Molecular prey identification in wild *Octopus vulgaris* paralarvae

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Abstract

The trophic ecology of *Octopus vulgaris* paralarvae collected in 2008 off the Ría de Vigo, NW Spain (42°12.80'N–9°00.00'W) was approached by both morphological and molecular methods. External digestion of prey and posterior suction of the liquefied contents by wild *O. vulgaris* paralarvae made the morphological identification of gut contents impossible. Thus, a PCR-based method using group specific primers was selected to identify prey consumed by *O. vulgaris* paralarvae in the pelagic realm. The mitochondrial ribosomal 16S gene region was chosen for designing group specific primers, which targeted a broad range of crustaceans and fishes but avoided the amplification of predator DNA. These primers successfully amplified DNA of prey by using a semi-nested PCR-based approach and posterior cloning. Homology search and phylogenetic analysis were then conducted with the 20 different operational taxonomic units (OTUs) obtained to identify the putative organisms ingested. The phylogenetic analysis clustered ingested prey into 12 families of crustaceans (11 belonging to the order Decapoda and 1 to the order Euphausiacea) and two families of fishes (Gobiidae and Carangidae). According to the Czekanowski's Index (CI), the trophic niche breadth of *O. vulgaris* paralarvae is low (CI=0.13), which means that these paralarvae are specialist predators at least during the first weeks of their life cycle. It is the first time that natural prey has been identified in *O. vulgaris* paralarvae collected from the wild and such knowledge may be critical to increasing the survival of *O. vulgaris* hatchlings in captivity, a goal that has been actively pursued since the 1960's by aquaculture researchers.

Key words: *Octopus vulgaris*, paralarval diet, group specific primers, gut content, PCR, trophic ecology, aquaculture.

Introduction

Dietary analysis in cephalopods is hampered by problems arising from the anatomy, physiology and mode of ingestion (Rodhouse and Nigmatullin 1996) of these organisms. The oesophagus diameter is limited physically as it passes through the brain, so the cephalopod beak bites small pieces of tissue to swallow. Rapid digestion rates in the stomach result in short residence times (two to six hours) making the prey remains visually unidentifiable (Altman and Nixon 1970; Andrews and Tansey 1983; Nixon 1985). The mode of prey ingestion can be internal, by biting with the beak, or external, where salivary enzymes paralyse and digest the flesh followed by the ingestion of the liquefied content (Nixon 1984; Guerra and Nixon 1987). These specialised feeding strategies largely avoid the ingestion of hard skeletal material and tend to bias data on both prey species and size when morphological analysis are used (Nixon 1985).

Cephalopods are known to be highly versatile predators with opportunistic predation behaviours (reviewed in Rodhouse and Nigmatullin 1996). While numerous works have focused on the trophic role of adults (Nixon 1987; Boyle et al. 1996; Rasero et al. 1996; Rodhouse and Nigmatullin 1996), the knowledge of diet in wild paralarvae is scarce due to the small size of this life history stage. The few attempts made to clarify the diet showed that paralarvae are mainly generalist feeders preying primarily on crustaceans, as observed by visual analysis by Passarella and Hopkins (1991) and Vecchione (1991). Further visual analysis made by Vidal and Haimovici (1998) showed that 11.4% of ommastrephid squid paralarvae contained copepod appendages. Additionally, Venter et al. (1999) developed an inmunoassay that detected copepods, euphausiaciids and polichaetes in the gut of six *Loligo reynaudii* paralarvae.

While some squid and cuttlefish paralarvae preying on pelagic crustaceans ingest exoskeleton pieces, thus allowing morphological analysis (Vecchione 1991; Passarella and Hopkins 1991; Vidal and Haimovici 1998); the external digestion exhibited in octopod paralarvae hatchlings rejects the entire crustacean zoeae exoskeleton therefore preventing morphological analysis of the dietary items (Hernández-García et al. 2000). Occasionally, the presence of thoracic appendages has been observed in the stomach of *Octopus vulgaris* hatchlings fed on *Artemia* under laboratory conditions, because *Artemia* has a thinner exoskeleton than other crustacean zoeae (Iglesias et al. 2006).

Octopus vulgaris is a generalist predator as both a juvenile and an adult, feeding upon a variety of organisms mainly within the class Crustacea, but also Gastropoda, Lamellibranchiata, Osteichthyes, Ophiuroidea, Polychaeta and Cephalopoda (Nigmatullin and Ostapenko 1976; Guerra 1978; Smale and Buchan 1981; Nixon 1987; Mather 1991). The industrial rearing of this octopus species has been hampered by the high mortality during the pelagic stage, despite the broad range of experimental diets

assayed throughout the past sixty years (reviewed in Iglesias et al. 2007). Although some authors have hypothesised that *O. vulgaris* prey upon crustaceans during the planktonic stage (Mangold and Boletzky 1973; Nixon 1985; Rodhouse and Nigmatullin 1996; Villanueva and Norman 2008), the feeding habits of wild *O. vulgaris* paralarvae are still unknown.

The trophic ecology of *Octopus vulgaris* paralarvae was tackled using both morphological and molecular methods, which have been shown to provide a comprehensive understanding of both invertebrate and vertebrate diets (Casper et al. 2007; Deagle et al. 2007, 2010; Braley et al. 2010). Given that *Artemia* was successfully detected in a single *O. vulgaris* paralarvae reared in laboratory by using species specific primers (Roura et al. 2010), the next step involved developing a molecular technique to detect the natural prey of wild paralarvae. This approach requires *a priori* knowledge of the fauna that coexist with paralarvae in the zooplankton. Hence, ten surveys were undertaken in the Ría de Vigo, a region of coastal upwelling off NW Spain (Otero et al. 2009), to obtain wild paralarvae as well as relative abundances of the different zooplankton species present in the area. Due to the enormous variety of suitable prey species in the zooplankton community; neither the species specific primer approach (King et al. 2008) nor the serological methods (Boyle et al. 1986, Venter et al. 1999) would be practical to identify prey. Therefore, we developed a technique to amplify small, multi-copy DNA fragments with universal primers for the 16S rRNA gene (Simon et al. 1994) in conjunction with group specific primers, designed within this gene, that anneal to short target templates of potential prey items (Deagle et al. 2005, 2007, 2009, 2010; Braley et al. 2010). The group specific primers were designed to amplify a wide range of crustaceans and fishes, likely the most suitable prey of wild *O. vulgaris* paralarvae, based upon reports that the feeding habits of cephalopods shift from crustacean feeders during early stages (Vecchione 1991; Vidal and Haimovici 1998; Venter et al. 1999) towards piscivory in juvenile and adult stages (Passarella and Hopkins 1991; Rasero et al. 1996).

The aim of this work was to identify natural prey of *Octopus vulgaris* paralarvae collected in the wild, using both morphological and molecular methods. Additionally, trophic selectivity of the paralarvae was addressed by comparing the composition of the zooplankton community they inhabit with the prey detected, under the assumption that cephalopod paralarvae are generalist predators. This molecular method is also immediately transferable to other oceanographic predator/prey scenarios as well as to other dietary studies on cephalopod paralarvae.

