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6 **Cryptic species and colonization processes in *Ophryotrocha* (Annelida,**
7 **Dorvilleidae) inhabiting vertebrate remains the shallow-water Mediterranean**

Abstract

Taboada, S., Leiva, C., Bas, M., Schult, N. & McHugh D. (2016). Cryptic species and colonization processes in *Ophryotrocha* (Annelida, Dorvilleidae) inhabiting vertebrate remains the shallow-water Mediterranean. *Zoologica Scripta*, 00, 000000.

Over the past several years, there has been growing interest in how bones of decaying mammals are colonized in the marine seabed. One of the most common opportunistic taxa occurring worldwide on bones are dorvilleid polychaetes of the genus *Ophryotrocha*. In a recent study in the Mediterranean, *Ophryotrocha puerilis* and *Ophryotrocha alborana* were two of the most abundant species occurring in experimentally deployed bones. These species have direct development and this make them a suitable model to study the mechanisms and processes allowing organisms lacking a dispersive larval phase to colonize new substrates. Here we address the colonization processes at the molecular level for the populations of *O. puerilis* and *O. alborana* on experimentally deployed mammal bones in the shallow-water Mediterranean collected over a year at 3-month intervals. High genetic distances between some of the *O. puerilis* organisms collected, indicated the occurrence of at least two cryptic sibling species (*O. puerilis* 'Shallow' and *O. puerilis* 'Deep') apart from *O. puerilis sensu stricto*. This was corroborated after phylogenetic analyses using an alignment of three concatenated genes (*COI, 16S, H3*) and after implementing species delimitation analyses using *COI*. The haplotype network inferred from *COI* sequences for *O. puerilis* 'Shallow', identified a few common haplotypes shared between the two trimesters analyzed and several other less represented haplotypes only present in one trimester. Thus, colonization of these experimental bones may have been achieved by a few organisms that arrived to the bones and were able to reseed, and by several individuals arriving to the experimental bones and not persisting across time. Contrastingly, *O. alborana* haplotype network revealed that none of the haplotypes present in three different trimesters were shared, suggesting that the populations arriving at the bones during each trimester were totally replaced by new individuals during the subsequent trimesters. Our study suggests that different species of shallow-water *Ophryotrocha* occurring in the Mediterranean may have different patterns of substrate colonization despite sharing similar life histories.

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Introduction

In the marine benthos, the establishment and maintenance of populations and assemblages of invertebrates on new free substrates typically rely on larval recruitment, or colonization by juveniles or adults in species that lack larval dispersal. For organisms with very specific habitat requirements, which narrow their possibilities to thrive to a limited range of habitats, the timing and sequence in which species appear in assemblages have been documented in some cases (*e.g.* Vrijenhoek *et al*. 2008; Fukasawa *et al*. 2015), but there is only limited understanding of the processes and dynamics of dispersal and colonization patterns by individual species. This is the case, for example, for organisms adapted to live in organically enriched habitats. Whether anthropogenically induced (*e.g.* sediment beneath fish farms, harbours) or naturally occurring (*e.g.* decaying mammal bones), organically enriched habitats harbour a plethora of opportunistic and specialist species that differ from those commonly found in background communities (Pearson & Rosenberg 1978; Rouse *et al*. 2004; Taboada *et al*. 2016).

Over the past several years, there has been growing interest in marine invertebrates associated with decaying mammal bones (*e.g.* whale-falls) and how the bones are colonized by a diversity of microbial and macrofaunal species (*e.g.* Rouse *et al.* 2004; Goffredi *et al*. 2005; Treude *et al.* 2009; Vrijenhoek *et al.* 2008; Wiklund *et al.* 2009a, b; Taboada *et al.* 2013, 2015b, 2016; Silva *et al.* 2016). Submerged bone remains of large whale carcasses become long-lasting islands of organic matter in an otherwise oligotrophic environment, giving shelter and providing nutrition to a variety of marine invertebrates (Smith 2006). Rich chemoautotrophic communities, including sulphide-oxidizing free-living bacteria and endosymbionts, thrive on these bones, making the organic matter retained in the bones available to a variety of macrofaunal invertebrates, the majority of which are polychaete annelids (Goffredi *et al*. 2005; Smith 2006; Treude *et al.* 2009). Due to the sparse and unpredictable occurrence of these substrates, several of these polychaete species rely on their larval dispersal capacities to colonize them and thus ensure their continuation in other distant habitats. Perhaps one of the most prominent examples of this are species of the genus *Osedax*. These siboglinid polychaetes, commonly known as bone-eating worms, are specialist organisms associated with bones; they have lecithotrophic trochophore larvae, that

presumably disperse across long distances to colonize bone habitats up to thousands of kilometers apart (Vrijenhoek *et al.* 2008; Taboada *et al.* 2015b).

