnification with an LAS image analysis system. Hatching ring Length (HL) was measured to the nearest 0.05 mm, and the total number of increments (d) was counted manually from the hatching ring to the tip of the rostrum (Moreno et al. 2012). When rings were not clear, they were estimated by extrapolation from the adjacent area. Statoliths from the oldest paralarvae were ground when necessary to reveal the rings (González et al. 2010). Hatching date was estimated by back-calculation assuming daily increment formation (Villanueva 2000) and hatching season was defined as either summer or early autumn, if the paralarvae had hatched before or after the 214th Julian day, respectively.

Final sample sizes for morphometric measurements were lower than for genetic identification due to damage to arms, tentacles and/or mantle in some specimens during sampling, and damage to some statoliths during manipulation.

Statistical analysis

Repeatability of the two measures per individual of tentacle length, statolith length, statolith width, hatching increment length and number of rings was tested following Lessells and Boag (1987), and the average value was considered for statistical analyses. All measures were log-transformed to achieve a normal distribution.

The three species of paralarvae did not differ in their average age measured as number of rings ($F_{2,122}=5.518$, $p=0.063$), thus allowing to analyse morphometric differences in squids with similar age range. Morphometric variables (DML, TL, HL, HW, TeL, A1, A2, SL, SW) were log transformed to achieve normality. All transformed morphometric variables showed linear and positive relationship with age (days, measured as number of increments, d) and with dorsal mantle length (DML) except hatching increment length (HL, $R^2 < 0.08$ in all cases) when considering the species separately and when data for all species were pooled together. The homogeneity of regression slopes for each morphometric variable, across species, was confirmed with an ANCOVA, including age as covariate and species as factor, or LnDML as covariate and species as factor.

In order to evaluate the differences of the three species at the same age, and to explore the existence of morphometric variables appropriate to distinguish the species, all the paralarvae collected over three years were pooled. Morphometric variables (DML, TL, HL, HW, TeL, A1, A2, SL, SW, HL) were analysed separately by means of generalised linear models (GLM) with species as a fixed factor and age (d) as a

covariate to control for age (HL was not evaluated with the covariate age due to the absence of linear and positive relationship). *Post hoc* comparisons among species were carried out using the least significant difference (LSD) test. Species averages measurements were expressed as estimated marginal means (EMM) $\pm$ standard error of the mean (sem) controlled by the covariate age.

Species morphometry was further evaluated using discriminant analysis (DA) with two different approaches: 1) to provide a discriminant function (DF) using all morphometric measures indicated above (DF A) in order to maximize the discrimination power, and 2) without including measurements obtained from the statoliths (DF B) (i.e. using only external characters), in order to see whether discrimination can be achieved without removing statoliths. In both approaches, linear regression was applied to every variable, using DML as the explanatory variable, to filter out the size effect (except to HL due to its independence from size), and the resulting residuals were introduced in the DA. In both cases, DA was used to test for differences firstly between the two genera (*Alloteuthis* and *Loligo*, DF-A1 and DF-B1) and afterwards to discriminate between *Alloteuthis* species (*A. media* and *A. subulata*, DF-A2 and DF-B2). Variables were eliminated with a stepwise backwards deletion approach by evaluating the significance of the functions with the Wilks's Lambda statistic (Λ) and evaluating the influence of each variable with the structure matrix. Eigenvalues and canonical correlation were calculated to assess the discriminant power of the functions. The structure matrix was employed to evaluate the correlation of each variable with the discriminant function. The standardised coefficients of all the different discriminant functions, with their corresponding group centroids (Table 5) are reported. A summary table (percentage of cases correctly and incorrectly assigned to each of the groups based on the discriminant analysis) is presented in supplementary material 3. GLM and DA statistical analyses were performed with IBM SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

Additionally, to explore seasonal and interannual size variability in *A. media* and *A. subulata* (González et al. 2010; Moreno et al. 2012), Gaussian generalised additive models (GAMs: Hastie and Tibshirani 1990) with identity link function were fitted to the following morphological measures: TL, HW, Lte and SL. Variability in TL, HW, Lte and SL was analysed separately. The effect of the explanatory variable DML was included in each model as a smoother to test for size variability, afterwards, the following explanatory variables were added one by one to avoid multicollinearity: hatching day, capture day (as smoothers), hatching season, capture season and year (as nominal). Differences between the basic model (with the response variable and DML as a smoother), and models with an extra explanatory response (hatching/capture day, hatching/capture season) were evaluated with the Akaike Information Criteria (AIC) and the variance explained by each model was compared. Subsequently, a similar approach was followed with the response variables DML, TL, HW, Lte and SL, but including the effect of the explanatory variable age (instead of DML) as smoother to test for size-at-age variability.

Variability in hatching ring length (HL) was analysed by fitting a GAM with the explanatory variables hatching day, capture day (both as smoothers), hatching season, capture season or year (as nominal), one by one separately to avoid multicollinearity. To avoid overfitting in smoothers, the number of knots (k) was

limited to 4. GAMs were not used to detect *Loligo vulgaris* variability among seasons or years because of the unbalanced representation of paralarvae across seasons and consequent small sample sizes in at least one group (summer $n = 6$, early autumn $n = 23$; $n = 16$ in 2012, $n = 8$ in 2013, and $n = 5$ in 2014, see Table 1). GAMs were performed in Brodgar 2.7.4 (Highland Statistics Ltd).

RESULTS

Genetic identification and molecular analyses

A total of 200 loliginid paralarvae was captured. Three different loliginid species were identified using molecular analysis: *A. media* ($n=115$), *A. subulata* ($n=42$) and *L. vulgaris* ($n=29$), corresponding to 57.5%, 21% and 14.5% of the total loliginid abundance respectively (Table 1). All of them were identified with GenBank similarities above 98%. Molecular identification was not possible in 14 paralarvae and, among the identified paralarvae, 24 sequences did not have sufficient quality to undertake population analysis. The remaining 101 *A. media*, 37 *A. subulata* and 24 *L. vulgaris* sequences were collapsed into haplotypes. Their most likely evolutionary relationships (log likelihood = -24117.48) are presented in **Fig. 4**.

Totals of 21, 3 and 10 haplotypes were found in *A. media*, *A. subulata* and *L. vulgaris*, respectively (Table 2). *Alloteuthis media* presented a star-like haplotype network, with two main haplotypes with high frequency (H1, 48.5% of individuals, and H5, 29.7%) and 19 haplotypes with low frequency (1-3% of individuals per haplotype) that presented one single nucleotide modification from these high frequent haplotypes (**Fig. 5**). *Alloteuthis subulata* had one main haplotype that was present in most individuals (H1, 83.78 %), and two less frequent haplotypes originated from it (**Fig. 5**). *Loligo vulgaris*, presented a complex haplotype network: a main haplotype (H1, 41% of individuals), a second-high frequency haplotype (H6 17% of individuals), 7 low frequent haplotypes originated from H6 and one low frequent haplotype originated from H1 (**Fig. 5**). However, all haplotypes were closely related, with one single mutation between haplotypes except in the case of H7 that presented 7 mutations. All the haplotypes were deposited in GenBank (accession number and sequences indicating variable sites can be found in supplementary material 2).