Methods

Sample collection, morphologic analysis and DNA extraction

Ten surveys to collect zooplankton and hydrographical data were undertaken at night during July and September-October 2008 in the Ría de Vigo, NW Spain (42º12.80'N–09º00.00'W) onboard RV "Mytilus". Biological sampling consisted of four transects as in González et al. (2005); three located outside the Cies Islands and one inside the Ría de Vigo (T2, T3, T4 and T5) parallel to the coast following an onshore-offshore depth gradient with an average depth of 26, 68, 85 and 110m, respectively. On each transect two double oblique trawls were deployed, one at the surface and one near the bottom, using a 75-cm diameter bongo net equipped with 375-µm mesh and a current meter. Zooplankton samples were fixed onboard with 96% ethanol and stored at -20ºC. In the laboratory, cephalopod paralarvae were separated and classified according to Sweeney et al. (1992) and our own reference collections. Zooplankton composition and abundance was estimated by Roura et al. (unpublished).

Morphological analyses of the gut contents were carried out from two batches of eighteen randomly selected *Octopus vulgaris* paralarvae, following two different procedures. In the first batch, the digestive tracts were removed and gut contents were distributed in water on a microscope slide and then examined under an inverted microscope at 100x to 400x magnification (Nikon Eclipse TS100) as in Passarella and Hopkins (1991). The second batch was prepared for routine histological analysis by staining with haematoxylin-eosin and examined under a microscope at 100x to 400x magnification (Nikon Eclipse 80i).

Genetic analysis was carried out with eighteen *O. vulgaris* paralarvae randomly sorted that were preserved in 70% ethanol at -20ºC. To avoid potential contaminants from the body surface before DNA extraction, individual paralarvae were washed with sterile distilled water, which was recovered and used as a negative control (Suzuki et al. 2006). Paralarvae were then dissected and their digestive system was removed and placed into DNA-free tubes. All dissections were performed in a UV-sterilized laminar flow hood with flame-sterilized dissection tools to avoid contamination. Gut and content DNA was extracted with a QIAamp DNA Micro Kit (QIAGEN), using RNA carrier in buffer AL. All steps followed manufacturer's instructions, with the exception of the 56ºC digestion step which was done overnight and the final elution step was done in two steps using 15 µl buffer AE in each elution.

Group specific primer design

Group specific primers were designed by obtaining 16S rRNA sequences from GenBank (Benson et al. 2002) corresponding to 30 taxonomically diverse crustaceans, 3 fishes, 2 echinoderms and 2 cephalopods

(one of them *Octopus vulgaris*) which are known to be present in the NE Atlantic Ocean (Table 5, supplementary material). These sequences were then aligned with MAFFT (Katoh et al. 2002). The software AMPLICON (Jarman 2004) was used to identify conserved regions within the target group of potential prey species, but with nucleotide mismatches at the 3' end of the *O. vulgaris* forward primer sequence to prevent its amplification (Deagle et al. 2007). Group specific primer specificity was tested by PCR using a gradient between 49ºC and 60ºC on known template DNA from across the Crustacea (the euphausiacid *Nyctiphanes couchii*, the crab *Necora puber*, the squat lobster *Galathea strigosa*, the hermit crab *Anapagurus laevis*, the prawn *Palaemon longirostris*, the mysid *Leptomysis gracilis* and the copepod *Calanus helgolandicus*), Chaetognata (*Sagitta elegans*) and *O. vulgaris*.

Genetic database of planktonic organisms from the Ría de Vigo

To ensure the correct identification of sequences obtained from the gut of *Octopus vulgaris* paralarvae, mtDNA16S sequences were obtained from 25 species of crustaceans collected in the zooplankton sampling done in the Ría de Vigo (Table 2). One individual of each species was visually identified, washed with distilled water to remove surface contaminants and DNA was extracted with the QIAamp DNA Micro Kit (QIAGEN), eluting the DNA in ultrapure water.

Due to difficulties amplifying crustacean 16S rRNA, PCR products were generated with different combinations of the universal primers 16Sar-16Sbr (Simon et al. 1994) and the designed group specific primers 16Scruf-16Scrur (Table 2). Copepod specific primers 16Sca and 16Scb (Braga et al. 1999) were needed to amplify a region that is nested in the 16S rRNA universal fragment and encompasses the sequence amplified with the designed group specific primers. Cycling conditions for the primers 16Sar-16Scrur and 16Scruf-16Sbr, consisted of an initial denaturation at 94ºC for 2 min followed by 39 cycles of: denaturation at 94ºC for 30 s, annealing at 57ºC for 35 s, extension at 72ºC for 40 s and a final step of 7 min at 72ºC. Cycling conditions for copepod primers 16Sca-16Scb consisted of an initial denaturation at 94ºC for 2 min followed by 38 cycles of: denaturation at 94ºC for 60 s, annealing at 50ºC for 60 s, extension at 72ºC for 60 s and a final step of 7 min at 72ºC.

All reactions were carried out in 25 µL, containing 10-100 ng of template 2.5 µL 10X PCR reaction buffer, 0.5 µL dNTPs, 0.75 µL each primer and 0.025 U μL^{-1} Taq polymerase (Roche). PCR amplifications were carried out in a TGradient thermocycler (Biometra). Aerosol resistant pipette tips were used to set up all PCR reactions. Negative controls, extraction controls and distilled water were included for each set of PCR amplifications. An aliquot of 1.5 µL from each PCR reaction was quantified

using Nanodrop 2000 spectrophotometer (Thermo Scientific), then electrophoresed on 1.75% agarose gel, stained with RedSafe™ (iNtRON biotechnology) and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories).

PCR products were purified with Exo-SAP (USB, Affymetrix) and sequencing reactions were carried out with an automated DNA sequencer (Applied Biosystems 3130), using the BigDyeTerminator V3.1 Cycle Sequencing Kit (Applied Biosystems) with forward primers. Chromatograms were examined using BioEdit Sequence Alignment Editor version 7.0.9 (Ibis Biosciences). All sequences were assessed for similarity using BLAST (Basic Local Alignment Search Tool) and were submitted to GenBank (Accession numbers in Table 2)

Identification of prey: semi nested PCR and cloning.

Two sets of semi-nested PCR amplifications were performed independently on the extracted DNA from the digestive tract of each *Octopus vulgaris* paralarvae (Fig. 1). In both sets, the first PCR was carried out with the universal primer 16Sar plus a reverse group specific primer (16Scrur for crustaceans/fishes and 16Scb for copepods) to increase the copies of prey DNA. The second PCR was carried out using 1 µL of the first PCR as a template, with forward and reverse group specific primers for crustaceans/fishes and copepods to amplify only prey DNA.

Cycling conditions for the primers 16Scruf-16Scrur consisted of an initial denaturation at 94ºC for 2 min followed by 33 cycles of: denaturation at 94ºC for 30 s, annealing at 57ºC for 35 s, extension at 72ºC for 40 s and a final step of 7 min at 72ºC. Cycling conditions for primers 16Sar-16Scb and subsequent 16Sca-16Scb as described above.