Apart from specialists, bones are also known to harbour several opportunistic polychaete species from different families (see Smith 2006; Wiklund *et al.* 2009a, b; Taboada *et al.* 2013, 2015a, 2016; Silva *et al.* 2016). Many of these opportunistic species seem adapted to thrive in extreme conditions of low oxygen and elevated H_2S concentrations. They often reach large population sizes, likely due to the fact that they usually reach sexual maturity relatively rapidly, have short generation times and undergo direct development (Pearson & Rosenberg 1978). Once they colonize a suitable habitat, these opportunistic species are able to exploit very efficiently organically rich substrates, which are often widely separated and ephemeral, thanks to their life history features. However, the mechanisms and processes that allow opportunistic organisms lacking a dispersive larval phase to colonize new substrates are not well understood.

One of the most common opportunistic taxa occurring worldwide on bones are members of the dorvilleid polychaete genus *Ophryotrocha*, which mostly feed on filamentous *Beggiatoa*-like bacteria developing on bones (Wiklund *et al.* 2009a, 2012; Taboada *et al.* 2013, 2016). In the Mediterranean, different species of *Ophryotrocha* have recently been reported in mammal bones (Taboada *et al.* 2016), although most of them are well known dwellers of anthropogenically enriched habitats (*e.g.* Simonini *et al.* 2010; Paxton & Åkesson 2011). In a recent study, Taboada *et al.* (2016) monitored the invertebrate communities colonizing experimentally deployed mammal bones in the shallow-water Mediterranean Sea over the course of a year, and found that *Ophryotrocha puerilis* Claparède & Metschnikow, 1869 and *Ophryotrocha alborana* Paxton & Åkesson, 2011 were two of the most abundant species occurring in the bones. *Ophryotrocha puerilis* appeared in the bones as an early colonizer, while the recently described *O. alborana* seemed to replace the former between the second and fourth trimesters after bone deployment. Interestingly, both species appeared to differ in the way they colonized bones: *while O. puerilis* appeared to colonize bones through multiple colonization events over the course of the year, only one independent event of colonization was inferred for *O. alborana* from the size-frequency analyses performed for both species (Taboada *et al.* 2016). *Ophryotrocha puerilis* is a protandrous, species with direct development and parental care, able to perform sex reversal several times

during its life (Åkesson 1973), while *O. alborana* is a simultaneous hermaphroditic species with direct development originally described from the SW Mediterranean Sea (Paxton & Åkesson 2011).

Here, we address the colonization processes of the populations of *O. puerilis* and *O. alborana* collected during the study by Taboada *et al*. (2016) using a molecular approach. Due to the lack of a larval phase, both species are presumed to have limited dispersal capabilities and represent an interesting case to investigate the patterns of colonization of new substrates. Drawing on phylogeographic analyses of a fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene for worms sampled from experimentally deployed bones at several sites across time, we aimed: (i) to establish whether both *O. puerilis* and *O. alborana* appeared in the experimentally deployed bones after one or more events of colonization; and (ii) to determine whether populations of these two species changed over time in the bones. Due to the relatively high genetic distances between some of the *O. puerilis* organisms collected for the molecular study, we also carried out species delimitation analyses to investigate the occurrence of cryptic species and performed phylogenetic analyses using three genes (*COI*, *16S* rDNA *–16S–*, *Histone H3 –H3–*) to place them within their phylogenetic context.

