Sepiolid paralarval diversity in a regional upwelling area of the NE Atlantic

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Abstract

Sepiolid paralarvae are poorly studied, at least in part, because of the difficulty of accurate identification using morphological analysis. To unravel the biodiversity of sepiolid paralarvae collected in the Ría de Vigo during the upwelling season (2012-2014), and to overcome the difficulties of traditional identification, sepiolid paralarvae were identified by amplifying the barcoding gene cytochrome *c* oxidase subunit I (COI). In addition, morphometric analysis (Generalised Lineal Models, GLM and Discriminant Analysis, DA) was used to identify morphometric patterns useful for paralarval species identification. Genetic barcoding successfully identified 34 *Sepiola pfefferi*, 31 *Rondeletiola minor*, 30 *Sepiola tridens*, 4 *Sepiola atlantica*, 2 *Sepietta neglecta* and 1 *Sepiola ligulata*. COI analysis also allowed us to infer that the paralarvae of the three most abundant species belonged to the same populations independently of the year sampled. GLM suggested that total length (statistically different among the three species) and tentacle length (statistically larger in *S. pfefferi* from the other two species) were good variables to distinguish among species. DA succeed in separating *S. pfefferi* from *S. tridens*, but *R. minor* overlapped along the first axes with both species, decreasing the accurate classification rate to 67%.

Keywords: sepiolidae; barcoding; morphometries; biodiversity.

INTRODUCTION

In the last few years, a wide variety of studies have examined how anthropogenic activity and global change are affecting marine ecosystems and marine biodiversity (Inniss et al., 2016). Species with short life cycles, like cephalopods, are especially sensitive to environmental conditions (Boyle & Rodhouse, 2005) and have been highlighted as indicators of local environmental changes (Pierce et al., 2008; Doubleday et al., 2016). However, most research on cephalopods has focused on commercially important species, in contrast with those with low commercial value, such as sepiolids (Jereb & Roper, 2005).

Moreover, less attention has been paid to cephalopod early life stages, named paralarvae (Boletzky, 1974, 2003; Young & Harman, 1988). However, interest and research on the ecology and biodiversity of paralarvae has increased lately (Piatkowski, 1998; Rocha et al., 1999; Falcon et al., 2000; Diekmann & Piatkowski, 2004). Research on paralarvae has been performed to evaluate the influence of ocean temperature changes on them (Moreno et al., 2012; Rosa et al., 2014), to understand the relationships between oceanographic conditions and paralarval abundance (Diekmann & Piatkowski, 2002; González et al., 2005; Moreno et al., 2009; Roura et al., 2016), and to predict adult populations size (Otero et al., 2008).

Previous studies have not identified all the sepiolid paralarvae to species level (Rocha et al., 1999; Moreno et al., 2009; Roura et al., 2016), because traditional identification with guides is based on adult characters that are often not visible in paralarvae (Bello & Salman, 2015) and are easily damaged during zooplankton towing (e.g. fins and tentacles) and preservation procedures (e.g. chromatophore pattern, Sweeney et al., 1992). However, accurate identification is essential to unravel the ecology of sepiolids (Roura et al. 2016).

The emergence of molecular tools, such as DNA barcoding, has facilitated the identification of many species in marine habitats (Bucklin et al., 2011). In cephalopods, it has been successfully employed to identify both adult sepiolid specimens (Groenenberg et al., 2009) and sepiolid paralarvae (Roura, 2013). Moreover, barcoding has unmasked an overlooked sepiolid species, *Sepiola tridens,* Heij & Goud, 2010, very similar to *Sepiola atlantica*. In addition, molecular analyses allow us to study phylogenetic relationships among species (Strugnell et al., 2005; Strugnell & Lindgren, 2007) and population structure (Bucklin et al., 2011). On the other hand, analyses of morphometric data with multivariate techniques have also been widely employed to differentiate closely related species (Conde-Padín et al., 2007) and populations (Silva, 2003), and to reveal sexual dimorphism (Galarza et al., 2008). Several studies have analysed adult cephalopod species morphologically (Borges, 1990; Pierce et al., 1994a, 1994b; Neige & Boletzky, 1997; Arkhipkin & Bjørke, 1999; Bello & Paparella, 2003; Anderson et al., 2008; Turan & Yaglioglu, 2009; Lefkaditou et al., 2012), and to our knowledge, these methods have not been tested in sepiolid paralarvae.

The Ría de Vigo (Galicia, NW Spain) is a highly diverse ecosystem where frequent nutrient enrichment inputs (Alvarez-Salgado et al., 2003) enhance productivity and important artisanal coastal fisheries, including fisheries for cephalopods (González et al., 1994; Guerra et al., 1994; Aristegui et al., 2004; Tasende et al., 2005; Otero et al., 2008; Pita et al., 2016). The Marine Protected Area located at the seaward end of the Ría de Vigo (Illas Atlánticas de Galicia National Park, PNIAG) has been recently identified as a preferred habitat for cephalopod spawning and juvenile recruitment (Guerra et al., 2014, 2015). A great effort has been made to study adult cephalopods - namely octopods, squids, and cuttlefishes - in this area, but research on sepiolids has been restricted to *Sepiola atlantica* (Rodrigues et al., 2011). Overall, 16 sepiolid species have been reported off the NW Iberian Peninsula (Jereb & Roper, 2005), only two of which are cited as occurring inside of the Ría: *Sepiola ligulata* and *S. atlantica* (Guerra, 1986; Rodrigues et al., 2011). Focusing on paralarvae, *Rondeletiola minor*, *S. atlantica*, *S. ligulata* and *S. tridens* have been reported in Galician waters (Rocha et al., 1999; Roura, 2013; Roura et al., 2016). All these paralarvae were identified with traditional methods, thus accurate-species level identification has never been addressed and their true diversity in the area remains unknown.

Understanding sepiolid diversity is essential to unravel the different life strategies that these paralarvae display in the coastal area of NW Iberian Peninsula. Thus, the aim of this work was to assess the biodiversity of sepiolid paralarvae present in zooplankton samples collected during the upwelling season between 2012-2014 in the Ría de Vigo. We identified all sepiolid paralarvae with molecular tools (COI gene) to overcome the difficulties of traditional taxonomic identification and provide some hints about their population structure. We also aimed to identify morphometric variables that could be useful for paralarval differentiation of sepiolids in the field.