The three species differed in their molecular diversity values (Table 2). Tajima's D value and Fu's F_s statistic were negative in all species but only significant for *A. media* (except in 2013). No statistical differences were found when comparing F_{ST} values among years or transects in each species.

Morphometric analyses

A. media paralarvae DML ranged from 1.2 to 7.4 mm (average $\pm$ standard error: 3.12 ± 0.13 $n = 105$), and were aged from 1 to 54 days old ($n = 82$). *A. subulata* paralarvae ranged from 1.7 to 6.7 mm (average $\pm$ standard error: 2.81 ± 0.16 $n = 41$), comprising paralarvae between 2 and 50 days old ($n = 23$). *L. vulgaris* paralarvae ranged from 1.8 to 5.3 mm (average $\pm$ standard error: 2.88 ± 0.15 $n = 27$), comprising paralarvae between 3 and 22 days old ($n = 12$).

GLM results of models fitted with species as factor and age as covariate, showed that dorsal mantle length, total length, head width and tentacle length for the same age paralarvae all differed significantly

among species (Table 3). Specifically, DML, total length and head width were all greater in *L. vulgaris* than in *A. media* and *A. subulata* (LSD *post hoc* tests, $p=0.003$ for both DML comparisons, $p<0.001$ in all other cases; see **Fig. 6**). In addition, TL in *A. media* was significantly larger than *A. subulata* ($p=0.027$; **Fig. 6**), tentacles of *A. subulata* were shorter than in *L. vulgaris* and *A. media* (LSD *post hoc* test; $p<0.001$ in both cases, **Fig. 6**). Arm lengths A1 and A2 did not differ significantly among species. Statolith measurements also differed significantly among species (Table 3): hatching increment length, statolith length and statolith width measures were longer in *L. vulgaris* than *A. media* and *A. subulata* (LSD *post hoc* tests, $p<0.005$ in all cases, **Fig. 7**).

All discriminant functions were statistically significant. Wilks' Lambda values (Λ), eigenvalues and canonical correlation are presented in Table 4. The discrimination between the genera *Loligo* and *Alloteuthis*, was 93.8% successful (61 out of 65) using the statolith and external morphometric variables and 75.5 % successful (71 out of 94) using only external morphometric variables. The differentiation between *A. media* and *A. subulata* was 82.3% successful (51 out of 59) with the discriminant function that included statolith measurements, and 72% (103 of $n=143$) without them (supplementary material 3). Only the paralarvae that presented all the morphometric variables employed in each discriminant function were retained in each analysis. The structure matrix revealed that the measurements which contributed the most to discriminate between genera, were HL and HW when employing the statoliths variables and HW when the statoliths were not considered. The structure matrix showed that Lte was the variable that contributed the most to discriminate between *A. media* and *A. subulata* regardless of whether statoliths variables were included (D.F.-A2, and D.F.-B2). The structure matrix, the Standardized Canonical Discriminant Function Coefficients and the corresponding group centroids are shown in Table 5.

GAM results are presented in full in the supplementary materials (4 and 5). In general, addition of a new explanatory variable (capture, hatching, year) to initial GAMs with DML or age (included as smoother to control for size or age variability respectively) and the response variable (DML, TL, HW, Lte, SL, HL), increased the variance explained by the model by less than 5% for all the response variables.

When analysing seasonal differences and DML was included as an explanatory variable to control for size, GAM results showed different seasonal variability for the response variables (TL, Lte, HW and SL) in both *A. media* and *A. subulata* (supplementary material 4 and 5): In *A. media*, paralarvae with the same dorsal mantle length had longer total length and tentacle length in summer than in early autumn. HW and SL (at the same DML) were greater in early autumn than in summer. In *A. subulata*, TL decreased with increasing capture day and hatching day. HW and SL were larger in *A. subulata* paralarvae captured in autumn.

When age instead of DML was included to control for ontogenetic changes in size in the different seasons, fewer significant differences or trends were revealed by the models: In *A. media* both HW and SL were larger at the same age in early autumn than in summer. In *A. subulata* HW was larger in early autumn than in summer.

In relation to hatching increment length, no variable was included to control for ontogenic change (DML or age). Thus, the effect on HL size of every explanatory variable was included separately one by one to detect seasonal variability: In *A. media*, GAMs revealed significantly larger HL (0.045%) in autumn than in summer, and did not show seasonal variability in *A. subulata* (supplementary material 4 and 5).

GAMs results did not reveal statistical differences between years for any response variable in *A. media*, but HW varied between years in *A. subulata*: When DML or d were included to control for body size, GAMs result showed shorter HW in 2013 than in 2014.

DISCUSSION

Molecular analyses successfully identified paralarvae of three loliginid species in the Ría de Vigo, revealing that the most abundant species was *A. media* rather than *L. vulgaris* as had previously been thought (González et al. 2010). Molecular analyses also provided information about the molecular diversity and genetic structure in the Ría de Vigo, revealing very different demographic patterns among the three species. Discriminant analysis performed on external and statolith morphometric characters permitted us to differentiate both genera (*Loligo* and *Alloteuthis*) with 94% accuracy, and between species with 82% accuracy. Results presented in this work suggest that the combination between genetic and morphometric approaches, could increase the possibilities to accurately identify loliginids paralarvae in the Ría de Vigo.

Genetic identification and molecular analyses

The phylogenetic reconstruction of COI mitochondrial haplotypes, clearly separated the three loliginid species present in Galician waters with high bootstrap values. Genetic divergence between the two *Alloteuthis* species was 5-6 %, which is similar to previously reported (Anderson et al. 2008) and also supports the existence of two different species based on the barcoding gap (Hebert et al. 2003b). Divergence between genera was bigger (13%, between *Loligo* and *Alloteuthis*) and agrees with divergences detected by Gebhardt and Knebelsberger (2015). Intraspecific divergence was also in the same range as found in previous research, between 0.2 and 0.5% for both *Alloteuthis* species (Anderson et al. 2008) and higher for *L. vulgaris*, between 0.2 and 2% (Gebhardt and Knebelsberger 2015).