Material and Methods

Sample collection and preservation

Details about the experimental design to obtain *Ophryotrocha puerilis* and *Ophryotrocha alborana* specimens associated with mammal bones are found in Taboada *et al.* (2016). Briefly, bones from a caudal fin of a common minke whale (*Balaenoptera acutorostrata* Lacépède, 1804) [Wh], and vertebrae from cows (*Bos taurus* Linnaeus, 1758) [Cw] and pigs (*Sus domesticus* Erxleben, 1777) [Pg] were experimentally deployed at about 20 m depth on the seabed of the Mediterranean coast of Blanes (NW Mediterranean, 41° 40.536' N, 2° 48.839' E) in March 2011 (Fig. 1, Table 1). Bones were deployed on three different habitats: rocky bottoms [Ro], sandy bottoms [Sa], and a *Posidonia oceanica* (Linnaeus) Delile, 1813 meadow [Po], and three replicates per type of bone were removed every three months (trimester 1–4 [T1–4]) at each of the substrates (Table 1). Bone samples were collected by SCUBA-diving and taken to the laboratory at the Department of Evolutionary Biology, Ecology and Environmental Science, Faculty of Biology, Universitat de Barcelona (Spain). Once there, bones were placed into individual containers with filtered seawater (0.22-μm) without supplementary oxygen forcing the system to become anoxic in order to force the animals to leave the bones and be easily collected. Individuals of *O. puerilis* and *O. alborana* inhabiting the bones were collected, transferred to petri dishes with filtered seawater and left overnight to eliminate gut content. Prior to preservation, organisms were anesthetized in a 7 % solution of $MgCl_2$ in freshwater, observed *in vivo* and photographed under a stereo-microscope, and finally fixed in absolute ethanol and stored at -20 °C. Not every sampling event yielded *Ophryotrocha* (see Table 1).

Although to our knowledge there is no information available about the duration of the life cycle for *O. puerilis* and *O. alborana*, for other congeneric species sexual maturity is attained after 18–51 days (*e.g.* Åkesson 1970; Paavo *et al*. 2000; Simonini & Prevedelli 2003). Hence it is likely that sampling every three-months would ensure that at least one new generation of worms was surveyed.

In addition, *O. puerilis* was collected from other either naturally-occurring or experimentally deployed bones (Fig. 1; Table 1). This included a seagull bone [Sg] collected in 2013 [13] at ~10 m on a sandy bottom [Sa] by SCUBA-diving close to the area where experimentally bones were deployed 41° 40.407' N, 2° 48.308' E (Seagull

Control: Fig. 1, Table 1). It also included whale bones [Wh] inside wire cages experimentally deployed at ~ 10 m in Blanes Harbour [Ha] in 2014 [14] and 2015 [15] 41° 40.468' N, 2° 47.943' E in July 2014 and January 2015, respectively (Harbour Control 14–15: Fig. 1 Table 1). Finally, whale bones [Wh] were also deposited at 53 m at the head of Blanes submarine canyon [Sc] 41°40.258' N 2°53.388' E in October 2013 (Blanes submarine canyon Control: Fig. 1, Table 1). Preservation of specimens was carried out as described above.

All the specimens identified as *O. puerilis* shared a similar body shape, two antennae and two palps, two slanted and bright eyes, a similar shape of parapodial lobes, and two anal cirri without median stylus. All the specimens identified as *O. alborana* also shared a similar body shape, two small antennae, two slanted red eyes, a similar shape of parapodial lobes, mammillate rosette glands in the posterior segments, and two anal cirri without median stylus.

DNA extraction and amplification

Total DNA was extracted using QIAamp DNA Micro Kit (Qiagen, www.qiagen.com) or the REDExtract-N-Amp kit (Sigma-Aldrich,www.sigma.com) from a few segments of the mid part of the body of *O. puerilis* and *O. alborana* (Table 1). About 600 bp of *cytochrome c oxidase subunit I* (*COI*) were amplified for 155 individuals of *O. puerilis* and 33 for *O. alborana* to conduct demographic analysis (see below). Primers used were those of Folmer *et al*. (1994) or the following previously unpublished primers: MegaCO1-F [5'-TAYTCWACWAAYCAYAAAGAYAATGG-3'] and MegaCO1-R [5'-TAKACTTCTGGRTGMCCAAARAATC-3']. In addition, for 28 individuals of *O. puerilis* and *O. alborana*, about 360 bp of *Histone H3* (*H3*) (Colgan *et al.* 2000) and 460 bp of *16S* rDNA (*16S*) (Palumbi 1996) were amplified and sequenced to corroborate their taxonomic identification and compared with the existing *Ophryotrocha* sequences available in the NCBI database (www.ncbi.nlm.nih.gov). Each PCR reaction mix contained 4 µl MgCl2, 0.2 mM dNTPs, 1 µl PCR buffer, 0.5 µl Taq DNA polymerase (Invitrogen), 0.125 µg/µl BSA, 1 µl primers (F and R), and H2O to reach a final reaction volume of 10 µl; 2 µl of genomic DNA was used in each reaction. For amplification, the following PCR protocols were used for *COI* [94 ºC/3 min – (94 ºC/30 s – 48 ºC/1 min – 72 ºC 1 min) x 40 cycles – 72 ºC/5 min], for *H3* [94 ºC/3 min –