MATERIAL 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 (Rocha et al., 1999). Additionally, four diurnal surveys were conducted, one in summer and the others in early autumn in both 2012 and 2013. For each survey, sampling was conducted along four transects following González et al. (2010), (Fig. 1a). Due to unfavourable weather conditions, transects 3,4 and 5 were cancelled on specific surveys: 2 nocturnal and the diurnal in autumn 2013, and one nocturnal survey in autumn 2014. Instead, the surveys were conducted to intercept the entrance and exit fluxes at north mouth (NM) and south mouth (SM) of the Ría (Fig. 1a). Moreover, two additional samplings of BN and BS were undertaken: one in autumn 2013 and the other in autumn 2014. For each transect, a

Multinet® Hydrobios Mammoth of 250 µm mesh size and aperture of 1 m^2, fitted with two electronic flow meters, was lowered to the sea floor. At the cruise velocity of 2.5 knots, the Multinet® was lifted up gradually to the surface, from one water layer to the next (from 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) passing through up to seven layers where depth was at the maximum. At each layer, the Multinet® filtered 200 m^3 and collected discrete samples. Then, zooplankton was fixed on board in 96% ethanol and frozen at -20ºC until sorting. In the laboratory, each sepiolid paralarva (N=105) was separated and preserved individually in 70% ethanol and stored at -20ºC.

Genetic identification and phylogenetic analysis

DNA from each mantle (N=105) was extracted with a QIAamp DNA Micro Kit (QIAGEN) following Roura et al. (2012). The barcoding region of the cytochrome c oxidase subunit I (COI) was amplified with the universal primers HCO2198 and LCO1490 (Folmer et al., 1994). Cycling conditions were: initial denaturation at 94ºC for 1 min, followed by 39 cycles of denaturation at 94ºC for 15 seconds, annealing at 48ºC for 30 seconds and extension at 72ºC for 45 seconds. The final elongation was at 72 ºC for 7 minutes. PCR amplification was performed in a total volume of 25µl with 1µL of forward and 1µL of 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 H_2O. Then, 2µl of each PCR product were checked on 1.5% agarose gels. Those presenting a clear band of the expected size (650bp) were cleaned using USB® ExoSAP-IT® PCR Product Cleanup (Affymetrix, Inc. USA) following the manufacturer's protocol, and sequenced by Sanger sequencing (Stab Vida, Portugal). Of an initial DNA sample of 105 paralarvae, five were not successfully sequenced.

Each sequence was compared to GenBank sequences using the BLAST algorithm (Altschul, 2014). For phylogenetic purposes only the sequences with perfectly clean chromatograms and desirable length (minimum 505 bp) were considered (n=88 sequences of a total of n=100). Sequences with poorly aligned positions were manually corrected comparing and compared with reference sequences from GenBank (see Results for accession numbers). In order to detect pseudogenes with stop codons, sequences were translated (Song et al., 2008) using EXPASY translate tool (http://www.expasy.org/).

All paralarval DNA sequences were collapsed into haplotypes with FaBOX (http://users-birc.au.dk/biopv/php/fabox/) and haplotypes were aligned using MUSCLE in MEGA 6.0 (Tamura et al., 2013). In order to infer the taxonomic level of the sequences that showed a BLAST homology below 98%, a phylogenetic tree was built with the following sequences: nine GenBank sequences (Groenenberg et al., 2009; Gebhardt & Knebelsberger, 2015) of sepiolids previously reported in the Eastern Atlantic (*Sepiola affinis* AY293716, *Sepiola*

aurantica AF035708, *Sepietta obscura* AY293723, *Sepiola intermedia* AF035709, *Sepiola rondeleti* AY293720, Sepietta oweniana FJ231297, *Sepiola robusta* AF035711, *Rossia macrosoma* KM517936 and *Stoloteuthis leucoptera* AF000068). In addition, one sequence of *Heteroteuthis dispar* from our reference library and the haplotypes obtained in our samplings were used. Evolutionary analyses were conducted in MEGA6.0. The analysis involved 38 nucleotide sequences and 505 positions. Models were fitted by maximum likelihood (ML) and models with the lowest Bayesian Information Criterion (BIC) scores were chosen. The evolutionary history was inferred by the Neighbour-Joining method (Saitou & Nei, 1987) and the evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei, 1993). The rate variation among sites was modelled with a gamma distribution (shape parameter= 79). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. Phylogenetic confidence in the tree was assessed with 1000 bootstrap replicates (Felsenstein, 1985).

Populations structure and genetic diversity parameters

In order to obtain indications of population structure of the most abundant species (i.e., those with N>5), a final alignment was built with 79 sequences of 505 bp. Variable sites (S), nucleotide diversity (π) and haplotype diversity (Hd), total number of mutations (Eta), Fu's Fs statistic (Fu, 1997) and Tajima's D (Tajima, 1989) values were estimated in DNAsp (Librado & Rozas, 2009). Positive Fu's Fs values indicate deficiency of alleles, due to a recent population bottleneck. Negative Fu's Fs values indicate an excess number of alleles, due to population expansion or genetic hitchhiking. Positive Tajima's D indicates population decreasing (or bottleneck) or over dominant selection at this locus. Negative Tajima's D indicate fewer haplotypes than the number of segregating sites, due to population size increasing or purifying selection at the locus. Nucleotide and haplotype diversity values in Goodall-Copestake et al., (2012) were considered as reference values to assess molecular diversity in our samples. A test of population differentiation among the three years was implemented in Arlequin 3.0, using Fu's Fst as a measure of genetic differentiation (Excoffier et al., 2005). For those sepiolid species with more than 20 individuals and a minimum of three haplotypes, a median joining haplotype network was built (Bandelt et al., 1999) using Nexus software (www.fluxus-engineering.com).