All species presented different patterns of molecular diversity but most abundant haplotypes for each species were consistent during the three years and in all transects suggesting that individuals of each species belong to the same population of that species:

A. media presented medium-high values of molecular and haplotype diversity. The most frequent haplotypes detected herein (H1/H5, MF157704/MF157710) had already been described in Galicia (EU668085.1/ EU668086.1 and EU668087.1/EU668084.1; Anderson et al. 2008) and presented between one and three different nucleotides from those reported in Mediterranean Sea (GU327600.1, GU327601.1, GU327602.1 and GU327603.1, Lefkaditou et al. 2012), agreeing with the subtle differences detected between Atlantic and Mediterranean populations (Anderson et al. 2008). Moreover, the two most frequent haplotypes (H1 and H5) matched with 8 (KM517867.1 KM517871.1 KM517873.1 KM517877.1 KM517879.1

KM517882.1, KM517890.1H3, KM517891.1) and 8 sequences respectively (KM517893.1, KM517865.1, KM517872.1, KM517878.1, KM517881.1, KM517886.1, KM517889.1, KM517892.1) that Gebhardt and Kneibelsberger, (2015) considered as *Alloteuthis* sp., being unable to classify them to species level based on morphological characters. *A. media* presented a star-like network characterized by two main haplotypes with many short branches emerging from them. This typical “star-like” network, agrees with the significant negative Tajima’s D and Fu’s Fs values, that indicate the existence of many segregating sites and low heterozygosity (i.e. one or two main haplotypes and single mutations are appearing and being accumulated from them).

A. subulata presented the lowest molecular diversity parameters among the three species in the Ría during the sampling period, with only three haplotypes generated by three nucleotide substitution (only one was non-synonymous). Of those haplotypes, the most frequently detected (H1) was the same that Anderson *et al.* (2008) and Lefkaditou *et al.* (2012) found in the Adriatic and in the Ionian Sea respectively (accession numbers EU668098-668100), thus suggesting small genetic differentiation for *A. subulata* across wide areas in the COI gene. Reduced genetic diversity could indicate a decrease of population size (Allcock and Strugnell 2012). However, it is possible that accurate estimation of the genetic population structure of this species could have been hindered due to the low number of *A. subulata* captured (haplotypes under-sampling) or that this species simply presents very low variability in mtDNA (Bucklin *et al.* 1997) as occurs in some other squids like *Architeuthis* (Winkelmann *et al.* 2013).

Loligo vulgaris presented the highest nucleotide diversity values, with high intraspecific variation despite the low number of paralarvae captured. Haplotype diversity was also the highest, due to multiple single haplotypes with many different mutations. The most frequently detected (H1) was identical to other individuals sequenced in Portugal (KF369144.1 and KF369144.1, Lobo *et al.* 2013) and presented one single modification from a sample captured in Plymouth (UK) (AF075397.1, Anderson 2000) and 6 nucleotide differences from haplotypes described in Turkey (GenBank accession number: KC789191.1). Additionally, the most different haplotype (H7) presents a single nucleotide difference with a previous haplotype described in Portugal (KF369141, Lobo *et al.* 2013). Tajima’s D values and Fu’s Fs presented negative but non-significant values, which indicates the existence of many silent mutations (does not change aminoacid sequence), thus producing low heterozygosity (in fact, there was only one non-synonymous mutations). These negative values agree with the high molecular and haplotype diversity, and generally indicate genetically-mixed populations in which mutations are accumulated over long periods at neutral markers, thus maintaining these high levels of diversity (Fauvelot *et al.* 2003).

In overall, the presence of the most abundant haplotypes in the Ría de Vigo across all transects and years, together with the lack of variability in F_{ST} values, suggested high gene flow, and null population differentiation of the different squid species inhabiting this small area (less than 20km). Genetic homogeneity in this study is not surprising, considering that loliginid adults can swim large distances (Sauer *et al.* 2000), and that long-term planktonic paralarvae (up to 3 months) present high capacity for dispersal, which might be

prompted by the Galician regional circulation system, dominated by the Portugal Current, which shows seasonal north/south-ward direction (Aristegui et al. 2009). This dynamic oceanographic circulation could maintain paralarval and genetic connectivity along Portugal and Spain coast as recently evidenced for *Octopus insularis* between Brazil and Ascension and St Helena Islands (Amor et al. 2017). Moreover, genetic homogeneity has been observed in marine invertebrates in Galician area (Sotelo et al. 2008), and has been also demonstrated in other squid species inhabiting areas over 200 km (Shaw et al. 2010; Winkelmann et al. 2013; Van Der Vyver et al. 2016). However, since subtle levels of genetic structure have been evidenced when employing microsatellites in other cephalopod species (Shaw et al. 1999; Pérez-Losada et al. 2007), and mitochondrial genes (COI and COIII) have shown significant genetic differentiation in some species among populations at small regional geographical scale, there could exist a combination of historical and/or current events that acted in the past restricting gene flow (Sales et al. 2014, 2017; Melis et al. 2018). Therefore, deeper analyses of genetic markers that are more variable than the COI gene (Bucklin et al. 1997), might reveal further population genetic structure of *Loligo vulgaris* and *Alloteuthis* spp. across their distribution range.

The decrease in abundance of loliginid paralarvae is correlated with the drop of official loliginid landings registered in Galicia during the period studied (*Loligo vulgaris* from 559.900,70 kg in 2011 to 137.467,62 in 2015 and *Alloteuthis* spp., from 74.351 kg in 2011 to 10,712 kg in 2015. <https://www.pescadegalicia.gal>). Cephalopod populations abundance, are highly variable and strongly affected by environmental conditions (Pierce et al. 2008; Rodhouse et al. 2014), and unfavourable oceanographic circumstances in 2013 and 2014, could had led to a higher mortality either of adults or paralarvae, or a distribution shift to other areas, as already occurred with *Loligo forbesii* (Chen et al., 2006). Previous researches have suggested that low number of individuals analysed (Zhang et al. 2010), can lead to under-sampling of rare haplotypes, thus the main reason of the reduction of molecular diversity levels in 2013 and 2014 could be the diminution of individuals captured.

Morphometric analyses

Significant size-at-age differences between the two genera, revealed by GLM analysis, were crucial to subsequently accomplish a successful discriminant analysis: *Loligo vulgaris* paralarvae were significantly bigger than those of *Alloteuthis* at the same age for all measurements. Differences were smaller between the two *Alloteuthis* species, and this was reflected in the lower discrimination power. Discriminant analysis did not allow a discrimination as good as has been achieved for adults (Lefkaditou et al. 2012), maybe due to the difficulties to obtain measures in paralarvae compared to adults. Notwithstanding, it provides new insights for a better identification of loliginid paralarvae previously identified only to family level (Rocha et al. 1999; González et al. 2005; Moreno et al. 2009; Otero et al. 2016; Roura et al. 2016).

The inclusion of the statolith variables increased the facility to distinguish between the two genera: the hatching increment length was the variable that contributed the most to discrimination. However, the use of statoliths, slows down the identification process by increasing the laboratory work. Furthermore, the

procedure of dissection, extraction and analysis of the statoliths requires and experienced person, thus being undesirable for a quick assessment in field work. The second most discriminative variable was head width, which is suggested to be employed for a quick evaluation, but decrease considerable the reliability of the identification.