All reactions were carried out in 25 µL, containing 50 ng of template the first PCR and the semi nested with 1 µL from the product of the first PCR 2.5 µL 10X PCR reaction buffer, 0.5 µL dNTPs, 0.3 µL $MgCl_2$, 0.5 µL each primer and 0.05 U μL^{-1} Taq polymerase (Roche).

Semi-nested PCR products from the digestive tract of the *Octopus vulgaris* paralarvae obtained with group specific primers (16Scruf-16Scrur) and copepod-specific primers (16Sca-16Scb) were ligated to a pCR 4-TOPO plasmid vector for 15 min at room temperature and cloned using TOPO TA Cloning kit (Invitrogen) with One Shot TOP10 chemically competent cells following the manufacturer's protocol. Plasmids were extracted from 10 colonies, when possible, with the Quick Plasmid Miniprep Kit (Invitrogen). Insert size was checked by PCR with universal vector specific T7 and T3 primers and

visualised by gel electrophoresis. Sequencing was carried out on 200 ng of plasmid DNA using primer T7.

Sequences recovered from clone libraries were edited and were considered to be part of the same "operational taxonomic unit" (OTU) if there was less than 1% sequence divergence, allowing for intra-specific variation and *Taq* polymerase errors (Braley et al. 2010). OTUs were compared to sequences found in GenBank using the BLAST algorithm. A phylogenetic tree was constructed to assign unknown sequences to the highest taxonomic level and to verify the OTU identifications. The tree contained all OTUs obtained from *Octopus vulgaris* with primers 16Scruf-16Scrur, together with the five closest matches of each OTU that were downloaded from GenBank. These sequences were aligned using MAFFT v5.7 (Katoh et al. 2002) with default settings. A substitution model was selected under the Akaike information criterion corrected for short sequences (AICc, Akaike 1974) as implemented in jModeltest (Posada 2008). The HKY + γ (Hasegawa et al. 1985) model was chosen to infer the evolutionary history by using the Maximum Likelihood (ML) method. The analysis involved 79 nucleotide sequences with a total of 164 positions in the final dataset. Bootstrap probabilities with 1000 replications were calculated to assess reliability on each node of the ML tree. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). If sequence similarity displayed in the BLAST was <98%, identification for the OTUs was restricted to the highest taxonomic lineage supported by bootstrap probabilities higher than 70% in the consensus tree.

Thophic niche breadth was calculated using Czekanowski's Index (CI) with the formula:

$$CI = 1 - 0.5 \Sigma_i | p_i - q_i |$$

where *pi* is the proportion of resource item *i* out of all items eaten by the paralarvae, and *qi* is the proportion of item *i* in the zooplankton available to the paralarvae (Feinsinger et al. 1981). Values for CI range from 1 for the broadest possible niche (a population uses resources in proportion to their availability) to [min *qi*] for the narrowest possible niche (a population is specialized exclusively on the rarest resource).

Results

Octopus vulgaris paralarvae and morphological analysis of the digestive tracts

All specimens used for morphological and genetic analysis were early hatchlings of less than 10 days according to the size (1.28-2.05 mm dorsal mantle length) and that each paralarva had 3 suckers per arm

(Villanueva 1995). Visual identification of the gut contents was inconclusive, because no solid remains were found. Histological sections made to the digestive tract also revealed empty digestive tracts (Fig. 2a) with the exception of two stomachs which were filled with liquefied material that was impossible to identify (Fig. 2b).

Group specific primers and genetic database

PCR tests using the designed group specific primers yielded a target band of the expected fragment size in all the crustaceans and chaetognat tested. However, copepods yielded only faint bands that did not correspond to copepod DNA when sequenced, so we decided to use the copepod specific primers (Braga et al. 1999) in conjunction with the designed group specific primers for dietary analysis and for submissions to the genetic database. No PCR products were obtained at any annealing temperature when *Octopus vulgaris* DNA was used as template. All sequences obtained from the zooplankton collected from the Ría de Vigo were submitted to GenBank (Accession numbers in Table 2).

Identification of preys in paralarvae by cloning

All octopus digestive tracts yielded amplifiable DNA when PCR was performed with the designed group specific primers 16Scruf-16Scrur. Although we intended to sequence 10 colonies per larvae, some samples did not yield the minimum number of colonies (Table 3). Overall, a total of 122 clones were sequenced, and 115 readable sequences were obtained. All sequences corresponded to prey species, with 114 clones corresponding to the semi-nested PCR band (16Scruf-16Scrur) and 1 clone corresponding to the first PCR (16Sar-16Scruf) identified as *Trachurus trachurus* (OTU 19, Table 3).

Cloning of the amplicons obtained with copepod specific primers 16Sca-16Scb in *Octopus vulgaris* gut contents resulted in 135 colonies, but all the sequences obtained from 125 readable clones corresponded to *O. vulgaris* except one that amplified the DNA of *Anapagurus laevis* (OTU 13, Table 3).

Prey detected consisted of 20 different OTUs with between 1 and 5 different OTUs per paralarvae (Table 3). Eight OTUs were assigned to species with 78 clones displaying 100% similarity, and 1 clone displaying 98% similarity to sequences from GenBank. Six OTUs showed similarities higher than 90% (13 clones), three were assigned to genus (94-95%), two to a subfamily (Gobiinae, 93 and 92%) and the last one to a family (Goneplacidae, 90%). The remaining four OTUs, corresponding to 22 clones, displayed between 76-81% similarities and were assigned to the familial level on the basis of their supported topographical position on the bootstrap consensus tree (Table 3, Fig. 3).

Summarizing, prey detected in *Octopus vulgaris* consisted mainly of crustaceans which accounted for 97.4% of the clones detected and the remaining 2.6% corresponded to fishes (Table 4). Three taxa accounted for 95% of the clones; prawns (37.1%), crabs (37.1%) and krill (19.8%). When considering the importance of these groups in the diet of *O. vulgaris*, it is remarkable that prawns and crabs are the most common prey species, detected in 14 and 12 paralarvae out of 18 respectively (Table 4). In spite of the high number of krill clones, these corresponded to only three paralarvae. The rest of the taxa were detected in only three paralarvae, or in just one in the case of the Thalassinidae. According to the CI the trophic niche breadth is low (0.13) indicating that *O. vulgaris* paralarvae are specialist predators. All OTUs were submitted to GenBank, accession numbers in Table 3.

Discussion

This is the first time that prey items have been identified in *Octopus vulgaris* paralarvae collected in the wild. This was approached by using two morphological techniques; visual analysis of the digestive tracts and histological sections, as well as one molecular technique using group specific primers. Although the combined approach of morphological and molecular methods has been documented as a more comprehensive way to understand the diet of both vertebrates and invertebrates (Casper et al. 2007; Deagle et al. 2007, 2010; Braley et al. 2010), only the molecular method succeeded identifying prey in *O. vulgaris* paralarvae. The small size of the paralarvae, the limitation of the oesophagus diameter, the high digestion rates, and the external digestion (Nixon 1985; Parra et al. 2000; Hernández-García et al. 2000), made it impossible to carry out morphological analyses of prey in *O. vulgaris* paralarvae during their first days of life in the pelagic realm.