Morphometric measurements and statistical analysis

Due to the damaged conditions of sampled paralarvae and bearing in mind the aim to identify measurements that could readily be taken in the field, we selected a small number of easy-to-measure features. Dorsal mantle length (DML), total length (TL) and eye diameter (ED) were measured on the dorsal side of each sepiolid paralarva. In addition, when present, the left tentacle length (TeL) was also measured (Supplementary Material SM.1). Measurements were taken for every individual (n=105) to the nearest 0.05 mm using a LEICA M205C stereomicroscope and LEICA Application

System image analysis software (Leica Microsystems). Only the species with more than five individuals were considered for morphometric analyses. Different sample sizes from those available for genetic identification were required because some paralarvae were damaged during sampling and fixing. Normality (n=85) was achieved with a Box-Cox transformation of all measurements. Box-Cox transformation is a mathematical procedure to optimize a power transformation. It aims to find a transformation coefficient (λ) that achieves the best approximation to a normal distribution (Box & Cox, 1964). The following λ were used for our variables: DML, -1.325; TL, -1.352; ED, -1.241; TeL, -1.139.

Dependent variables (TL, ED and TeL) were analysed separately by generalised linear models (GLM) with species as a fixed factor and DML as a covariate to control for body size differences. Post-hoc comparisons among species were carried out using least significant difference (LSD) tests.

Species morphometry was evaluated using discriminant analysis (DA) for *S. tridens* (n=29), *S. pfefferi* (n=34) and *R. minor* (n=22). To remove the size effect, linear regression was applied in every variable using DML as the explanatory variable, and the resulting residuals were introduced in the DA. Canonical correlations were calculated to test the relative importance of each function to identify the different sepiolids. The discriminant power of the functions was evaluated with the Wilks's Lambda statistic. The structure matrix was employed to evaluate the importance of each variable in the discriminant functions. Probabilities were calculated according to group sizes. All statistical analyses were performed with IBM SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as means ± standard error of the mean (s.e.m.).

RESULTS

Identification and phylogenetic analysis

A total of 105 paralarvae was captured in the three years of sampling (53 paralarvae in 2012, 33 in 2013 and 19 in 2014) (Table 1), and barcoding revealed seven sepiolid species (seven species in 2012, five in 2013 and six in 2014), six of them identified with sequence similarities to GenBank references above 98% (Fig. 2). In order of abundance, the species found were: *Sepiola pfefferi* (reference sequence: KM517947) with 34 individuals, *Sepiola tridens* (KM517961) with 30, *Rondeletiola minor* (AY293725) with 25 individuals, *Sepiola atlantica* (FJ231317) with 4 individuals, *Sepietta neglecta* (FJ231301) with 2 individuals and *Sepiola ligulata* (AY293717) with 1 individual. The last four paralarval sequences had homologies between 89 - 90% in GenBank, and their taxonomic level was assessed according to their relative position in a phylogenetic tree (Fig. 3). This tree included the haplotypes of the paralarval sepiolids found in the Ría de Vigo (13 *Rondeletiola minor,* 8 *Sepiola pfefferi,* 2 *Sepiola tridens*, 1 *Sepiola atlantica*, 2

191 unknown sepiolid, 1 *Sepietta neglecta*, 1 *Sepiola ligulata*) plus 10 sequences of sepiolids downloaded from GenBank
192 (Fig. 3) and a sequence of *Heteroteuthis dispar* from our own collection. The Neighbour-Joining tree showed the
193 unknown sepiolids within a clade that includes the subfamily Heteroteuthidinae, poorly supported by bootstrap values
194 (Fig. 3).
195
196 **Population structure**
197
198 Haplotype and nucleotide diversity values differed between the three most abundant species: *Rondeletiola minor*
199 presented the largest haplotype diversity, with all the specimens analyzed each year having a different haplotype (Table
200 2). In contrast, *Sepiola tridens* showed only one variable site and a single mutation found only in 2013 in the 29
201 sequences analyzed. *Sepiola pfefferi* presented intermediate values of nucleotide and haplotype diversity. In the three
202 sepiolids, the nucleotide diversity decreased from 2012 to 2014, in proportion with the lower number of paralarvae
203 collected. In all the species, Tajima's D values were negative and not statistically significant. Fu's Fs statistic was
204 negative for all species and statistically significant in *R. minor* and *S. pfefferi*. A detailed analysis of Fu's Fs for each
205 species each year revealed that these differences were only significant in 2012 in *R. minor* (Table 2).
206
207 Only *Sepiola pfefferi* and *Rondeletiola minor* provided enough data for the network analysis (more than 20
208 sequences and 3 haplotypes, Fig. 4). Results for *S. pfefferi* revealed the existence of two main haplotypes: H1, found
209 in all three years and shared by 72.72% of individuals, and H2, a less frequent haplotype sampled only in 2012 in 3
210 individuals. There were 6 haplotypes with one single mutation, which were sampled only once. In *R. minor* the most
211 frequent haplotype (H5) was present in one individual each year. Two other haplotypes were sampled twice in different
212 years (H8 and H9), and the rest of the haplotypes (in total 10) were sampled only once.
213
214 **Morphometric analysis**
215
216 Average DML measurements (in mm) for the most abundant species are shown in Table 1. The biggest paralarvae
217 sampled were those of *Sepiola atlantica* which also showed the largest size range (DML between 2.49 and 9.22 mm)
218 and the smallest were *Rondeletiola minor* (DML between 1.15 and 2.69 mm).
219
220 Generalized linear models, considering DML as a covariate, revealed that TL statistically differed among the
221 three most abundant sepiolids (Table 3). Thus, TL in *Sepiola. pfefferi* was 2.16 % larger than *Sepiola tridens (*LSD
222 *post hoc* test, $p<0.001$) and 4.16 % larger than *Rondeletiola minor* (LSD *post hoc* test, $p<0.001$; Fig. 5). *S. tridens was*
223 2.05 % larger than *R. minor* (LSD *post hoc* test, $p=0.004$). TeL also differed statistically among the three species (Table
224 3): *S. pfefferi* tentacles were 79.78 % larger (LSD *post hoc* test; $p<0.001$) than *S. tridens* and 81.48 % larger than *R.*

minor (LSD *post hoc* test; $p<0.001$). *S. tridens* and *R. minor* tentacles did not statistically differ (LSD *post hoc* test, $p=0.959$; Fig. 5). There were no significant differences among species for ED ($_{F2,81}=1.982$, $p=0.144$).