Discrimination between the two species of *Alloteuthis* (*A. media* and *A. subulata*) was less successful. This was also evident when the statoliths were not included, as was also the case when distinguishing among genera. The structure matrix revealed that tentacle length was, by far, the variable that permitted better discrimination between *A. media* and *A. subulata*. Tentacle length was also used to achieve successful discrimination in adults in previous studies (Lefkaditou et al. 2012) in which separation among species was perfect. Unfortunately, in this study we could not employ other characters that helped to separate the species using adult specimens, like tentacular club width and central club sucker diameter (Anderson et al. 2008; Lefkaditou et al. 2012), because those measurements on paralarvae are difficult, due to the small size, damage that occurs during capture and deformation during preservation. However, we included Arm Length, which in adults was found to be very informative (Lefkaditou et al. 2012). Unfortunately, in our case, Arm Length did not contribute greatly to the discriminant function, suggesting that the difference is too small in paralarvae, perhaps due to measurement error, and/or because this difference becomes more pronounced later in life as it occurs with other body parts as fins length (Anderson et al. 2008).

Body shape variability due to external abiotic factors has been observed in cephalopods, in adults and in paralarvae, in soft parts and in hard structures (Pierce et al. 1994a, b; Jackson et al. 1997; Villanueva 2000; Forsythe 2004; Moreno et al. 2007, 2012; Van Der Vyver et al. 2016). Thus, this body variability needs to be taken into account to establish a reliable morphometric approach to distinguishing among paralarvae. Both *Alloteuthis* species, seem to have wider head and larger statoliths in autumn than in summer. Statoliths larger length in *Alloteuthis media* could be related to the size at hatching, since HL was also longer in autumn, and thus, due to different oceanographic conditions during embryonic development (Villanueva et al. 2003; Moreno et al. 2012). Instead, in *A. subulata*, although SL was longer in autumn, GAMs did not reveal differences in statoliths size at hatching.

Bigger paralarvae, and larger statoliths later in the year, could be explained as due to oceanographic conditions, for example increasing water temperature or variability in salinity levels. However, the change in body shape, with shorter and wider individuals later in the year, rather than bigger individuals, could be related with other factors such as feeding: This has been suggested in previous works, in which arm and tentacle length variation, was proposed as an adaptation to plankton size spectrum, thus producing high levels of intraspecific variability (Rodhouse and Piatkowski 1995).

Loliginid squid systematics has been constantly review during recent years, and remains unresolved for *Alloteuthis* (Anderson 2000; Anderson et al. 2008). *A. subulata* and *A. media* are sympatric in the main part of their distribution range (Jereb and Roper 2010; Oesterwind et al. 2010; Pierce et al. 2010; Jereb et al. 2015), and the identification of both species has been controversial during recent years, because characters

traditional employed to differentiate among them, have shown little reliability when comparing them with molecular data. Genetic identification supports the existence of two different species (Anderson et al. 2008), however, the observation of a single species according to molecular taxonomy, with different, and intermediate morphotypes has raised many doubts about their true distribution (Laptikhovsky et al. 2005; Anderson et al. 2008; Lefkaditou et al. 2012; Gebhardt and Knebelsberger 2015). Our data support the hypothesis of the existence of two different and coexisting species based on the barcoding 3% threshold (Hebert et al. 2003a) and the morphological difference detected in the tentacle length. We consider that reliable identification to distinguish between *A. media* and *A. subulata* is currently possible only with molecular methods, but the exploration of other characters rather than those previously employed, might reveal more taxonomic information. Additionally, a higher number of individuals from different areas and seasons should be analysed to account for morphological variability to completely differentiate adults and paralarvae.

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Fig. 1

1A) Map of the study area showing the locations of the seven plankton transects performed in 2012, 2013 and 2014, with bottom depths ranging from 20 to 105 m. 1B) Depth layers sampled for each transect.

Fig. 2

External measurements undertaken in all Loliginids paralarvae: Dorsal Mantle Length (DML), Total Length (TL), Head Width (HW) and Tentacle Length (TeL1, TeL2).

Fig. 3

Measurements and counts undertaken in statoliths. Above: Statolith Length (SL) and Statolith Width (SW). Below: Hatching ring Length (HL) and number of daily growth increments (d).

Fig. 4

Optimal (highest log likelihood) phylogenetic tree of the haplotypes of the loliginid paralarvae found in the Ría de Vigo. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths representing the number of substitutions per site. Branch lengths longer than 0.005 are indicated below the branch in italics

Fig. 5

Median joining network based on 505 base pairs of the COI mtDNA fragment for (a) 101 individuals of *Alloteuthis media*, (b) 37 individuals of *Alloteuthis subulata* and (c) 24 individuals of *Loligo vulgaris* collected in the Ría de Vigo during 2012 (grey), 2013 (white), 2014 (black). Size of circles is proportional haplotype frequency. Numbers in branches represent the position of the nucleotide substitution between haplotype sequences. The black dot indicates a missing or not sampled haplotype

Fig. 6

GLM results showing size differences in the body measurements dorsal mantle length (DML), total length (TL), head width (HW) and tentacle length (Lte) (mm) for each loliginid paralarvae species, when age was included as covariate. All variables are log transformed. Circles represent estimate marginal mean (EMM, for each morphometric variable) in each species, holding the covariate (age) constant as its mean value. Error bars represent $\pm$ standard error. Numbers indicate sample size. Multiple comparisons are based on EMM and performed with LSD post hoc test. Significantly different comparisons are presented above each figure ($p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.001$ ***).

Fig. 7

GLM results showing size differences in measurements undertaken on the statoliths of the three loliginid paralarvae species (statolith length (SL), statolith width (SW) and statolith hatching ring length (HL) (μm)), when age was included as covariate. All variables are log transformed. Circles represent estimate marginal mean (EMM, for each morphometric variable) in each species, holding the covariate (age) constant as its mean value. Error bars represent $\pm$ standard error. Numbers indicate sample size. Multiple comparisons are based on EMM and performed with LSD post hoc test. Significantly different comparisons are presented above each figure ($p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.001$ ***).

A)

B)

Fig. 1

Fig. 2

833 Fig. 3
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. Fig. 4

838 **Fig. 5**

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842 **Fig. 6**

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Fig. 7

Table 1

Loliginid paralarvae (Am: *Alloteuthis media*, As: *Alloteuthis subulata*, Lv: *Loligo vulgaris*) captured in the Ría de Vigo during 2012, 2013 and 2014 in all the transects (2, 3, 4, 5, BN: north mouth, BS: south mouth, C: centre) and in both seasons (S: Summer, A: early Autumn). NA: individuals for which sequencing did not succeed. Total number of paralarvae captured are indicated for each species, transect, year and season.

Year	2012					2013							2014							S	A	Total
Transect	2	3	4	5	Total 2012	2	3	4	5	BS	C	Total 2013	2	3	4	5	BN	BS	Total 2014			
Am	31	19	13	3	66	1	5	4	1	3	7	21	16	3	3	3	1	2	28	58	57	115
As	1	13	17	4	35				5	2		7								17	25	42
Lv	3	6	4	3	16		1	3	1	2	1	8	3	1		1			5	6	23	29
NA	6	5	1	1	13		1					1								7	7	14
Total	41	43	35	11	130	1	7	12	4	5	8	37	19	4	3	4	1	2	33	88	112	200

Table 2

Results of the analysis of molecular diversity parameters for *Alloteuthis media*, *Alloteuthis subulata* and *Loligo vulgaris*: number of sequences analysed (n), variable sites (S), total number of mutations (Eta), number of haplotypes (h), haplotype diversity (Hd), haplotype diversity standard deviation (s.d. Hd), nucleotide diversity (π), nucleotide diversity standard deviation (s.d. π), Fu's Fs statistic (Fu's Fs) and Tajima's D value (D). Significant values of neutrality statistics (Fu's Fs p -value <0.02, D p -value <0.05) and high molecular diversity values (Hd and π according to Goodall-Copestake et al, 2012) are in bold type.