The advantage of molecular methods is that when morphological methods were ineffective, i.e. digestive tract is empty or filled with unidentifiable remains, prey cells with sufficient DNA to be detected by PCR are able to be recovered (King et al. 2008). The main obstacle in employing molecular techniques in small animals is distinguishing prey DNA among the overall volume of host DNA (Symondson 2002). To overcome this obstacle we designed group specific primers within the 16S rRNA region for crustaceans and fishes, which selectively avoided amplification of *Octopus vulgaris* DNA. Other studies previously used this region of the 16S rRNA to design group specific primers for dietary purposes (Deagle et al. 2005, 2007, 2009; Braley et al. 2010). Braley et al. (2010) designed a reverse group specific primer for crustaceans used in conjunction with the universal 16Sar, but only 11 of 184 PCR attempts produced

successful amplifications of krill and shrimp. In contrast, the group specific primers designed in this study effectively amplified DNA, both alone and in conjunction with the universal 16Sar-16Sbr, from a wide range of crustacean taxa: cladocerans, crabs, prawns, thalassinids, krill, hermit crabs, porcellanids, carideans (Palaemonidae, Crangonidae and Alpheidae), mysids as well as fishes.

The unexpected failure to amplify copepod DNA is a potential consequence of using group specific primers (Jarman et al. 2004; Deagle et al. 2005, 2007; Braley et al. 2010), which have been designed to exclude from amplification *Octopus vulgaris* DNA. For this reason PCR had to be run with the copepod specific primers 16Sca -16Scb (Braga et al. 1999) both in copepods and octopus paralarvae. These primers effectively amplified copepod DNA for the genetic library (Table 2), however failed to amplify copepod DNA from the digestive tract of *O. vulgaris* paralarvae. This suggests that early hatchlings of *O. vulgaris* do not eat copepods, despite their presence as one of the main zooplankton taxa (table 4) and being the most common prey in previous studies undertaken with other cephalopod paralarvae (Passarella and Hopkins 1991; Vecchione 1991; Vidal and Haimovici 1998; Venter et al. 1999). Nonetheless, the erratic movements and the extremely fast escape responses that copepods display (Yen and Fields 1992) potentially pose a challenge for the early *O. vulgaris* hatchlings when compared with the predictable swimming behaviour of crab and prawn zoeae or krill calyptopis. Indeed, Chen et al. (1996) found in *Loligo opalescens* paralarvae that copepod capture is a skill acquired in an experience-dependent manner during the post-hatchling stage.

In the current study, seven OTUs (29 clones) could not be identified to species or genus because no similar sequences were present in GenBank. Phylogenetic relatedness was used to assign the unidentified sequences to the highest taxonomic lineage based on the bootstrap values of the consensus tree nodes. This reflects the difficulty when working with the diet of an expected generalist predator, due to the limited sequence information available to target the large diversity of potential prey taxa (Blankenship and Yayanos 2005; Suzuki et al. 2006, 2008). A prerequisite for resolving the diet of any predator living in such a complex environment is the extensive characterization of the system (Sheppard et al. 2005; King et al. 2008). In this work, five sequences that were submitted to GenBank from zooplankton species found in the Ría de Vigo, were detected in the gut of the paralarvae, which highlights the importance of an appropriate genetic database to obtain the highest level of identification and to reduce the uncertainty of any species identification.

While previous work on cephalopod paralarvae diet found that paralarvae are generalist predators, prey species detected in early hatchlings of *Octopus vulgaris* suggest that they are actually specialist predators according to the CI obtained (0.13). Among the crustaceans, the group that primarily contribute to the total abundance of zooplankton in the Ría de Vigo are krill, or Euphausiacea, which were only detected in three paralarvae (Table 4). By contrast, all the paralarvae analysed ate some Decapoda, which include Brachyura (crabs), Caridea (shrimps), Anomura (hermit crabs) and Thalassinidea (mud shrimps), despite their much smaller contribution to the total abundance of zooplankton which was less than 4.26% (Table 4). In fact, the trophic selection is quite evident for carideans, which were the most abundant prey present in 14 out of 18 *O. vulgaris* paralarvae, but whose contribution to the total zooplankton abundance was only 0.28%.

The specialist trophic strategy during the first days in the pelagic ecosystem could be a consequence of a lack of skills to capture fast moving and more abundant prey, as proved in paralarvae of *Loligo opalescens* (Chen et al. 1996). As it occurs in the former species, an ontogenic switch into a generalist predation strategy would be expected as the *Octopus vulgaris* paralarvae grow and gain experience, but further research is needed to test this hypothesis. On the other hand, if paralarvae were truly specialists throughout the planktonic phase, this might explain the high mortality of *O. vulgaris* hatchlings both under culture and in the wild, due to prolonged starvation periods (Vecchione 1991).

In conclusion, up to 20 prey species have been detected in *Octopus vulgaris* paralarvae obtained from the wild with a PCR-based method. This is the first successful attempt to unravel the complex trophic interactions that occur in the pelagic ecosystem for *O. vulgaris* paralarvae. Based on the prey species detected and their relative abundances in the zooplankton, *O. vulgaris* paralarvae can be considered specialist predators during their first days of life in the pelagic ecosystem. Such knowledge can be critical to solving the primary problems associated with the integral culture of this species, which is the low survival of the paralarvae likely due to inadequacy of food supplied (Iglesias et al. 2007). Further effort will progress in this direction to enhance the knowledge of this species during its planktonic phase.

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References

Akaike H (1974) A new look at the statistical model identification. IEEE Trans Automat Contr 19:716-723

Altman JS, Nixon M (1970) Use of beaks and radula by *Octopus vulgaris* in feeding. J Zool Lond 161:25-38

Andrews PLR, Tansey EM (1983) The digestive tract of *Octopus vulgaris*:the anatomy, physiology and pharmacology of the upper tract. J Mar Biol Assoc UK 63:109-135

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA, Wheeler DL (2002) GenBank. Nucleic Acids Res 30:17-20

Blankenship LE, Yayanos AA (2005) Universal primers and PCR of gut contents to study marine invertebrate diets. Mol Ecol 14:891-899

Boletzky SV (2003) Biology and early life stages in cephalopod molluscs. Adv Mar Biol 44:144-202

Boucher-Rodoni R, Boucaud-Camou E, Mangold K (1987) Feeding and digestion. In: Boyle PR (ed) Cephalopod Life Cycles, vol. 2, Comparative reviews, Academic Press, London, pp 85-108

Boyle PR, Grisley MS, Robertson G (1986) Crustacea in the diet of *Eledone cirrhosa* (Mollusca: Cephalopoda) determined by serological methods. J Mar Biol Assoc UK 66: 867-879

Braga E, Zardoya R, Meyer A, Yen J (1999) Mitochondrial and nuclear rRNA based copepod phylogeny with emphasis on the Euchaetidae (Calanoida). Mar Biol 133:79-90