(94 ºC/30 s – 60–53 ºC/1 min – 72 ºC 1 min) x 7 cycles – (94 ºC/30 s –53 ºC/1 min – 72 ºC 1 min) x 20 cycles 72 ºC/5 min], and for *16S* [94 ºC/3 min – (94 ºC/30 s – 60–48 ºC/1 min – 72 ºC 1 min) x 12 cycles – (94 ºC/30 s –48 ºC/1 min – 72 ºC 1 min) x 20 cycles 72 ºC/5 min]. PCR products were sequenced at the facilities of the Scientific and Technological Centers, Universitat de Barcelona (CCiT-UB) and at Colgate University on an ABI 3130A Genetic Analyser (Applied Biosystems), using the primers mentioned above. All the new sequences obtained in this study used for phylogenetic and demographic analyses are deposited in NCBI GenBank (accession numbers KY378402–KY378645).

Phylogenetic analysis

Molecular phylogenetic analyses of *Ophryotrocha* were conducted using data sets for *COI*, *16S*, and *H3* using sequences available in NCBI and sequences obtained in this study (Suppl. Mat. Table S1). In total, 67 terminal taxa were used in the analysis including 59 *Ophryotrocha*, *Iphitime hartmanae* Kirkegaard, 1977 and *Exallopus jumarsi* Blake, 1985; seven taxa representing the dorvilleid genera *Parougia*, *Dorvillea*, and *Protodrovillea*; and the eunicid *Eunice pennata* (Müller, 1776) as an outgroup for tree rooting. For *O. puerilis* and *O. alborana* in our study we selected the organisms displaying the most divergent *COI* haplotypes to be included in the phylogenetic analyses. Overlapping sequence fragments were assembled into consensus sequences using the software Geneious vs. 8.1.7 (Drummond *et al*. 2010), and aligned using Q-INS-I option of MAFFT (Katoh *et al*. 2002). The most appropriate evolutionary model for each gene (GTR+I+G for all the markers genes) was obtained by running the alignments in jModelTest (Posada 2008) via the Akaike Information Criterion (AIC). A combined analysis using the three concatenated genes was conducted using Maximum Likelihood analyses (ML) with RAxML (Stamatakis 2006; Stamatakis *et al.* 2008) and Bayesian inference analyses (BI) with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). ML were run using 10 heuristic searches (SPR and NNI) and robustness of the nodes was determined with 10 runs and 500 replicates using the GTR+I+G evolutionary model; concatenated sequences were partitioned by gene and protein coding genes (*H3* and *COI*) were partitioned into codon positions. BI analyses were run twice for each dataset with four chains for 2.5 million generations (25 % trees discarded as burn-in)

sampling a tree every 1,000 generations; partition codons were used for *H3* and *COI* and the best evolutionary models previously inferred for every gene were applied. Convergence among chains, mixing within chains (i.e., ESS values) and the number of burn-in generations were monitored with the program TRACER 1.6 (Rambaut *et al*. 2014). Results were visualized in FigTree v.1.4.2 (Rambaut 2006).

Species delimitation analysis

Since our phylogenetic results pointed to the possible occurrence of three different species among the specimens identified as *O. puerilis* (*O. puerilis sensu stricto*, *O. puerilis* 'Shallow' and *O. puerilis* 'Deep'; see Results section), we tested the molecular boundaries of these groups of organisms through two commonly used species delimitation analyses: the Poisson Tree Processes model (PTP; Zhang *et al*. 2013) and the Automatic Barcode Gap Discovery (ABGD; Puillandre *et al*. 2012). For PTP analyses we used a rooted BI phylogenetic tree (analyses were run twice for each dataset with four chains for 10 million generations, 25 % trees discarded as burn-in, sampling a tree every 1,000 generations) for the *COI* partition including all the terminal taxa included in the phylogenetic analyses, all the haplotypes of *O. puerilis* and *O. alborana* used in our study, and a selection of *COI* sequences for the species *Ophryotrocha cyclops* Salvo, Wiklund, Dufour, Hamoutene, Pohle & Worsaae, 2014, *O. orensanzi*, and *Ophryotrocha labronica* Bacci & La Greca, 1961 available in NCBI; for *O. labronica* we also included three *COI* sequences from individuals collected in Blanes harbour (*O. labronica* WhHa14_13-15). PTP was run in the bPTP server (http://species.h-its.org/ptp/), a Bayesian implementation of the PTP model for species delimitation, using 100,000 generations for the Markov chains with a thinning of 100 and a burn-in of 0.1. Convergence was checked using the log likelihood for the MCMC iterations after thinning in the above mentioned web server. For ABGD analyses we used an unrooted alignment including all the haplotypes of *O. puerilis* and *O. alborana* used in our study, together with all the available *Ophryotrocha COI* sequences in NCBI having more than one sequence per species (also including the three individuals of *O. labronica* collected in Blanes harbour mentioned earlier). ABGD was run in the ABGD web server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) using the default