Discriminant functions are shown in Table 4. Canonical correlation for the first function was 0.364 and it explained 72.6 % of the variance. The canonical correlation of the second function was lower (0.137) and it explained 27.4 % of the variation. Wilks´ Lamba statistic indicated that the first function had higher discriminant power (0.645) than the second function (0.876), but both functions were statistically significant (X^2 =35.551, d.f.= 6, $p<0.001$; X^2 =10.415, d.f.= 2 $p=0.005$, respectively). Discriminant power values for the variables in the first function were: 0.999 for TeL, 0.671 for TL and 0.253 for ED. In the second function, the TL value was 0.726, ED was 0.247, and TeL was -0.034. The discriminant functions allowed us to classify 67.1 % of the individuals correctly: 36.4 % accuracy for *R. minor* (n=22), 94.1 % for *S. pfefferi* (n=34) and 58.6 % for *S. tridens* (n=29) (Table 5). The first function assigned positive values to *S. pfefferi* and negative values to *S. tridens* and *R. minor*. Function 2 separated *R. minor* (negative values) from *S. tridens* and *S. pfefferi* (with positive values). See Supplementary material SM.2 for further details.

DISCUSSION

Genetic barcoding has revealed an unexpected diversity of sepiolid paralarvae in the zooplankton communities of the Ría de Vigo (NW Spain). We successfully identified 6 species of sepiolid paralarvae and detected the existence of an unknown sepiolid, which would have been impossible to identify with traditional methods. The two most abundant sepiolid paralarvae had never been reported in the Ría de Vigo: *Sepiola pfefferi* and *Sepiola tridens*. Population structure analyses revealed that all individuals of the three most abundant species belonged to the same populations, although a different genetic signal was detected in each one. Morphometric analyses of the three most abundant species, based on 4 measurements, allowed us to correctly assign 67.1 % of the individuals, evidencing a substantial degree of overlap in body shape based on the measurements considered.

Our results showed that the most abundant sepiolid species among the paralarvae sampled in the Ría de Vigo was *Sepiola pfefferi*, with individuals captured in all the transects. Previous studies of *S. pfefferi* showed that its distribution extended from the Faeroe Islands and southern Norway to Brittany (France) (Grimpe, 1921; Jereb & Roper, 2005). Thus, this is the first report of *S. pfefferi* at this latitude. The second most abundant species was *Sepiola tridens*, a recently described species frequently confused with *Sepiola atlantica* (Groenenberg et al., 2009; Heij & Goud, 2010) that has never been found inside the Ria de Vigo but was detected in the continental shelf of Galicia (Roura, 2013). Habitat separation between *S. tridens* and *S. atlantica* was reported (Groenenberg et al., 2009; Heij & Goud, 2010), with *S. tridens* inhabiting deeper waters than *S. atlantica*. Our results are consistent with this, although sample sizes were small: only two specimens of *S. tridens* were captured inside the Ría, and the rest were captured in deeper waters.

All specimens of *S. atlantica* (n=4) were found in shallow and inner waters, and their low abundance greatly contrasts with the high abundance of *S. atlantica* adults reported inside of the Ría (Guerra, 1986; Rodrigues et al., 2011). Our results might explain why previous reports (Guerra, 1986) showed great variability in the hectocotylus of *S. atlantica* captured in this area, likely owing to misidentification of other coexisting sepiolids.

The other three species found had been previously reported in Galician coastal waters: *Sepietta neglecta* (Pérez-Gándaras Pedrosa, 1980), *Sepiola ligulata* (Guerra, 1986) and Rondeletiola minor (Pérez-Gándaras Pedrosa, 1980), which was the third most abundant species in our study. The large quantity of *R. minor* captured agrees with Czudaj et al. (2013), who suggested that this species is the most abundant sepiolid along the Atlantic Iberian coast. Previous studies have always captured *R. minor* together with *Sepietta oweniana* (Orsi Relini & Bertuletti, 1989; Jereb et al., 1997; Lefkaditou & Kaspiris, 2005). Interestingly, we did not find any specimen of *S. oweniana* in our surveys, despite adult species had previously been reported in the area (Pérez-Gándaras Pedrosa, 1980; Jereb & Roper, 2005).

The four unknown sepiolids were tentatively assigned to the subfamily Heteroteuthidinae, according to their position in the phylogenetic tree Fig. 3). The closest available sequence from any member of this family previously reported in the Eastern Atlantic belongs to *Heteroteuthis dispar,* but the long genetic distance observed between these unknown sepiolids and the sequence of *H. dispar* (even higher than that observed between *Sepietta* and *Rondeletiola,* Fig. 3) suggests that the unknown sepiolids might not belong to the Heteroteuthidinae. Another *Heteroteuthis* has also been described in the Eastern Atlantic *Heteroteuthis atlantis* by Voss (1955), but this report was based only on two individuals and no genetic data was available for comparison. In fact, other authors (Nesis, 1982) consider *H. atlantis* to be the same species as *H. dispar.* Additional evidence against the inclusion of these unknown sepiolids within the subfamily Heteroteuthidinae is that the genus *Heteroteuthis* is the most oceanic member of sepiolids (Nesis, 1993) with planktonic juveniles and adults found far away from the coast (Lefkaditou et al., 1999). However, these unknown sepiolids were collected in shallow waters. Unfortunately, the lack of many sepiolids in the genetic database, together with the misidentifications present in GenBank (Heij & Goud, 2010), precluded the identification of these sepiolids and further research is needed.