Braley M, Goldsworthy S, Page B, Steer M, Austin JJ (2010) Assessing morphological and DNA-based diet analysis techniques in a generalist predator, the arrow squid *Nototodarus gouldi*. Mol Eco Res 10:466-474

Casper R, Jarman S, Gales N, Hindell M (2007) Combining DNA and morphological analyses of faecal samples improves insight into trophic interactions: a case study using a generalist predator Mar Biol 152:815-825

Chen DS, VanDykhuizen G, Hodge J, Gilly WF (1996) Ontogeny of copepod predation in juvenile squid (*Loligo opalescens*) Biol Bull 190:69-81

Deagle BE, Jarman SN, Pemberton D, Gales NJ (2005) Genetic screening for prey in the gut contents from a giant squid (*Architeuthis sp*). J Hered 96:417–423

Deagle BE, Gales NJ, Evans K, Jarman SN, Robinson S, Trebilco R, Hindell M (2007) Studying seabird diet through genetic analysis of faeces: a case study on macaroni penguins (*Eudyptes chrysolophus*). *PLoS ONE* 2, e831

Deagle BE, Kirkwood R, Jarman SN (2009) Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Mol Ecol 18:2022–2038

Deagle B, Chiaradia A, McInnes J, Jarman S (2010) Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? Cons Genet 11:2039-2048

Feinsinger P, Spears EE, Poole RW (1981) A simple measure of niche breadth. Ecology 62:27–12

González AF, Otero J, Guerra A, Prego R, Rocha FJ, Dale AW (2005) Distribution of common octopus and common squid paralarvae in a wind-driven upwelling area (Ría de Vigo, northwestern Spain). J Plankton Res 27:271–277

Guerra A (1978) Sobre la alimentación y el comportamiento alimentario de *Octopus vulgaris*. Inv Pesq 42:351–364

Guerra A and Nixon M (1987) Crabs and mollusc shells drilling by *Octopus vulgaris* (Mollusca: Cephalopoda) in the Ría de Vigo (NW Spain). J Zool 211:515-523

Hasegawa M, Kishino H, Yano T (1985) Dating the human-ape split by a molecular clock of mitochondrial DNA. J Mol Evol 22:160-174

Hernández-García V, Martín AY, Castro JJ (2000) Evidence of external digestion of crustaceans in *Octopus vulgaris* paralarvae. J Mar Biol Ass UK 80:559–560

Iglesias J, Fuentes L, Sánchez J, Otero JJ, Moxica C, Lago MJ (2006). First feeding of *Octopus vulgaris* Cuvier 1797 paralarvae using *Artemia*: Effect of prey size, prey density and feeding frequency. Aquaculture 261:817-822

Iglesias J, Sánchez FJ, Bersano JGF, Carrasco JF, Dhont J, Fuentes L, Linares F, Muñoz JL, Okumura S, Roo J, Meeren T van der, Vidal EAG, Villanueva R (2007) Rearing of *Octopus vulgaris* paralarvae: Present status, bottlenecks and trends. Aquaculture 266:1-15

387 Itami K, Izawa Y, Maeda S, Nakai K (1963) Notes on the laboratory culture of the octopus larvae. Bull
388 Jap Soc Sci Fish 29:514-520
389 Jarman S (2004) AMPLICON: Software for Designing PCR Primers on Aligned DNA Sequences.
390 Bioinformatics 20:1644-1645
391 Jarman S, Deagle B, Gales NJ (2004) Group specific polymerase chain reaction for DNA-based analysis
392 of species diversity and identity in dietary samples. Mol Ecol 13:1313-1322
393 Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence
394 alignment based on fast Fourier transform. Nucleic Acids Res 30:3059-3066
395 King RA, Read DS, Traugott M, Symondson WOC (2008) Molecular analysis of predation: a review of
396 best practice for best DNA-based approaches. Mol Ecol 17:947-963
397 Mather JA (1991) Foraging, feeding and prey remains in middens of juvenile *Octopus vulgaris*
398 (Mollusca: Cephalopoda). J Zool Lond 224:27-39
399 Nigmatullin CM, Ostapenko AA (1976) Feeding of *Octopus vulgaris* Lam. from the northwest Africa
400 coast. ICES CM 1–15
401 Nixon M (1984) Is there external digestion by *Octopus*? J Zool Lond 202:441-447.
402 Nixon M (1985) Capture of prey, diet and feeding of *Sepia officinalis* and *Octopus vulgaris* (Mollusca:
403 Cephalopoda) from hatchling to adult. Vie Milieu 35:255-261
404 Nixon M (1987) Cephalopods diet. In: Boyle PR (ed) Cephalopod Life Cycles, vol. 2, Comparative
405 reviews, Academic Press, London, pp 201-219
406 Otero J, Álvarez-Salgado XA, González AF, Gilcoto M, Guerra A (2009) Influence of high-frequency
407 coastal upwelling events on *Octopus vulgaris* larval dynamics in the NW Iberian shelf. Mar Ecol
408 Prog Ser 386:123-132
409 Parra G, Villanueva R, Yufera M (2000) Respiration rates in late eggs and early hatchlings of the
410 common octopus, *Octopus vulgaris*. J Mar Biol Ass UK 80:557–558
411 Passarella KC and Hopkins TL (1991) Species composition and food habits of the micronektonic
412 cephalopod assemblage in the eastern Gulf of Mexico. Bull Mar Sci 49:638–659
413 Posada D (2008) jModelTest: Phylogenetic Model Averaging. Mol Biol Evol 25:1253-1256
414 Rasero M, González AF, Castro BG, Guerra A (1996) Predatory relationships of two sympatric
415 ommastrephids species *Todaropsis eblanae* and *Illex coindetii* (Mollusca, Cephalopoda) off
416 Galician Waters (NW Spain). J Mar Biol Assoc UK 76:73-87

417 Rodhouse PG, Nigmatullin CM (1996) Role as consumers. Phil Trans R Soc Lond B 351:1003-1022
418 Roura A, González AF, Pascual S, Guerra A (2010) A molecular approach to identifying the prey of
419 cephalopod paralarvae. ICES J Mar Sci 67:1408-1412
420 Sheppard SK, Harwood JD (2005) Advances in molecular ecology: tracking trophic links through
421 predator-prey foodwebs. Funct Ecol 19:751–762
422 Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and
423 phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase
424 chain reaction primers. Ann Entomol Soc Am 87:651-701
425 Smale MJ, Buchan PR (1981) Biology of *Octopus vulgaris* off the east coast of South Africa. Mar Biol
426 65:1–12
427 Suzuki N, Murakami K, Takeyama H, Chow S (2006) Molecular attempt to identify prey organisms of
428 lobster phyllosoma larvae. Fish Sci 72:342-349
429 Suzuki N, Hoshino K, Murakami K, Takeyama H, Chow S (2008) Molecular diet analysis of phyllosoma
430 larvae of the japanese spiny lobster *Panulirus japonicus* (Decapoda: Crustacea). Mar Biotech
431 10:49-55
432 Sweeney MJ, Roper CFE, Mangold K, Clarke MR, Boletzky SV (1992) 'Larval' and juvenile
433 cephalopods: a manual for their identification. Smithsonian Contributions to Zoology
434 Symondson W (2002) Molecular identification of prey in predator diets. Mol Ecol 11:627-641
435 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5: Molecular
436 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum
437 parsimony methods. Mol Biol Evol 28:2731-2739
438 Vecchione M (1991) A method for examining the structure and contents of the digestive tract in
439 paralarvae squids. Bull Mar Sci 49:300-308
440 Venter JD, Wyngaardt Sv, Verschoor JA (1999) Detection of zooplankton prey in squid paralarvae with
441 Inmunoassay. J Inmunoassay 20:127-149
442 Vidal EAG, Haimovici M (1998) Feeding and the possible role of the proboscis and mucus cover in the
443 ingestion of microorganisms by rhynchoteuthion paralarvae (Cephalopoda: Ommastrephidae). Bull
444 Mar Sci 63:305–316
445 Villanueva R (1995) Experimental rearing and growth of planktonic *Octopus vulgaris* from hatching to
446 settlement. Can J Fish Aquat Sci 52:2639–2650