Species	Year	n	S	Eta	h	Hd	s.d. Hd	π	s.d. π	Fu's Fs	Fu's Fs p -value	D	D p -value
<i>Alloteuthis media</i>	2012	53	14	14	14	0.712	0.048	0.0020	0.0003	-11.419	0.000	-2.017	0.006
	2013	20	4	4	5	0.558	0.114	0.0015	0.0004	-1.893	0.043	-0.989	0.192
	2014	28	8	8	8	0.706	0.061	0.0020	0.0610	-4.045	0.003	-1.558	0.043
	all	101	21	21	21	0.680	0.037	0.0019	0.0002	-22.355	0.000	-2.221	0.001
<i>Alloteuthis subulata</i>	2012	32	3	3	3	0.287	0.088	0.0008	0.0003	-0.342	0.293	-1.079	0.171
	2013	5	1	1	2	0.400	0.237	0.0008	0.0005	0.090	0.284	-0.817	0.288
	2014	-	-	-	-	-	-	-	-	-	-	-	-
	all	37	3	3	3	0.287	0.088	0.0008	0.0003	-0.305	0.288	-1.025	0.190
<i>Loligo vulgaris</i>	2012	11	11	13	7	0.873	0.089	0.0058	0.0023	-1.737	0.111	-0.949	0.135
	2013	8	12	13	4	0.643	0.184	0.0067	0.0034	-1.938	0.026	-1.048	0.129
	2014	5	3	3	4	0.900	0.161	0.0024	0.0006	1.274	0.747	-1.343	0.058
	all	24	13	15	10	0.808	0.071	0.0053	0.0016	-2.543	0.098	-0.786	0.236

Table 3

Results of GLM model analysis presenting the significant differences among the three species in body characters. A single model for each of the following response variables was built: Dorsal mantle length (DML), total length (TL), head width (HW), tentacle length (TeL), statolith length (SL), statolith width (SW) and hatching increment length (HL). Species was included in each model as explanatory variable, and age was included as a covariate except in that with HL (due the absence of relationship with age). The interaction term age*species was not statistically significant in any models. All continuous variables in the table are log transformed and effects of all explanatory variables were statistically significant. The reference level is *Loligo vulgaris*.

Dependent variable	Source of variation	Species	d.f.	Estimate	$\pm$ s.e.m.	F	P
DML	Intercept			0.812	0.047		
	Species		2, 112			5.243	0.006
		<i>A. media</i>		-0.148	0.049		
		<i>A. subulata</i>		-0.174	0.056		
	Age		1,112	0.024	0.001	435.650	<0.001
TL	Intercept			1.372	0.048		
	Species		2, 113			12.496	<0.001
		<i>A. media</i>		-0.202	0.051		
		<i>A. subulata</i>		-0.289	0.058		
	Age		1,113	0.026	0.001	527.107	<0.001
HW	Intercept			0.315	0.051		
	Species		2, 112			10.087	<0.001
		<i>A. media</i>		-0.240	0.054		
		<i>A. subulata</i>		-0.233	0.062		
	Age		1,112	0.019	0.001	253.965	<0.001
Lte	Intercept			-0.160	0.075		
	Species		2, 109			12.527	<0.001
		<i>A. media</i>		-0.130	0.078		
		<i>A. subulata</i>		-0.388	0.090		
	Age		1,109	0.030	0.002	306.307	<0.001
SL	Intercept			-1.865	0.045		
	Species		2, 113			7.007	0.001
		<i>A. media</i>		-0.177	0.048		
		<i>A. subulata</i>		-0.180	0.055		
	Age		1,113	0.023	0.001	484.485	<0.001
SW	Intercept			-2.457	0.055		
	Species		2, 110			10.234	<0.001
		<i>A. media</i>		-0.254	0.057		
		<i>A. subulata</i>		-0.182	0.064		
	Age		1,110	0.022	0.001	380.621	<0.001
HL	Intercept			4.852	0.027		
	Species		2, 118			13.129	<0.001
		<i>A. media</i>		-0.145	0.029		
		<i>A. subulata</i>		-0.109	0.031		

Table 4

The statistic Wilks' Lambda (Λ), chi square value (χ^2), degrees of freedom (d.f), and the statistical significance (Sig.) for each discriminant functions (D.F.) are presented. Moreover, the eigenvalue and the canonical correlation are also shown for each D.F.

D.F.	(Λ)	χ^2	d.f.	Sig.	Eigenvalue	Canonical Correlation
A1	0.561	34.368	7	0.000	0.782	0.662
A2	0.635	24.754	5	0.000	0.575	0.604
B1	0.806	19.415	4	0.001	0.241	0.441
B2	0.840	24.342	2	0.000	0.190	0.399

Table 5

Variables employed in each discriminant function (D.F.): total length (TL), head width (HW), tentacle length (TeL), Arm1 (A1), statolith length (SL), statolith width (SW), hatching increment length (HL). All variables are transformed with natural logarithms. D.F. -A included statolith variables, and D.F. -B did not include statolith variables. Their corresponding standardized coefficients and the structure matrix are also presented for each function. Group centroids values for each D.F. are also presented: The possible groups in D.F-A1 and D.F-B1 are: *Alloteuthis* (1) and *Loligo* (2). In A2 and B2 the possible groups are: *A. media* (1) and *A. subulata* (2).

Statolith	Discriminate between	D.F.	Discriminating variable	Structure matrix	Standardized Canonical Coefficients	Functions at Group Centroids Belonging group	
						1	2
With	Genera	A1	HL	0.727	0.863	-0.278	2.730
			HW	0.416	0.708		
			SW	0.088	0.130		
			SL	-0.034	-0.535		
			Lte	-0.043	0.156		
			A2	-0.100	-0.024		
	Species	A2	A1	-0.169	-0.310	0.357	-1.557
			Lte	0.681	1.472		
			A2	0.209	0.250		
			TL	0.164	-0.881		
Without	Genera	B1	HL	-0.191	-0.032	-0.228	1.033
			SW	-0.23	-0.364		
			TL	0.487	0.631		
			HW	0.861	0.817		
	Species	B2	Lte	0.097	-0.291	0.265	-0.707
			A1	-0.079	-0.218		
			Lte	0.994	1.010		
			HW	0.033	-0.110		