The three most abundant species revealed very different patterns of molecular diversity: *Rondeletiola minor*, despite being the less abundant and with the lowest numbers of sequences analysed, presented the highest values of molecular diversity: haplotype diversity close to 1 and very high nucleotide diversity compared with other marine animals (Goodall-Copestake et al., 2012), thus showing a very high degree of polymorphism. However, the lower paralarval catch in 2013 and 2014 led to a drop in nucleotide diversity to median-low values (Table 1). In contrast, *Sepiola tridens* showed very low haplotype and nucleotide diversities during the period studied. The Ría de Vigo (42ºN) is the southernmost limit of its distribution and this low diversity may be the result of strong environmental selection

293 acting on this species. Finally, *Sepiola pfefferi* presented values of molecular diversity between the other two species,
294 that are intermediate among those typical in marine species (Goodall-Copestake et al., 2012). As in *Rondeletiola minor*,
295 all the molecular diversity values decreased from 2012 to 2014 probably related with the lower abundance of paralarvae
296 collected. The evaluation of the population structure, with both Tajima's D value and the statistic Fu's Fs, led us to
297 reject the hypothesis of constant population sizes (Table 2): negative values indicated that there were an excess number
298 of alleles, (i.e. many mutations at silent sites not contributing to heterozygosity), suggesting either that the populations
299 might be increasing or are being purified at a specific locus (Tajima, 1989; Fu, 1997). Significant values of Fu's Fs
300 were only found for *R. minor* and *S. pfefferi* in 2012, perhaps due to the decrease in the number of paralarvae captured
301 in the following two years.
302
303 The population structure analysis is corroborated by the haplotype networks: *S. pfefferi* presented a clear star-
304 like haplotype network (one main haplotype sampled every year and many single haplotypes modified by only
305 mutation). The central and most frequent haplotype is the ancestral one, and the other haplotypes are mutations from it
306 (Posada & Crandall, 2001). The many low-frequency haplotypes of *R. minor*, together with the high nucleotide diversity,
307 suggest a more recent exchange with other populations. The existence of only two haplotypes (one of them unique) in
308 *Sepiola tride*ns suggest a young population resulting from a recent bottle neck or due the founder effect followed by an
309 expansion from scarce haplotypes. It could be that the species came from the North Sea and all the individuals are
310 descended from this founder. Since this species has recently been discovered (Heij & Goud, 2010) and its habitat
311 distribution has not been studied in detail, this question remains unsolved. However, the hypothesis of increasing
312 population size in all species disagrees with the lower paralarval catch and fewer haplotypes found in 2013 and 2014
313 compared to 2012. Selection pressure towards more appropriate haplotypes and consequently purification at a specific
314 locus could be the best explanation.
315
316 Marine animals with a planktonic stage, usually show low genetic differentiation (Bucklin et al., 1997). Our
317 molecular analysis results of the three most abundant species agree with it and also agree with other studies on
318 cephalopods (loliginids and cuttlefish), that have shown genetic homogeneity over great distances (Shaw et al., 1999;
319 Pérez-Losada et al., 2007). However, no conclusive statements about population genetics of sepiolids can be made
320 because comparisons with individuals captured over greater distances and with adults is necessary.
321
322 The morphometric approach was not very successful for identifying the different sepiolid paralarvae.
323 Although the discriminant analysis showed a promising percentage of correct assignment, above 94%, for *S. pfefferi*,
324 it was considerably less successful for *S. tridens* and *R. minor*, thus showing some degree of overlap in shape. Ideally,
325 more measurements are needed. However, the frequent damage of specimens during capture and body contraction
326 during fixation procedures, make it difficult to identify additional useful measurements or to apply a morphometric

approach. Further research should be attempted with hard parts (i.e. statoliths, stylets or beaks) instead of soft tissues and external morphometries. Another reason for the failure of morphometric discrimination could be the wide range of sizes recorded for each species; ontogenetic changes in body shape within species may mask differences between species. In fact, the wide range of sizes found in almost all planktonic sepiolid paralarvae (except *R. minor* and *S. neglecta*) supports the hypothesis of Roura et al. (2016), that sepiolid paralarvae are retained over the continental shelf and coastal waters during their planktonic stage. In addition, the capture of large-sized juveniles in the water column, together with the burying behaviour observed in recently hatched paralarvae (Rodrigues et al., 2010), suggest a merobenthic lifestyle (Bello & Biagi, 1995; Roura et al., 2016) rather than an holobenthic lifestyle.

Knowledge of biodiversity, ecology and genetic structuring of marine organisms is crucial to understand their responses to changing environments. The Marine Protected Area (Illas Atlánticas de Galicia National Park, PNIAG) studied here, has already been identified as an important habitat for *Octopus vulgaris* juvenile settlement (Guerra et al., 2014) and this work suggests it is also a hotspot for sepiolids. Overall, seven sepiolid species have been identified in the planktonic communities around the PNIAG. This study emphasizes that genetic identification remains the most appropriate tool to identify young stages of wild sepiolids, given that visual and morphometric analyses failed to accurately identify them. The high biodiversity of sepiolids detected in the Ría de Vigo is remarkable and an excellent starting point to keep studying the different strategies followed by these small cephalopods. Further research is needed to understand if the decline in abundance and genetic diversity observed in the last few years (2013 and 2014), was a result of unusual oceanographic conditions or related to other environmental pressures on their habitat.

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Species	2012				2013						2014					Total captured	DML Measurements (mm)
	2	3	4	5	2	3	4	5	SM	NM	2	3	4	5	BS		
Sepiola pfefferi	4	4	7		4	2			6		1	2	1	2	1	34	2.79 ± 0.21 (n=34)
Sepiola tridens	1	1	9	6		3	3	1	1	1		1		2	1	30	2.31 ± 0.22 (n=29)
Rondeletiola minor		2	11	1	1	2	1	1	3			1	1	1		25	1.67 ± 0.065 (n=22)
NA	1		1						1		1				1	5	-
Heteroteuthidinae	1	1							1		1					4	3.07 ± 0.96 (n=4)
Sepiola atlantica	1				2						1					4	5.25 ± 1.61
Sepietta neglecta			1											1		2	2.95± 0.11 (n=2)
Sepiola ligulata				1												1	2.90
Total	8	8	29	8	7	7	4	2	12	1	4	4	2	6	3	105	

Table 1

Species of sepiolids captured in the Ría de Vigo during the three years in all the transects (NM: North Mouth, SM: South Mouth). NA: individuals whose sequencing did not succeed. Heteroteuthidinae, paralarvae with GenBank homologies between 89 - 90%. Dorsal mantle length (DML) measurements (mm) expressed as mean ± standard error are shown for every species.