Villanueva R, Norman M (2008) Biology of the planktonic stages of benthic octopuses. Oceanogr Mar Biol Annu Rev 46:105-202

Yen J, Fields DM (1992) Escape responses of *Acartia hudsonica* (Copepoda) nauplii from the flow field of *Temora longicornis* (Copepoda). Arch Hydrobiol Beih 36:123-134

Fig.1 Diagram of the two semi nested PCR undertaken on each paralarvae, showing the prey targeted and the primers used on each PCR

Fig.2 Histological sections of *Octopus vulgaris* paralarvae stained with haematoxylin-eosin showing (a) an empty stomach and (b) a stomach filled with undefined material (*) impossible to recognise. Abbreviations, br: brain; di gl: digestive gland; oe: oesophagus; ra: radula; st: stomach; su: sucker. Scale bars 100 nm.

Fig.3 Maximum Likelihood tree for affiliating 18 operational taxonomic units (OTUs) obtained from the digestive tract *Octopus vulgaris* paralarvae. OTUs obtained from the digestive tract are shown in bold. Eukaryote rRNA sequences obtained by the BLAST searches are in italics with accession numbers. Only bootstrap probabilities higher than 60 after 1000 replications are shown in the branches

Figure 1
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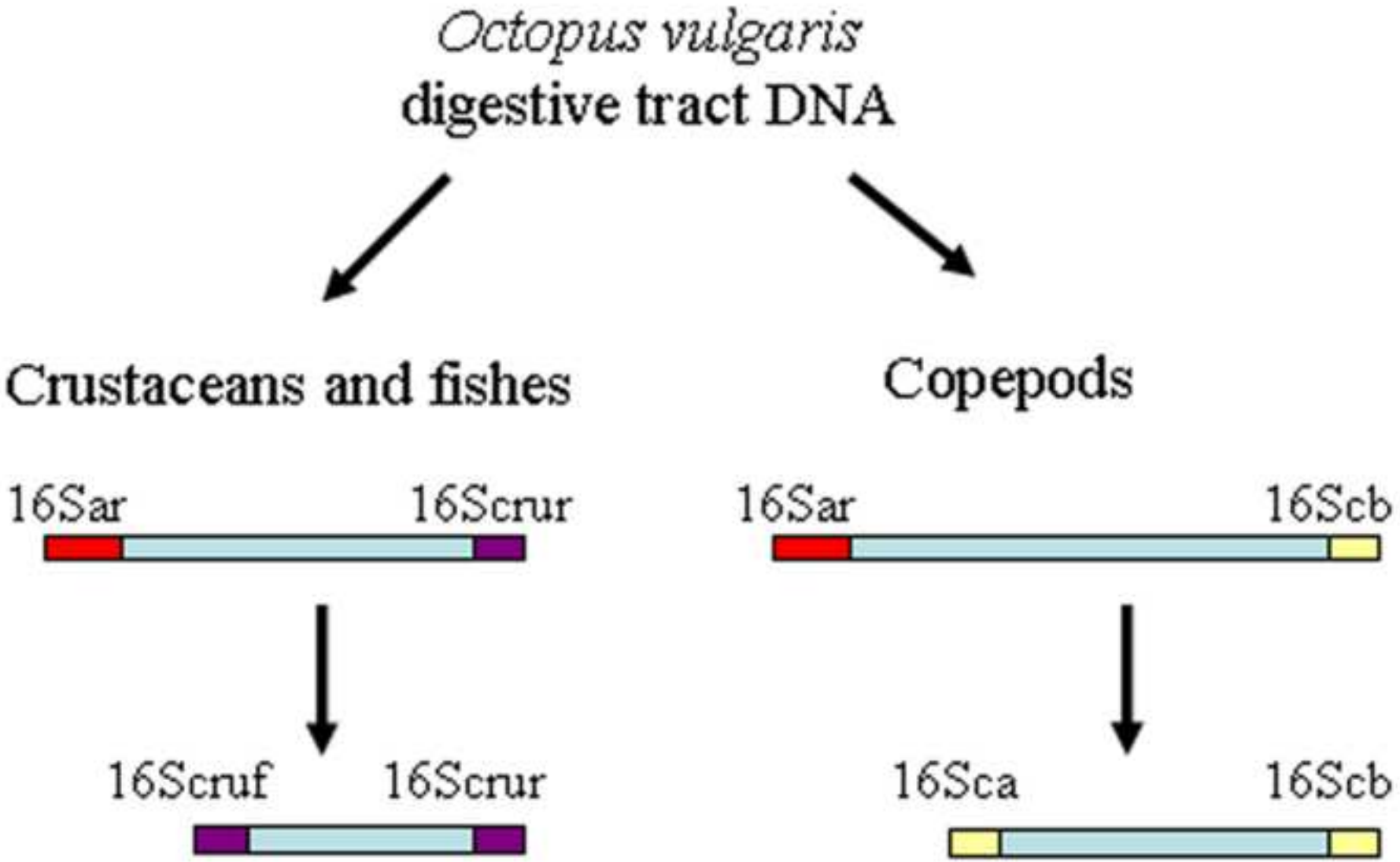