SUPPLEMENTARY MATERIAL

Survey date			Transect						
Year	Month	Day	T2	T3	T4	T5	SM	C	NM
2012	7	17	x	x	x	x			
2012	7	19	x	x	x	x			
2012	7	24	x	x	x	x			
2012	7	26	x	x	x	x			
2012	9	12	x	x	x	x			
2012	9	16	x	x	x	x			
2012	9	17	x	x	x	x			
2012	9	20	x	x	x	x			
2012	10	1	x	x	x	x			
2012	10	5	x	x	x	x			
2013	7	9	x		x	x			
2013	7	11	x	x	x	x			
2013	7	16	x	x	x	x			
2013	7	18	x	x	x	x			
2013	9	17	x				x	x	x
2013	9	24		x	x	x			
2013	10	4		x		x	x	x	x
2013	10	8		x	x	x	x	x	x
2013	10	10	x	x	x	x			
2013	10	15		x	x	x	x		
2014	7	16	x	x	x	x	x		
2014	7	17	x	x	x	x			
2014	7	24	x	x	x	x			
2014	7	31	x	x	x	x			
2014	9	23	x	x	x	x			
2014	9	25	x	x	x	x			
2014	9	30	x	x	x	x			
2014	10	2	x	x	x	x			
2014	10	10	x				x		x
2014	10	23	x	x	x	x	x		x

Supplementary material 1

Survey dates specifying transects sampled in each survey. 24h surveys are presented in bold type (same transects were sampled during the day and again during the night). SM (South mouth), C (Centre), NM (North mouth).

Species	Haplotype	GenBank Accession number	Nucleotide position																				
			15	21	81	102	105	123	135	180	204	231	249	255	259	309	324	333	363	406	450	477	489
A. media	H1	MF157704	T	A	T	G	T	A	A	T	A	T	G	T	C	C	G	A	G	T	A	A	A
	H2	MF157705	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
	H3	MF157706	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H4	MF157707	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
	H5	MF157708	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
	H6	MF157709	.	.	.	.	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
	H7	MF157710	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
	H8	MF157711	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.
	H9	MF157712	.	.	.	.	.	.	.	.	.	C	.	C	T	.	G	.	.	.	.	G	.
	H10	MF157713	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	.
	H11	MF157714	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
	H12	MF157715	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H13	MF157716	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
	H14	MF157717	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.
	H15	MF157718	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.
	H16	MF157719	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.
	H17	MF157720	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H18	MF157721	.	.	.	.	.	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.
	H19	MF157722	.	.	.	A	C	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
	H20	MF157723	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G
	H21	MF157724	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.
A. subulata			34	35	357																		
	H1	MF157701	G	T	T																		
	H2	MF157702	.	.	C																		
	H3	MF157703	T	G	C																		
L. vulgaris			15	21	72	132	177	189	192	249	303	315	412	420	454								
	H1	MF157725	C	A	G	G	A	G	A	C	C	C	C	A	T								
	H2	MF157726	.	.	.	A	G	.	.	.	.	.	.	.	C								
	H3	MF157727	.	.	.	A	.	.	.	.	.	.	.	.	C								
	H4	MF157728	.	.	A	.	.	.	.	.	.	.	.	.	C								
	H5	MF157729	.	.	.	.	C	.	.	.	.	.	.	.	C								
	H6	MF157730	.	.	.	.	.	.	.	.	.	.	.	.	C								
	H7	MF157731	T	.	.	C	G	A	G	G	.	T	T	C	C								
	H8	MF157732	.	T	.	A	.	.	.	.	.	.	.	.	C								
	H9	MF157733	.	.	.	.	.	.	.	T	.	.	.	.	.								
	H10	MF157734	.	.	.	.	G	.	.	.	.	.	.	.	C								

Supplementary material 2

Alignment of variable sites of haplotypes (H) of the three loligid species. Conserved sites are indicated with a dot, variable sites are indicated with the nucleotide base (A: adenine, C: cytosine, T: thymine, G: guanine). Nucleotide positions are indicated for each species. Haplotype 1 (H1) is employed as the reference sequence for each species. GenBank accession number for each haplotype is also shown

DF	Original group membership	N original	Predicted Group Membership (%)		Overall successful classification (%)
			1	2	
A1	<i>Alloteuthis</i>	59	94.9	5.1	93.8
	<i>Loligo</i>	6	16.7	83.3	
A2	<i>A. media</i>	48	89.6	10.4	82.3
	<i>A. subulata</i>	11	27.3	72.7	
B1	<i>Alloteuthis</i>	77	74.0	26.0	75.5
	<i>Loligo</i>	17	17.6	82.4	
B2	<i>A. media</i>	104	73.1	26.9	72.0
	<i>A. subulata</i>	39	30.8	69.2	

Supplementary material 3

Classification results of the discriminant analysis showing the number of specimens in the original groups (N original) and the percentage of individuals classified in each group (Predicted Group Membership). In A1 and B1 the groups are: *Alloteuthis* (1) and *Loligo* (2). In A2 and B2 the groups are: *A. media* (1) and *A. subulata* (2).