values for the Jukes-Cantor and Kimura distances and also applying a relative gap width (X) of 10.

In addition, minimum genetic distances based on uncorrected *p*-distance and Kimura 2 parameters (K2p) models were calculated using MEGA vs. 5.2.2 (Tamura *et al.* 2011); the default parameters were used to calculate distances between and within the three lineages originally identified as *O. puerilis* but phylogenetically separated (*O. puerilis sensu stricto*, *O. puerilis* 'Shallow' and *O. puerilis* 'Deep') and within *O. alborana*. Also, genetic distances were calculated for the three lineages of *O. puerilis* with respect to the other species of *Ophryotrocha* in the analysis. These distances were calculated using the *COI* alignment used in the phylogenetic analyses and also the *COI* alignment used for the demographic analysis (see Demographic analysis section below).

Demographic analysis

COI sequences of *O. alborana* and *O. puerilis* (including separately *O. puerilis sensu stricto*, *O. puerilis* 'Shallow' and *O. puerilis* 'Deep'; see *Species delimitation analysis* section above) were aligned in Geneious vs. 8.1.7 using Q-INS-I option of MAFFT and checked manually. Polymorphic sites and levels of DNA polymorphism were calculated for each lineage on each substrate using DnaSP vs. 5.10.1 (Librado & Rozas 2009), and included number of haplotypes (*H*), private haplotypes (*Hp*), haplotype diversity (*Hd*), and nucleotide diversity (π). Haplotype richness (*Hr*) was also calculated with the program CONTRIB (Petit *et al*. 1998) after rarefaction using the minimum sample size for each population.

COI alignments of *O. alborana*, *O. puerilis sensu stricto*, *O. puerilis* 'Shallow' and *O. puerilis* 'Deep' were used to construct unrooted haplotype networks with the program PopART (http://popart.otago.ac.nz) using the Median Joining network option (Bandelt *et al*. 1999).

A hierarchical analysis of molecular variance (AMOVA) using genetic distances was used to assess differentiation between the trimesters in *O. puerilis* 'Shallow' samples from the first and second trimester (WhRoT1 and WhSaT1; WhRoT2 and CwRoT2). Significance was tested by running 20,000 permutations in *Arlequin* vs. 3.5.2.2 (Excoffier & Lischer 2010) using the standard AMOVA computation.

Finally, in order to test whether bones from the head of the Blanes canyon

289 (WhSc13) had a different pattern of number of individuals per haplotype we ran a set of
290 one-way ANOVAs using the program *StatPlus:mac* vs. 5.9.92 (AnalystSoft Inc.
291 www.analystsoft.com/en/products/statplusmac/). Comparisons were made after (i)
292 aggregating the haplotypes from the different bones for the three lineages (*O. puerilis*
293 *sensu stricto*, *O. puerilis* 'Shallow' and *O. puerilis* 'Deep'), (ii) considering all bones
294 from the different lineages separately, and (iii) considering all bones from the different
295 lineages separately but only using the ones with more than 10 individuals per bone.