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
R. minor	2012	9	10	10	9	1	0.052	0.00572	0.00118	-7.090	**0.001**	-1.000	> 0.10
	2013	5	6	6	5	1	0.126	0.00475	0.00105	-2.680	0.064	-1.145	> 0.10
	2014	3	2	2	3	1	0.272	0.00264	0.00088	-1.216	0.229	-	-
	All	17	11	11	13	0.963	0.033	0.00472	0.00077	-9.988	**0.001**	-0.993	> 0.10
S. pfefferi	2012	15	4	4	5	0.629	0.125	0.00181	0.00044	-1.754	0.104	-0.823	> 0.10
	2013	11	3	3	3	0.345	0.172	0.00108	0.00061	-0.537	0.254	-1.599	0.10 > p > 0.05
	2014	7	1	1	2	0.286	0.196	0.00057	0.00039	-0.095	0.367	-1.006	> 0.10
	All	33	7	7	8	0.472	0.011	0.00144	0.00039	-5.236	**0.004**	-1.670	0.10 > p > 0.05
S. tridens	2012	16	0	0	1	-	-	-	-	-	-		
	2013	9	1	1	2	0.222	0.166	0.00044	0.00033	-0.263	0.342	-1.088	> 0.10
	2014	4	0	0	1	-	-	-	-	-			
	All	29	1	1	2	0.069	0.063	0.00014	0.00013	-1.183	0.207	-1.149	> 0.10

Table 2

Analysis of molecular diversity parameters for the more abundant species (*Rondeletiola minor*, *Sepiola pfefferi* and *Sepiola tridens*) with 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 (p <0.05) are in bold type.

Dependent variable	Source of variation	Species	*d.f.*	Estimate ± s.e.m.	*F*	*P*
Total Length	Intercept			0.509±0.008		
	Species	*R. minor*	2, 81	-0.013±0.004	200.853	<0.001
		S. pfefferi		0.014±0.004		
	Dorsal Mantle Length		1, 81	0.273±0.018	242.586	<0.001
Tentacle Length	Intercept			-0.434±0.089		
	Species	*R. minor*	2, 81	-0.002±0.045	60.459	<0.001
		S. pfefferi		0.233±0.038		
	Dorsal Mantle Length		1, 81	1.231±0.185	44.125	<0.001

Table 3

Final models from GLM analysis of the differences among the species in body characters: Total Length and Tentacle Length. All continuous variables in the table are Box-Cox transformed and effects of all explanatory variables were statistically significant.

	Function	
	1	2
TL	2.311	87.287
ED	0.579	3.165
TeL	6.412	-5.959
(Constant)	0.000	0.000

Table 4

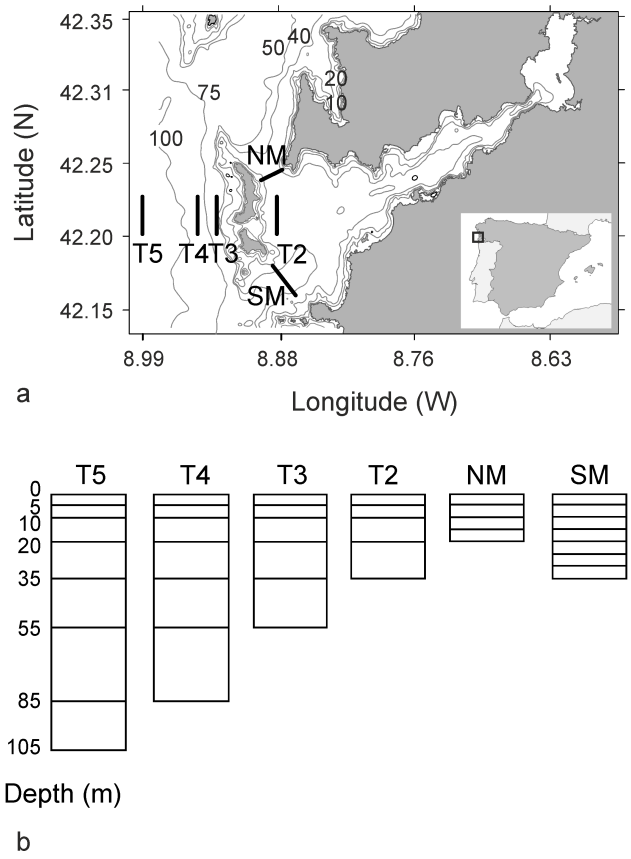
Discriminating variables (Total Length (TL), Eye Diameter (ED) and Tentacle Length (TeL) are Box-Cox transformed) and the coefficients of the two discriminant functions.

	Species	Predicted group			
		R. minor	*S. pfefferi*	*S. tridens*	*Total*
Original group	*R. minor*	8	7	7	22
	S. pfefferi	1	32	1	34
	S. tridens	5	7	17	29

Table 5

Classification results of the discriminant analysis showing the number of specimens in the original groups and the individuals classified in each predicted group. 67.1% of the original cases were correctly classified.

553 **Figures**



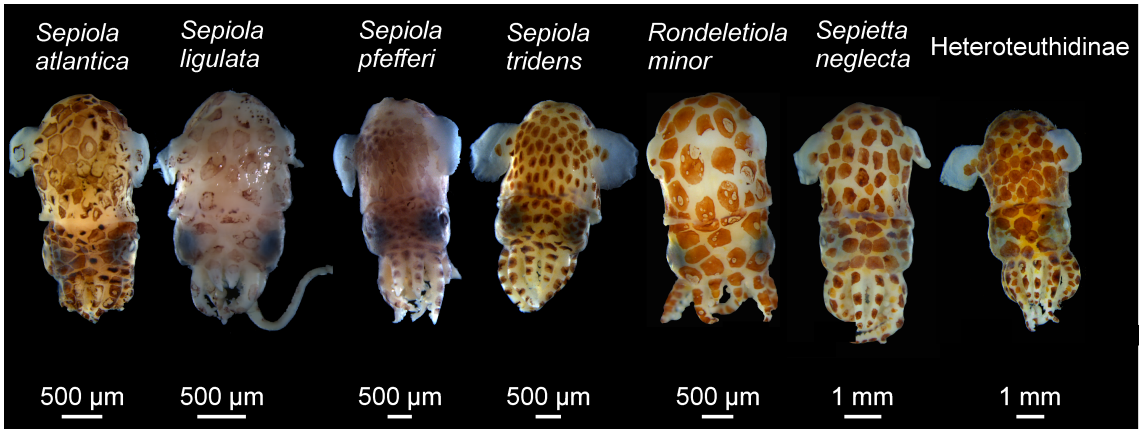
554

555 **Fig. 1**

556 1a) Map of the study area showing the six plankton transects performed in 2012, 2013 and 2014, in locations with

557 bottom depths ranging from 20 to 105 m.