Alloteuthis media

Response	Models with AGE to control for ontogenic changes							Models with DML to control for ontogenic changes						
	Explanatory	df	Est.	±se	p value	Dev. Ex. (%)	AIC	Explanatory	df	Est.	±se	p value	Dev. Ex. (%)	AIC
LnDML	age(s)	1.253 (+)			<0.001	78.4	-45.81							
LnDML	age(s)	1.185			<0.001	79.3	-47.39							
	Capture day (s)	1 (+)			0.063									
LnDML	age(s)	1.185			<0.001	79.3	-47.39							
	Hatching day (s)	1 (+)			0.063									
LnDML	age(s)	1.312			<0.001	79.4	-47.8							
	Hatching season	1	0.077	0.039	0.051									
LnDML	age(s)	1.312			<0.001	79.4	-47.8							
	Capture season	1	0.077	0.039	0.051									
LnDML	age(s)	1.335			0.660	78.7	-42.73							
	year	2			0.660									
	2013		-0.051	0.057	0.368									
	2014		-0.021	0.049	0.667									
LnTI	age(s)	1.807 (+)			<0.001	82.3	-51.84	LnDML(s)	2.649 (+)			<0.001	94.1	-185.78
LnTI	age(s)	1.785			<0.001	82.6	-51.38	LnDML(s)	2.411			<0.001	95.4	-189.41
	Capture day (s)	1.000 (+)			0.218			Capture day (s)	2.049(-)			0.058		
LnTI	age(s)	1.785			<0.001	82.6	-51.38	LnDML(s)	2.797			<0.001		-162.59
	Hatching day(s)	1.000 (+)			0.218			Hatching day (s)	1.000 (-)			0.056	95.4	
LnTI	age(s)	1.806			<0.001	82.6	-51.33	LnDML(s)	2.803			<0.001	95.4	-162.44
	Hatching season	1	0.046	0.038	0.233			Hatching season	1	-0.047	0.019	0.013		
LnTI	age(s)	1.806			<0.001	82.6	-51.33	LnDML(s)	2.559			<0.001	94.4	-189.99
	Capture season	1	0.046	0.038	0.233			Capture season	1	-0.037	0.019	0.063		
LnTL	age(s)	1.769			<0.001			LnDML(s)	2.534			<0.001	94.2	-183.55
	year	2			0.524	82.5	-49.19	year	2			0.387		
	2013		-0.057	0.054	0.297			2013		-0.013	0.028	0.629		
	2014		0.002	0.047	0.959			2014		0.027	0.024	0.272		
LnHW	age(s)	1.000 (+)			<0.001	68.7	-38.83	LnDML(s)	2.386 (+)			<0.001	89.5	-157.44
LnHW	age(s)	1.000			<0.001	71.4	-44.19	LnDML(s)	2.532			<0.001	90.3	-162.97
	Capture day (s)	1.000 (+)			0.008			Capture day (s)	1.000 (+)			0.008		
LnHW	age(s)	1.000			<0.001	71.4	-44.19	LnDML(s)	2.478			<0.001	90.5	-131.83
	Hatching day(s)	1.000 (+)			0.008			Hatching day (s)	1.000 (+)			0.019		
LnHW	age(s)	1.000			<0.001	71.3	-44.15	LnDML(s)	2.435			<0.001	90.4	
	Hatching season	1	0.108	0.040	0.008			Capture season	1	0.052	0.024	0.030		-130.87
LnHW	age(s)	1.000			<0.001	71.3	-44.15	LnDML(s)	2.570			<0.001	90.2	-162.12
	Capture season	1	0.108	0.040	0.008			Hatching season	1	0.054	0.021	0.013		
LnHW	age(s)	1.000			<0.001	68.7	-34.9	LnDML(s)	2.443				89.7	-155.75
	year	2			0.970			year	2					
	2013		-0.003	0.059	0.957			2013		0.046	0.032	0.152		
	2014		-0.012	0.051	0.808			2014		0.008	0.028	0.773		
LnLte	age(s)	1.528 (+)			<0.001	75.4	9.4	LnDML(s)	2.214 (+)			<0.001	89.6	-75.19
LnLte	age(s)	1.506			<0.001	75.5	11.07	LnDML(s)	1.962			<0.001	90.4	-79.96
	Capture day (s)	1.000 (+)			0.565			Capture day (s)	1.908 (-)			0.031		
LnLte	age(s)	1.506			<0.001	75.5	11.07	LnDML(s)	2.194			<0.001	89.6	-57.93
	Hatching day(s)	1.000 (+)			0.565			Hatching day (s)	1.000 (-)			0.134		
LnLte	age(s)	1.526			<0.001	75.4	11.21	LnDML(s)	2.207			<0.001	89.7	-58.65
	Hatching season	1	0.024	0.056	0.670			Hatching season	1	-0.063	0.037	0.088		
LnLte	age(s)	1.526			<0.001	75.4	11.21	LnDML(s)	2.106			<0.001	90.2	0.0108
	Capture season	1	0.024	0.056	0.670			Capture season	1	-0.082	0.032	0.011		
LnLte	age(s)	1.769			<0.001	82.5	-49.19	LnDML(s)	2.155			<0.001	90.2	-77.33
	year	2			0.524			year	2			0.054		
	2013		-0.057	0.054	0.297			2013		0.055	0.046	0.233		
	2014		0.002	0.047	0.959			2014		0.098	0.041	0.019		
LnSL	age(s)	2.025 (+)			<0.001	83.2	-75.75	LnDML(s)	2.866 (+)			<0.001	91.1	-133.91
LnSL	age(s)	2.087			<0.001	87.2	-94.6	LnDML(s)	2.895			<0.001	92.2	-143.69
	Capture day (s)	1.947 (+)			<0.001			Capture day (s)	1.000 (+)			0.001		
LnSL	age(s)	2.127			<0.001	87.2	-94.65	LnDML(s)	2.886			<0.001	93.5	-142.67
	Hatching day (s)	1.914 (+)			<0.001			Hatching day(s)	2.065 (+)			0.015		
LnSL	age(s)	2.106			<0.001	86.9	-94.64	LnDML(s)	2.895			<0.001	92.1	-142.09
	Hatching season	1	0.139	0.029	<0.001			Capture season	1	0.070	0.022	0.002		
LnSL	age(s)	2.106			<0.001	86.9	-94.64	LnDML(s)	2.902			<0.001	93.4	-143.36
	Capture season	1	0.139	0.029	<0.001			Hatching season	1	0.068	0.022	0.002		
LnSL	age(s)	2.035			<0.001	84.0	-75.66	LnDML(s)	2.844			<0.001		-134.16
	year	2			0.168			year	2			0.127		
	2013		-0.083	0.044	0.064			2013		-0.051	0.032	0.116	91.5	
	2014		-0.017	0.041	0.676			2014		0.020	0.028	0.476		
LnHL								Capture day (s)	1.437 (+)			0.083	6.16	-172.13
LnHL								Hatching day (s)	2.088 (+)			0.043	9.73	-174.19
LnHL								Capture season	1	0.045	0.019	0.019	6.3	-173.13
LnHL								Hatching season	1	0.045	0.019	0.019	6.3	-173.13
LnHL								year	2			0.164	4.2	-169.21
								2013		-0.037	0.025	0.144		
								2014		0.016	0.022	0.481		