Figure 2
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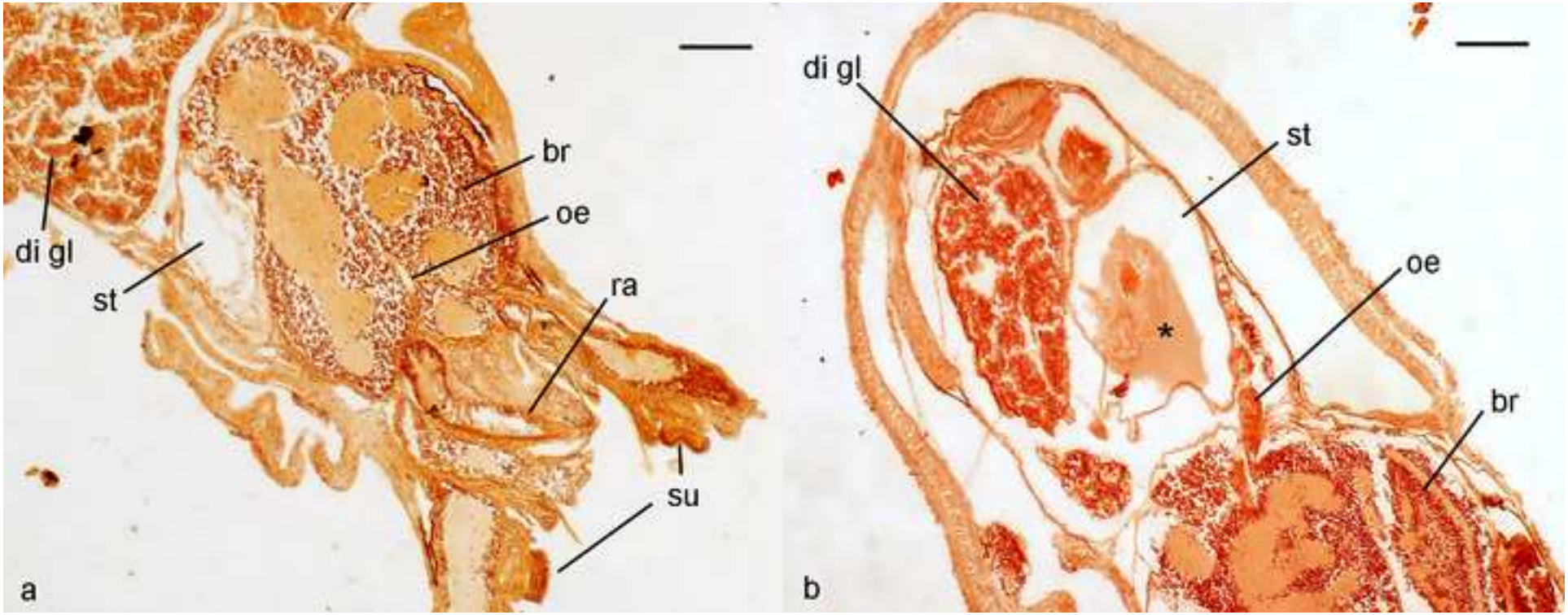

Figure 3

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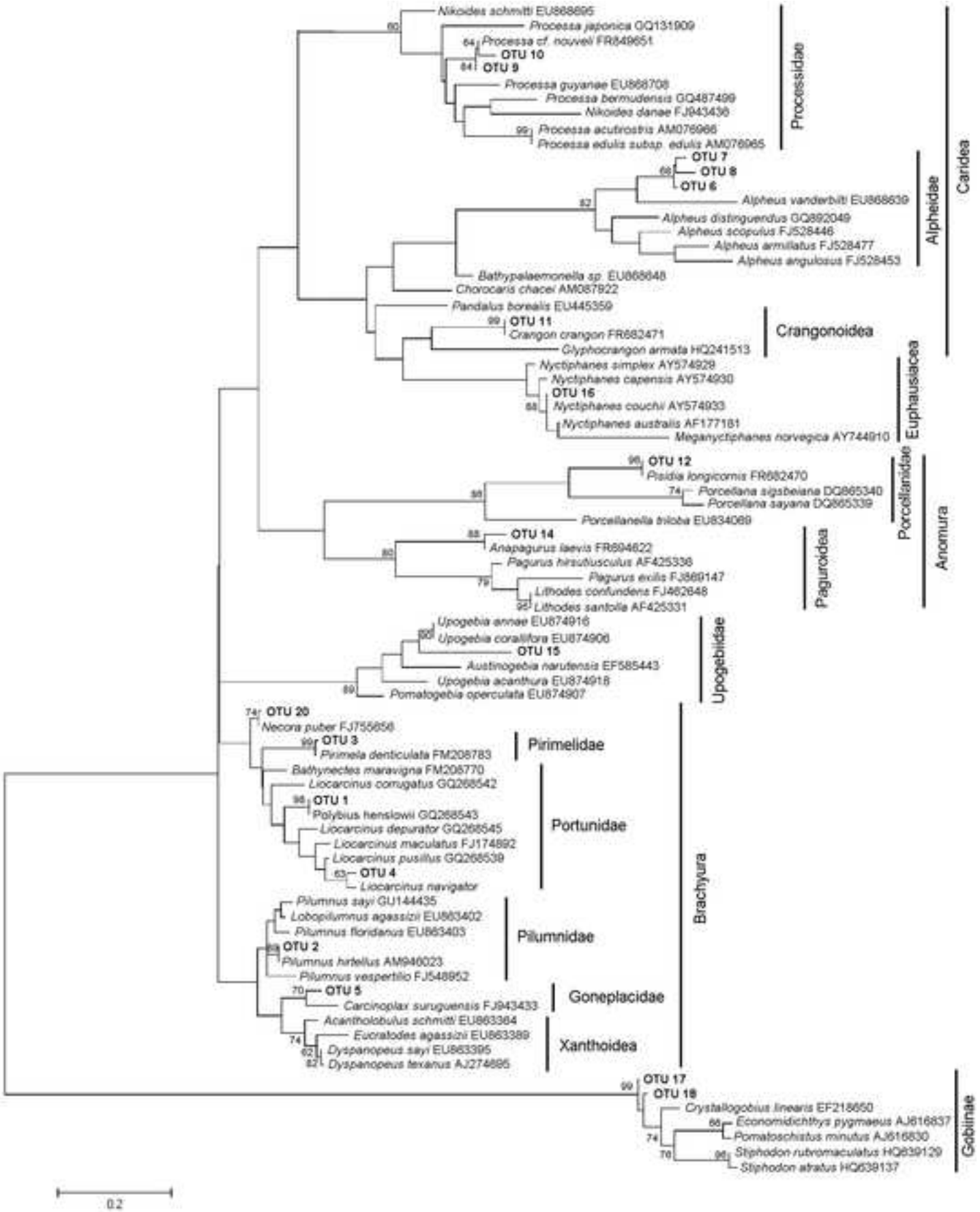

Table 1. Primers used in the current study showing the sequence of forward and reverse primers, the annealing temperature of each primer and the sizes of the amplified PCR products.

Target taxon	Forward primer (5´- 3´)	Reverse primer (5´- 3´)	Annealing Temperature	Product size (bp)
Universal	16Sar CGCCTGTTTATCAAAAACAT	16Sbr CCGGTCTGAACTCAGATCACGT	50 ºC	550-620
Eucarida	16Scruf GACGATAAGACCCTATAA	16Scrur CGCTGTTATCCCTAAAGTAA	57 ºC	194-204
Copepod	16Sca TGTTAAGGTAGCATAGTAAT	16Scb ATTCAACATCGAGGTCACAA	50 ºC	356-387

Table 2. List of species sequenced to create a 16S rRNA library of zooplankton present in the Ría de Vigo including GenBank Accession numbers, size of PCR amplicons in base pairs and PCR primers used to amplify each species.