558 1b) Depth layers sampled for each different transect. Within each layer 200 m^3 of water were filtered.



559

560 **Fig. 2**

561 All the sepiolid paralarvae species identified in the surveys.

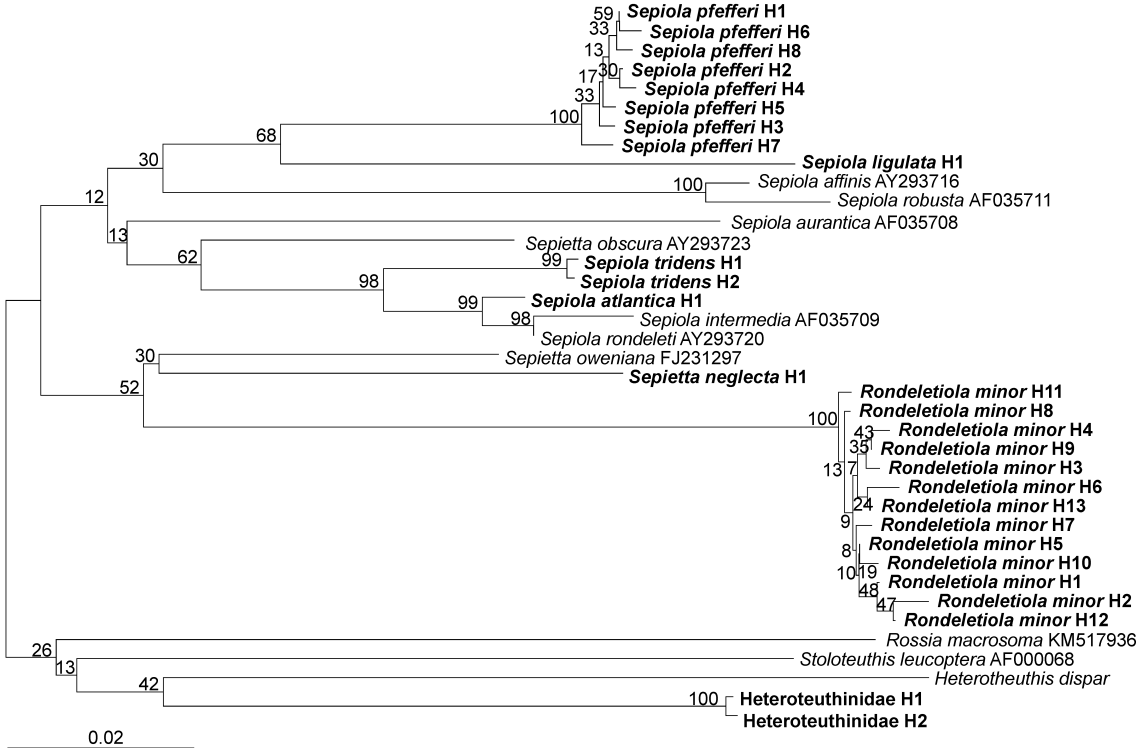
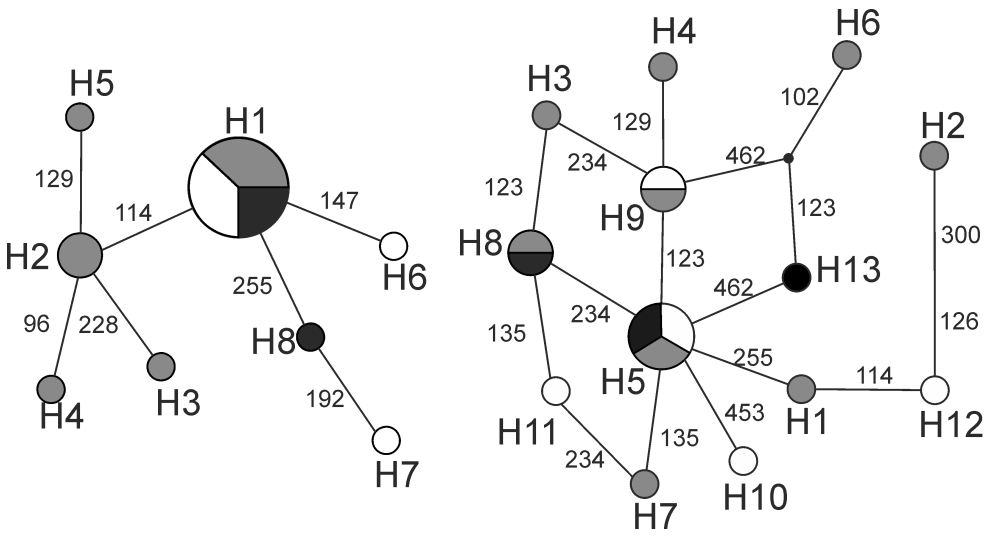


Fig.3

Optimal Neighbour-Joining phylogenetic tree of the haplotypes of the sepiolids paralarvae found in the Ría de Vigo and the GenBank sequences of sepiolids previously described in the North Atlantic area (sum of branch length = 0.918). 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 measured in the number of substitutions per site. Captured paralarvae in our samples are indicated in bold type. Sequences downloaded from GenBank and reference sequences are presented in regular type.



570

571 **Fig.4**

572 Median Joining network based on 505 base pairs of the COI mtDNA fragment for 33 individuals of *Sepiola pfefferi*
573 (left) and 17 individuals of *Rondeletiola minor* (right) collected in the Ría de Vigo during 2012 (grey), 2013 (white),
574 2014 (black). Circle size is proportional to the number of individuals belonging to the same haplotype (H1- H13).
575 Numbers in branches represent the position of the nucleotide substitution between haplotype sequences. The black dot
576 indicates a missing or not sampled haplotype.