296 **Results**

297 *Phylogenetic analysis*

298 The consensus tree obtained from the Bayesian (BI) analysis of the concatenated
299 alignment (Fig. 2) also summarizes the support recovered from the Maximum
300 Likelihood (ML) analysis. The concatenated alignment consisted of 1,309 bp (528 bp of
301 *COI*, 460 bp of *16S*, and 321 bp of *H3*). Both BI and ML analyses recovered similar
302 topologies and the three major clades previously defined within *Ophryotrocha*, namely
303 '*labronica*', '*hartmanni*' and '*lobifera*' clades (see Taboada *et al*. 2013), were
304 recovered, although with moderate support. The newly sequenced *O. alborana*
305 specimens included in our study clustered together with the previously available
306 sequence for the species. Samples morphologically identified here as *O. puerilis* formed
307 a monophyletic group of three distinct well-supported lineages, with *Ophryotrocha*
308 *eutrophila* as the sister group (Fig. 2). All the samples collected from the Blanes
309 harbour and one sample from the shallow experimental bones clustered together with *O.*
310 *puerilis* from NCBI (lineage '*O. puerilis sensu stricto*'), and this group was sister to *O.*
311 *puerilis siberti* (Fig. 2). The lineage '*O. puerilis sensu stricto*' together with *O. puerilis*
312 *siberti* formed the sister group to the majority of the samples collected from the shallow
313 experimental bones and the ones from the Seagull control –SgSa13– (lineage '*O.*
314 *puerilis* Shallow'). Finally, the lineage '*O. puerilis* sensu stricto' together with *O.*
315 *puerilis siberti* and the subclade '*O. puerilis* Shallow' formed the sister group to the
316 lineage '*O. puerilis* Deep', which comprised all the samples collected at the head of the
317 Blanes submarine canyon –WhSc13– (Fig. 2).
318
319 *Species delimitation analysis*

320 To test the possible occurrence of different species within the *O. puerilis* complex
321 identified in the phylogenetic tree (lineages '*O. puerilis sensu stricto*', '*O. puerilis*
322 Shallow' and '*O. puerilis* Deep'; Fig. 2), we used ABGD, bPTP, and also calculated
323 pairwise genetic distances within and between the lineages. ABGD clearly
324 differentiated the three lineages of *O. puerilis* identified in our phylogenetic tree as
325 three different species and also supported all individuals of *O. alborana* as members of
326 the same species. ABGD additionalyy recovered other *Ophryotrocha* species included
327 in the analyses as separate species, and separated as two different species the two

lineages of *O. labronica* corresponding to the two haplogroups defined by Cossu *et al*. (2015) including also the three individuals we collected in Blanes harbour (*O. labronica* Wh-Ha-14_13-15). Interestingly, these two *O. labronica* species inferred here do not cluster together with the original *COI* sequence available in NCBI for *O. labronica* (GQ415479) (Suppl. Mat. Table S2).

bPTP analysis identified *O. puerilis* 'Shallow' as a separate species with moderate support, and displayed low support identifying two different species within *O. puerilis* 'Deep' and eight within *O. puerilis sensu stricto* (Suppl. Fig. 1). bPTP also recovered up to eight species within *O. alborana* with moderate support. As for the rest of the *Ophryotrocha* species included in the analyses, bPTP consistently recovered all the species as separate species (with moderate support) except for the case of *O. labronica*. In this case, the two species inferred here corresponded to the two haplogroups identified in the study by Cossu *et al*. (2015) and also include the three individuals of specimens collected in Blanes harbour (*O. labronica* Wh-Ha-14_13-15). As for ABGD analyses, these two *O. labronica* species inferred here do not cluster together with the original *COI* sequence available in NCBI for *O. labronica* (GQ415479) (Suppl. Fig. 1).

The within-species genetic divergence was the lowest for individuals of *O. puerilis* 'Shallow' (ranging from 0.0 to 2.6 % –average 0.85 %– and from 0.0 to 2.5 % –average 0.83 %– for K2p and *p*-distance, respectively), while it was the highest for individuals of *O. puerilis sensu stricto* (ranging from 0.0 to 8.5 % –average 3.52 %– and from 0.0 to 7.4 % –average 3.16– for K2p and *p*-distance, respectively) (Table 2). Average pairwise genetic distances (K2p and *p*-distance, respectively) were 28.3 % and 19.8 % between *O. puerilis* 'Shallow' and *O. puerilis sensu stricto*, 30.3 % and 21.0 % between *O. puerilis* 'Shallow' and *O. puerilis* 'Deep', and 34.5 % and 22.8 % between *O. puerilis sensu stricto* and *O. puerilis* 'Deep' (Table 2). This leaves gaps in genetic distances within and between these lineages of greater than 25 % (K2P) or 15 % (*p*-distance). When comparing these three species with their closest phylogenetically related taxa, the lowest genetic distances were detected between *O. puerilis siberti* and *O. puerilis sensu stricto* (25.5 % and 18.4 %), and *O. eutrophila* and *O. puerilis sensu stricto* (29.5 % and 20.7 %), while the highest genetic distances were detected between