Accession number	Species	Taxon	Length (bp)	Primer set	Homology (%)
FR851238	*Jaxea nocturna*	Thalassinidae	361	*16Sar-16Scrur*	99
FR851240	*Callianasa subterranea*	Thalassinidae	365	*16Sar-16Scrur*	99
FR851239	*Podon intermedius*	Cladocera	357	*16Sar-16Scrur*	99
FR682469	*Nyctiphanes couchii*	Euphausiacea	356	*16Sar-16Scrur*	99
FR849634	*Galathea strigosa*	Galatheidae	338	*16Sar-16Scrur*	
FR682470	*Pisidia longicornis*	Porcellanidae	380	*16Sar-16Scrur*	
FR849633	*Solenocera membranacea*	Penaeidae	367	*16Sar-16Scrur*	
FR682471	*Crangon crangon*	Crangonidae	371	*16Sar-16Scrur*	
FR694622	*Anapagurus laevis*	Paguridae	363	*16Sar-16Scrur*	
FR849637	*Cestopagurus timidus*	Paguridae	276	*16Scruf-16Sbr*	
FR849651	*Processa cf. nouveli*	Processidae	170	*16scruf-16Scrur*	
FR849636	*Leptomysis gracilis*	Mysidacea	198	*16Scruf-16Sbr*	
FR849648	*Calanus helgolandicus*	Copepoda	349	*16Sca-16Scb*	99
FR849642	*Calanoides carinatus*	Copepoda	346	*16Sca-16Scb*	
FR849638	*Mesocalanus tenuicornis*	Copepoda	341	*16Sca-16Scb*	
FR849639	*Paraeuchaeta hebes*	Copepoda	340	*16Sca-16Scb*	
FR849643	*Paracalanus parvus*	Copepoda	365	*16Sca-16Scb*	
FR849645	*Pseudocalanus elongatus*	Copepoda	275	*16Sca-16Scb*	
FR849646	*Metridia lucens*	Copepoda	372	*16Sca-16Scb*	99
FR849641	*Pleuromamma gracilis*	Copepoda	329	*16Sca-16Scb*	
FR849650	*Diaixis pygmaea*	Copepoda	206	*16Sar-16Scb*	
FR849649	*Acartia clausii*	Copepoda	323	*16Sca-16Scb*	96
FR849634	*Clausocalanus sp.*	Copepoda	284	*16Sca-16Scb*	
FR849640	*Oithona sp.*	Copepoda	397	*16Sca-16Scb*	
FR849647	*Candacia armata*	Copepoda	350	*16Sca-16Scb*	

Table 3

Table 3. Prey DNA (OTUs 1-20) detected in the eighteen *Octopus vulgaris* paralarvae (Oc1 to Oc18) by cloning the PCR products obtained with group specific primers (16Scruf-16Scrur), including closest matches, their GenBank Accession numbers and percentages of similarity obtained from BLAST.

OTU*	Taxon	Species	Ac. number	(%)	Oc 1	Oc 2	Oc 3	Oc 4	Oc 5	Oc 6	Oc 7	Oc 8	Oc 9	Oc 10	Oc 11	Oc 12	Oc 13	Oc 14	Oc 15	Oc 16	Oc 17	Oc 18
OTU 1	Brachyura	*Polybius henslowii*	DQ388059	100				6				2	1	1								
OTU 2	Brachyura	*Pilumnus hirtellus*	AM946023	100					3		3		2								1	8
OTU 3	Brachyura	*Pirimela denticulata*	FM208783	100		3																
OTU 20	Brachyura	*Necora puber*	FJ755656	100																4		
OTU 4	Brachyura	*Liocarcinus sp.*	GQ268541	95														4				
OTU 5	Brachyura	Goneplacidae	FJ943433	90	5																	
OTU 6	Caridea	Alpheidae 1	FJ528488	80		2						2						3	1			
OTU 7	Caridea	Alpheidae 2	DQ682879	79	1		3							1								
OTU 8	Caridea	Alpheidae 3	DQ682895	76					1			1	1					3	2			
OTU 9	Caridea	*Processa nouveli*	FR849651	100			1			1			1	3	1	3	9				1	
OTU 10	Caridea	*Processa sp.*	FR849651	94									1									
OTU 11	Caridea	*Crangon crangon*	FR682471	100					1													
OTU 12	Anomura	*Pisidia longicornis*	FR682470	98																1		
OTU 13[a]	Anomura	*Anapagurus laevis*	FR694622	98		1																
OTU 14	Anomura	*Anapagurus sp.*	FR684622	94										1								
OTU 15	Thalassinidea	Upogebiidae	EU874916	81			1															
OTU 16	Euphausiacea	*Nyctiphanes couchii*	AY574933	100											9	7			7			
OTU 17	Teleostei	Gobiinae	EF218650	93													1					
OTU 18	Teleostei	Gobiinae	EF218650	92																		1
OTU 19[b]	Teleostei	*Trachurus trachurus*	AB096007	99																	1	
		Trachurus japonicus	AP003092	99																		

*Each Operational Taxonomic Unit (OTU) has been submitted to GenBank, accession numbers: FR849614-849632 and HE586322. [a] Obtained with primers 16Sca-16Scb. [b] Obtained with primers 16Sar-16Scrur

Table 4. Composition of the zooplankton community during the study expressed as the percentage of each taxon to the total abundance and the diet in *Octopus vulgaris* paralarvae by the number and percentage of clones corresponding to a given taxon and the number of paralarvae where those taxa were detected.

Phyla	Taxon	Wild Zooplankton Abundance (%)	Clones detected and percentage (%)	Number of paralarvae
Crustacea	Euphausiacea	27.8765	23 (19.8)	3
Echinodermata	Ofiuroidea	20.3526		
Crustacea	Copepoda	19.0708		
Chordata	Thaliacea	15.2601		
Crustacea	Cirripeda	3.9272		
Chaetognatha	Sagittidae	2.7184		
Crustacea	Cladocera	2.2304		
Crustacea	Anomura	2.1644	3 (2.6)	3
Crustacea	Brachyura	1.8174	43 (37.1)	12
Cnidaria	Cnidaria	1.5349		
Echinodermata	Equinoidea	1.2949		
Mollusca	Gastropoda	0.8575		
Crustacea	Caridea	0.2777	43 (37.1)	14
Chordata	Teleostei	0.2518	3 (2.6)	3
Crustacea	Misidacea	0.2352		
Crustacea	Amphipoda	0.0297		
Platemintha	Turbellaria	0.0215		
Annelida	Polychaeta	0.0203		
Mollusca	Bivalvia	0.0144		
Briozoa	Ciphonaute	0.0126		
Crustacea	Cumacea	0.0088		
Crustacea	Thalassinoidea	0.0084	1 (0.9)	1
Crustacea	Stomatopoda	0.0068		
Crustacea	Dendrobranchiata	0.0030		
Crustacea	Isopoda	0.0018		
Mollusca	Cephalopoda	0.0016		
Cephalochordata	Branchiostomidae	0.0009		
Crustacea	Ostracoda	0.0007		

Table 5

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