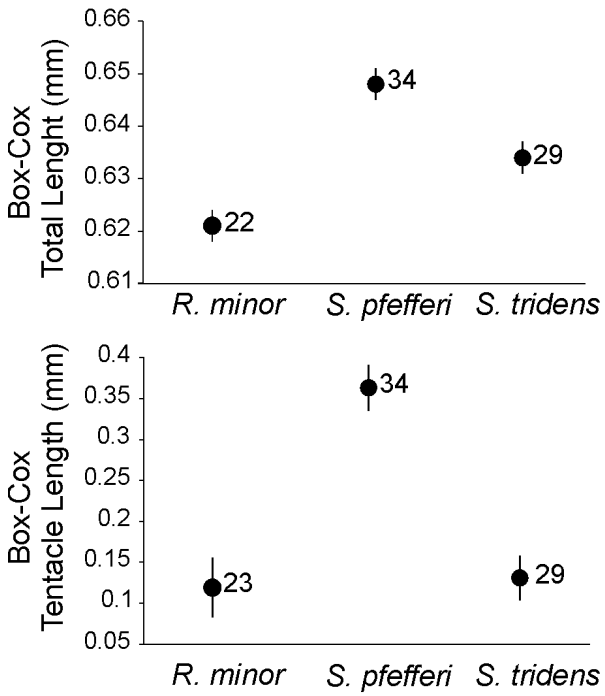
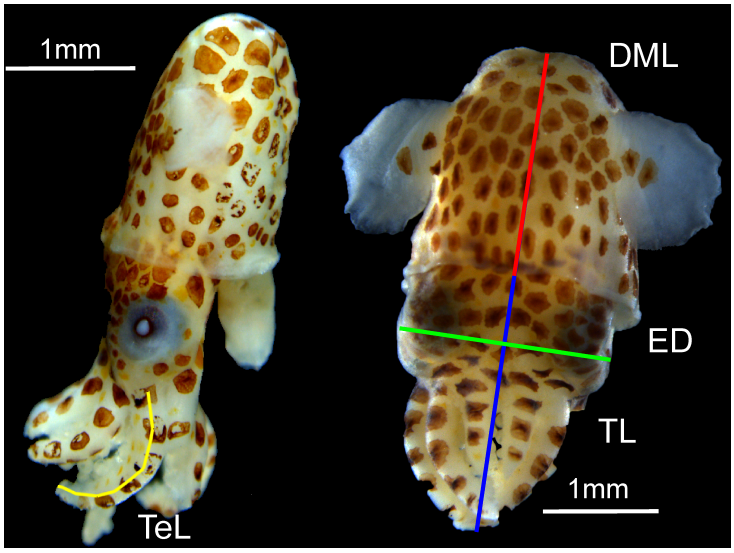


Fig.5

Sizes differences for the measures total length and tentacle length (mm) of the three most abundant species (*Rondeletiola minor*, *Sepiola pfefferi* and *Sepiola tridens*). Both variables are Box-Cox transformed. TL was statistically different among the three species. TeL of *S. pfefferi* significantly differed from *S. tridens* and *R. minor*. Length values are least square means ± s.e.m. Numbers indicate sample size.

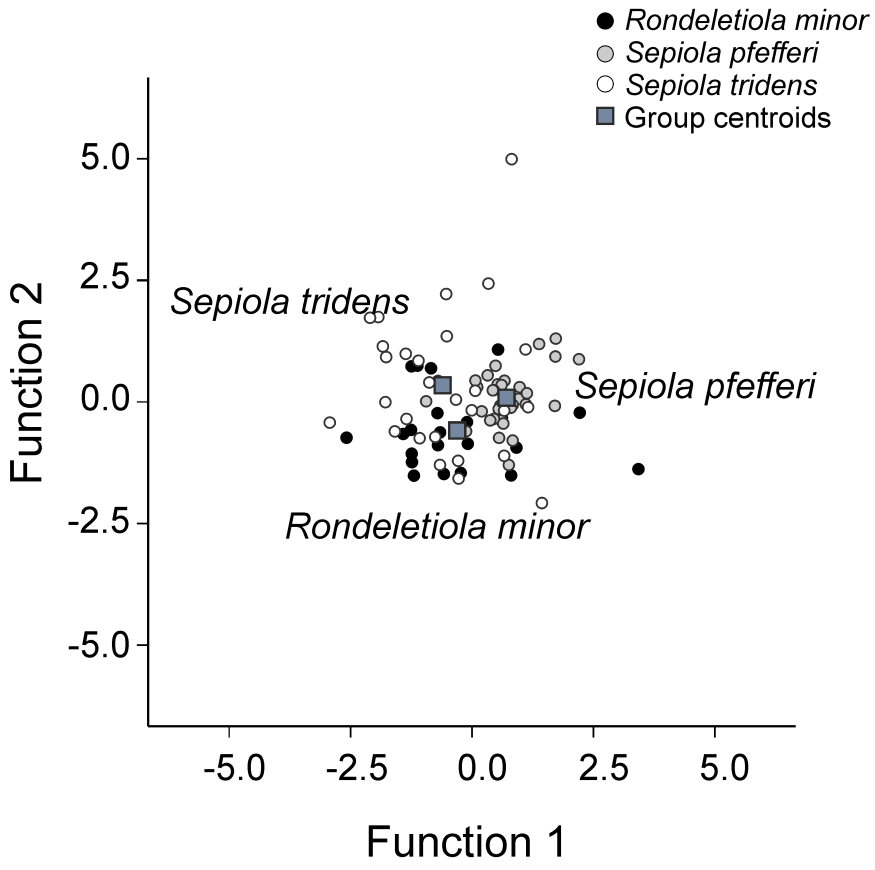


583

584 **Supplementary Material 1**

585 Measures undertaken in all sepiolid paralarvae: dorsal mantle length (DML), total length (TL), eye distance (ED) and

586 tentacle length (TeL).



587

588 **Supplementary Material 2**

589 Graphic representation of the values of the two discriminant equations and the group centroids