359 *O. puerilis siberti* and *O. puerilis* 'Deep' (34.7 % and 23.0 %) and *O. eutrophila* and *O.*
360 *puerilis* 'Shallow' (31.4 % and 21.6 %) (Table 2).
361
362 *Demographic analysis*
363 A 573 bp fragment (571 bp excluding missing data) of *COI* was analyzed for 33
364 individuals of *O. alborana* collected from three experimental bones from three different
365 trimesters (PgRoT2, CwPoT3, CwRoT4; Table 3). In total, 27 polymorphic sites (4.7
366 %), 13 of them parsimony informative, and 18 different haplotypes were found in the
367 dataset: H1–H5, H7–H8, H10–H11 (private haplotypes in PgRoT2), H12 (private
368 haplotype in CwPoT3), and H9 (2 individuals in CwRoT4) (Fig. 3A). The five most
369 common haplotypes accounted for 66 % of the total number of individuals and were
370 particular from each substrate (H1 and H2 in CwPoT3, H4 in CwRoT4, and H11 and
371 H17 in PgRoT2; Fig. 3A). Haplotype diversity was 0.936±0.024 and nucleotide
372 diversity 0.009±0.001 (Table 3).
373 A total of 482 bp of *COI* were obtained from 100 individuals of *O. puerilis*
374 'Shallow' from four experimental bones from two different trimesters (WhSaT1,
375 WhRoT1, WhRoT2 and CwRoT2) and two controls (SgSa13 and WhHa15; Table 3). In
376 total, 57 polymorphic sites (10.8 %), 25 of them parsimony informative, and 41
377 different haplotypes (28 of which were private: 18 in the organisms from the
378 experiments and 10 in the controls; Table 3) were found in the dataset. The five most
379 common haplotypes in *O. puerilis* 'Shallow' (H1–H5), all of them having more than 5
380 individuals per haplotype, accounted for 52 % of the total sample (Fig. 3B). Three of
381 the most common haplotypes (H1–H3) were present in the majority of substrates from
382 both experiments (sharing samples from different trimesters) and controls, while the
383 other two (H4 and H5) were present only in two substrates; haplotypes H1–H4 were
384 present in samples from the two trimesters (Fig. 3B). Haplotype diversity ranged from
385 0.930±0.015 to 0.965±0.024 when considering separately the samples from the
386 experiments and the controls, and was 0.937±0.013 for the whole dataset (Table 3).
387 Nucleotide diversity was 0.008±0.001 in the experiments, 0.009±0.001 for the controls,
388 and 0.008±0.001 for the whole dataset (Table 3). AMOVA results comparing the
389 samples from the two trimestres (WhRoT1 and WhSaT1; WhRoT2 and CwRoT2)

showed no significant structuring across sites or trimesters and the greatest genetic variability was retained within samples (89.62 %; $p < 0.00001$).

For *O. puerilis sensu stricto* a *COI* alignment of 482 bp was obtained from a total of 30 individuals, three of which belonged to one experimental bone (WhRoT1) and the rest to the controls at Blanes harbour (WhHa14 and WhHa15; Table 3). A total of 64 polymorphic sites (13.3 %), 37 of them parsimony informative and 13 haplotypes were found in the dataset. Two haplotypes (H1–H2) accounted for 50 % of the total individuals in the sampling and only H3 was shared between two different substrates but from the same locality (WhHa14 and WhHa15; Fig. 3C). Haplotype diversity and nucleotide diversity varied greatly between samples from the experimental bone and the controls and was 0.871±0.043, and 0.0311±0.00468 for the whole dataset (Table 3).

Finally, for *O. puerilis* 'Deep' a *COI* alignment of 482 bp was obtained from a total of 25 individuals collected from a single whale bone (WhSc13; Table 3). A total of 29 polymorphic sites (6.0 %), 25 of them parsimony informative, and five haplotypes were found. Two of the haplotypes were private (H4 and H5) and the most common haplotype (H1) accounted for 60 % of the total individuals (Fig. 3D). Haplotype diversity and nucleotide diversity was 0.610±0.094 and 0.017±0.005, respectively (Table 3). No significant difference was found when comparing the pattern in the number of haplotypes between *O. puerilis* 'Deep' and the rest of samples from the two other species.