<i>Alloteuthis subulata</i>														
Response.	Models with AGE to control for ontogenic changes							Models with DML to control for ontogenic changes						
	Explanatory	df	Est.	±se	p value	Dev. Ex. (%)	AIC	Explanatory	df	Est.	±se	p value	Dev. Ex. (%)	AIC
LnDML		1.000 (+)			<0.001	90.1	-33.69							
LnDML	age(s)	1.000			<0.001	91.2	-33.25							
	Capture day (s)	1.57 (-)			0.431									
LnDML	age(s)	1.000			<0.001	91.5	-33.06							
	Hatching day (s)	2.103 (-)			0.495									
LnDML	age(s)	1.000			<0.001	90.1	-33.69							
	Hatching season	1	-0.019	0.049	0.495	91.2	-33.25							
LnDML	age(s)	1.57			<0.001									
	Capture season	1	-0.019	0.049	0.431	91.5	-33.06							
LnDML	age(s)	2.103												
	year	1	-0.003	0.061	0.495	90.2	-31.87							
LnTL	age(s)	1.000 (+)			<0.001	88.7	-27.59	LnDML(s)	1.000 (+)			<0.001	92.8	-73.2
LnTL	age(s)	1.000			<0.001			LnDML(s)	1.000			<0.001		
	Capture day (s)	1.000 (-)			0.647			Capture day (s)	2.887 (-)			0.011	94.8	-80.79
LnTL	age(s)	1.000			<0.001	90.0	-26.9	LnDML(s)	1.000			<0.001	93.8	-36.81
	Hatching day (s)	1.669 (-)			0.479			Hatching day (s)	2.223 (-)			0.048		
LnTL	age(s)	1.000			<0.001	89.0	-26.05	LnDML(s)	1.000			<0.001	92.9	-71.37
	Capture season	1	-0.036	0.056	0.532			Capture season	1	-0.013	0.032	0.700		
LnTL	age(s)	1.000			<0.001	89.0	-26.05	LnDML(s)	1.499			<0.001	91.0	-29.82
	Hatching season	1	-0.036	0.056	0.532			Hatching season	1	-0.031	0.052	0.554		
LnTL	age(s)	1.000			<0.001	89.8	-27.81	LnDML(s)	1.000			<0.001	93.3	-73.88
	year	1	-0.094	0.066	0.170			year	1	-0.065	0.041	0.118		
LnHW	age(s)	2.564 (+)			<0.001	81.9	-24.85	LnDML(s)	1.000 (+)			<0.001	83.5	-48.48
LnHW	age(s)	1.000			<0.001	82.6	-26.81	LnDML(s)	1.000			<0.001	86.3	-54.31
	Capture day (s)	1.000 (+)			0.040			Capture day (s)	1.000 (+)			0.007		
LnHW	age(s)	2.634			<0.001	85.9	-28.37	LnDML(s)	1.693			<0.001	88.5	-33
	Hatching day (s)	1.000 (+)			0.0374			Hatching day (s)	2.006 (+)			0.028		
LnHW	age(s)	1.000			<0.001	82.2	-26.31	LnDML(s)	1.000			<0.001	86.1	-53.64
	Capture season	1	0.116	0.056	0.051			Capture season	1	0.108	0.040	0.011		
LnHW	age(s)	1.000			<0.001	82.2	-26.31	LnDML(s)	1.871			<0.001	86.1	-30.34
	Hatching season	1	0.116	0.056	0.051			Hatching season	1	0.119	0.051	0.031		
LnHW	age(s)	1.178			<0.001	84.6	-29.37	LnDML(s)	1.000			<0.001	85.4	-51.53
	year	1	-0.177	0.063	0.0118			Year	1	-0.120	0.054	0.031		
LnLte	age(s)	1.000			<0.001	77.0	-7.21	LnDML(s)	1.000 (+)			<0.001	76.9	-9.27
LnLte	age(s)	1.000			<0.001	77.5	-5.67	LnDML(s)	1.000			<0.001	81.1	-12.37
	Capture day (s)	1.000			0.535			Capture day (s)	2.418 (-)			0.086		
LnLte	age(s)	1.000			<0.001	80.2	-7.02	LnDML(s)	1.601			<0.001	86.5	-12.36
	Hatching day (s)	1.693			0.410			Hatching day (s)	2.393 (-)			0.159		
LnLte	age(s)	1.255			<0.001	79.1	-6.68	LnDML(s)	1.876			<0.001	82.7	-9.44
	Hatching season	1	-0.100	0.089	0.277			Hatching season	1	-0.110	0.083	0.201		
LnLte	age(s)	1.255			<0.001	79.1	-6.68	LnDML(s)	1.000			<0.001	78.2	-9.62
	season	1	-0.100	0.089	0.276			Capture season	1	-0.104	0.070	0.143		
LnLte	age(s)	1.053			<0.001	79.3	-7.32	LnDML(s)	1.000			<0.001	78.0	-9.22
	year	1	-0.159	0.116	0.186			Year	1	-0.132	0.097	0.182		
LnSL	age(s)	1.000 (+)			<0.001	82.7	-25.54	LnDML(s)	1.000 (+)			<0.001	91.2	-42.6
LnSL	age(s)	1.000			<0.001	84	-25.23	LnDML(s)	1.000			<0.001		
	Capture day (s)	1.000			0.233			Capture day (s)	1.000 (+)			0.031	93.0	46.02
LnSL	age(s)	1.000 (+)			<0.001	84.0	-25.23	LnDML(s)	1.000					-43.23
	Hatching day (s)	1.000			0.233			Hatching day (s)	1.478 (+)			0.036	93.2	
LnSL	age(s)	1.000 (+)			<0.001	84.0	-25.26	LnDML(s)	1.000			<0.001	92.6	
	Capture season	1	0.069	0.056	0.228			Capture season	1	0.076	0.039	0.064		-44.63
LnSL	age(s)	1.000			<0.001	84.0	-25.26	LnDML(s)	1.000			<0.001		
	Hatching season	1	0.069	0.056	0.228			Hatching season	1	0.086	0.039	0.040	92.1	-40.91
LnSL	age(s)	1.000			<0.001	83.7	-24.86	LnDML(s)	1.000			<0.001		
	year	1	-0.074	0.068	0.292			Year	1	-0.066	0.049	0.199	91.9	-42.53
LnHL								Capture day (s)	1.000			0.315	4.8	-56.03
LnHL								Hatching day (s)	1.000			0.185	8.22	-56.87
LnHL								Capture season	1	-0.033	0.027	0.249	6.26	-56.38
LnHL								Hatching season	1	-0.033	0.027	0.249	6.26	-56.38
LnHL								Year	1	0.018	0.036	0.612	1.24	-55.18

Supplementary material 4

Summary of the results for the GAM models for *Alloteuthis media* and *Alloteuthis subulata*. For each morphometric response variable (Total Length (TL), Head Width (HW), Tentacle Length (TeL), Statolith Length (SL), Statolith Width (SW)), the models included as smoothers either dorsal mantle length (DML, left side of table) or age (right side of table) to control for ontogenetic changes in size (except for and Hatching increment Length (HL) because it is independent of size). The effects of capture day, hatching day, capture season, hatching season and year were then tested in separate models. Estimate (Est.), ± Standard Error (se) are given for nominal explanatory variables. The reference level of hatching and capture season is summer. The reference level for year is 2012. Degrees of freedom (df) are given for explanatory variables fitted as smoothers(s). Smoother trend is indicated with a positive or negative sign between brackets next to df. Statistical significant *p*-values are in bold type. The explanatory variables dorsal mantle length, age, capture day and hatching day were fitted as smoothers (indicated with s after the variable). The explanatory variables capture season hatching season and year were fitted as nominal. Deviance explained (Dev. Ex.) and Akaike Information Criteria (AIC) are presented for every model.

Alloteuthis media

Alloteuthis media

(A)

Alloteuthis subulata

SMOOTHER

AGE

DML

Explanatory variable

Hatching day

Capture day

Hatching day

Capture day

Response variable

LnDML

LnTL

LnHW

LnLte

LnSL

LnHL

(B)

Supplementary material 5

Generalized additive models results: smoothing curves for partial effect of hatching day or capture day on the morphometric measurements of *Alloteuthis media* (A) and *Alloteuthis subulata* (B) (Dorsal Mantle Length (DML), Total Length (TL), Head Width (HW), Tentacle Length (TeL), Statolith Length (SL), Statolith Width (SW) and Hatching increment Length (HL)). Dashed lines indicate 95% confidence intervals around the main effect. In each model, age (left) or DML (right) were included as smoothers to control for age or size effect (except for HL because it is size independent). Significant p -values of the smoothers are indicated in bold below each model. See previous table for further information of complete models.