Unraveling squid paralarvae diversity off a coastal upwelling ecosystem (Ría de Vigo)

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Summary
Cephalopod paralarvae abundance may be difficult to estimate because their low occurrence in the zooplankton. Moreover, paralarvae alteration during trawl and conservation could hinder their identification to species level. The use of molecular tools based on genetic sequences is a more suitable approach to identify cephalopod paralarvae. Thus, the aim of this work was to identify squid paralarvae (family Loliginidae) with molecular techniques. In summer and early autumn in 2012, we undertook ten oceanographic surveys in the Ria de Vigo (Galicia, Spain) with a multinet trawl gear. A total of 129 loliginid paralarvae were captured, DNA was extracted and a fragment of cytochrome oxidase subunit I (COI) gen was amplified. The results showed 59 *Alloteuthis media*, 32 *A. subulata*, 13 *Loligo vulgaris* and 25 samples that did not amplify COI gen. Our results show that loliginid paralarvae diversity have been underestimated off the Ría de Vigo, and highlights the integration of molecular techniques with morphological studies for a more realistic estimation of squid paralarvae population.

Introduction
The planktonic stage of neritic cephalopods, known as paralarvae, remains as the most uncertain part of their life cycle since they are scarce in the plankton and, after collection, discriminating among species is not accurate because morphological identification relies in characters present only in alive paralarvae, which partially dissapear in preserved individuals (Vecchione 1986). Several surveys were undertaken off the Ría de Vigo to estimate the distribution of cephalopod paralarvae (González et al. 2010; Otero et al. 2008; Rocha et al. 1999; Roura 2013) and the most exhaustive work about loliginid paralarvae (González et al. 2010), considered all the loliginid found as *Loligo vulgaris* based on morphological characters. In recent years, DNA-based identification (barcoding) has revealed as an alternative approach for paralarvae identification, uncovering higher cephalopod paralarvae diversity than expected using morphological features (Roura 2013). Understanding the early life ecology of cephalopod and their real abundance is of vital importance for fishery management. Consequently, the aim of this research is to identify with molecular tools the loliginid paralarvae found off the Ría de Vigo.

Material and methods
Ten zooplankton samplings were carried out in the Ría de Vigo (NW Spain) in summer and early autumn 2012. Zooplankton collection was undertaken onboard the R/V "Mytilus" in four parallel transect following a bathymetric gradient (40, 60, 80, 110 m) with a multinet trawl gear of 200 μm mesh size. Samples were fixed on board with 96% ethanol to allow DNA preservation. Loliginid paralarvae were sorted and classified. Mantle was separated and DNA extracted. Universal primers HCO and LCO (Folmer et al 1994) were employed to amplify a 710-bp fragment of the mitochondrial COI gene. When electrophoresis did not show an appropriate band, a touch-down PCR was done to amplify a shorter DNA region of 313 bp of the mitochondrial COI gene with primers mlCOIintF and jgHCO2198 (Leray et al. 2013). Positive PCR were sequenced and all sequences were assessed for similarity using BLAST (Basic Local Alignment Search Tool) against the GenBank Database.

Results and Discussion
Overall, 129 loliginid paralarvae were found off the Ría de Vigo. A total of 91 paralarvae successfully amplified with universal primers; 13 samples that did not yield bands with the universal primers, successfully amplified the shorter fragment; 17 samples did not amplify and 8 samples were

completely dry hampering DNA extraction. The molecular identification showed that 59 paralarvae were *Alloteuthis media*, 32 *A. subulata* and 13 *Loligo vulgaris* (Figure 1).

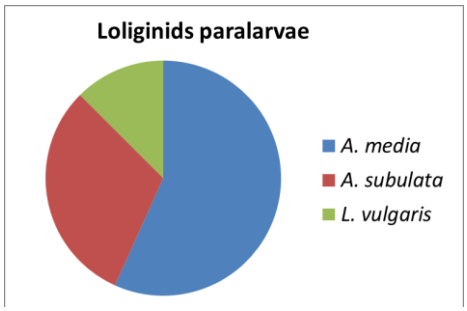

Figure 1. Loliginid paralarvae diversity revealed by genetic barcoding off Ría de Vigo.

Our results agree with the diversity of squid paralarvae found by Roura (2013) in the Galician and Portuguese shelf, and reveal the dominance of *Alloteuthis* species in the coastal environment off the Ria de Vigo. These results suggest that the real diversity of loliginids have been underestimated in previous studies that relied on morphological characters (González et al. 2005, 2010; Moreno et al. 2009), where loliginid paralarvae were mostly considered as *Loligo vulgaris.*

Our finding contrasts with the squid landings in our region, which are mainly represented by *L. vulgaris*. Moreover, eggs masses from *L. vulgaris* have been found previously (Guerra and Rocha 1994), but eggs masses from *A. media* or *A. subulata* have never been reported in our area (Guerra pers.com.). Nevertheless, the low abundance of *L. vulgaris* paralarvae found herein could be attributed to biological conditions: spawning of *L. vulgaris* is more intensive from December to April, so the highest paralarvae abundance should be in May-June (González et al. 2010; Moreno et al. 2009). Another possibility would be that *L. vulgaris* is changing its distribution area, as previously happened with *Loligo forbesi* (Chen et al. 2006; Guerra et al. 2004).

This work highlights the importance of molecular tools to identify loliginid paralarvae. Our results evidence that the squid biodiversity has been underestimated and further efforts should be done to unravel the ecology of the different squids found off the Ría de Vigo. Such knowledge will then be used to develop specific models for each squid species to understand how environmental conditions affect their planktonic stages and its repercussion to fishery landings and management.

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