Discussion

Phylogenetic and species delimitation analyses

Both our phylogenetic and species delimitation analyses unambiguously indicate that two new *Ophryotrocha* species closely related to *O. puerilis* occur in the shallow NW Mediterranean. On the basis of our results, we propose that the two reciprocally monophyletic lineages referred to here as *O. puerilis* 'Shallow' and *O. puerilis* 'Deep' represent two new cryptic species of the genus *Ophryotrocha.* These two new *Ophryotrocha* species should be regarded as sibling species of *O. puerilis* and *O. puerilis siberti* based on the strongly supported sister-species relationships among them (Fig. 2), which suggests relatively early divergence within *Ophryotrocha*. To our knowledge, this brings the number of *Ophryotrocha* species known from the Mediterranean (excluding the introduced species *Ophryotrocha adherens* Paavo, Bailey-Brock & Åkesson, 2000, *Ophryotrocha diadema* Åkesson, 1976 and *Ophryotrocha japonica* Paxton & Åkesson, 2010; see Simonini *et al*. 2009) to nine and includes, apart from the four species studied here (*O. alborana*, *O. puerilis sensu stricto*, *O. puerilis* 'Shallow' and *O. puerilis* 'Deep'), the shallow-water *O. labronica*, *Ophryotrocha macrovifera* Paxton & Åkesson, 2010, *Ophryotrocha robusta* Paxton & Åkesson, 2010 and *Ophryotrocha rubra* Paxton & Åkesson, 2010, and the deep-water *Ophryotrocha mediterranea* Martin, Abello & Cartes, 1991 (La Greca & Bacci 1962; Martin *et al*. 1991; Paxton & Åkesson 2007, 2010; Paxton *et al*. 2011).

Future studies will reveal whether the new sibling cryptic species within *O. puerilis* species complex (*O. puerilis* 'Shallow' and *O. puerilis* 'Deep') that we inferred based on molecular data have in fact distinct diagnostic morphological characters. Our preliminary examination of several preserved individuals of the different species did not identify any clear macroscopical/microscopical difference, but we cannot discard that SEM imaging might show microscopical differences in the jaw apparatus and/or ciliary patterns along their bodies. Other than morphological differences, recognition through chemical signals might play an important role in mate choice for these species, which is especially relevant for closely related species sharing habitat (see Knowlton 1993). In this sense, previous studies in the hermaphroditic *O. diadema* showed that group size is assessed by chemical cues and that a reduction in the number of eggs is induced when the number of partners increases, which suggests that these hermaphroditic worms

perceive social cues and adjust sex allocation accordingly (Schleicherová *et al*. 2006, 2010). It is thus possible that given the hermaphroditic condition of *O. puerilis*, a condition likely shared with the undescribed sibling species identified here, chemical recognition might also occur in these species, something that should be investigated in future studies.

Closely related species living in sympatry

The different substrates analyzed in this study harboured several different species of *Ophryotrocha* occurring in sympatry. Apart from *O. alborana*, *O. puerilis sensu stricto* and the two proposed cryptic species (*O. puerilis* 'Shallow' and *O. puerilis* 'Deep'), at least *Ophryotrocha labronica*, *Ophryotrocha robusta* and another potential species from the '*labronica*' clade (authors' pers. obs.), also appeared in the bones. Species co-occurrence is not uncommon in *Ophryotrocha* and has been previously reported in different studies using mammal bones from a range of geographic areas (*e.g.* Wiklund *et al*. 2009; Taboada *et al*. 2013; Ravara *et al*. 2015). Just focusing on the *O. puerilis* species complex uncovered here, two species (*O. puerilis sensu stricto* and *O. puerilis* 'Shallow') appear to be syntopic *sensu* Rivas (1964) since they occur in the same locality and share the same habitat, while *O. puerilis* 'Deep' should be considered as a parapatric species with respect to the other two since its distribution does not significantly overlap although it is immediately adjacent to the distribution of the shallower species. Although further similar studies in other areas and depths should be conducted to confirm this, it appears that there may be a bathymetric and/or temperature-related segregation between *O. puerilis* 'Deep' and the other two sibling species (*O. puerilis sensu stricto* and *O. puerilis* 'Shallow'). The proximity of *O. puerilis* 'Deep' to the Blanes submarine canyon (Fig. 1), an area influenced by deeper and colder water masses (Ahumada *et al*. 2013), suggests that this species might be more common in deeper and colder habitats. Examples of cryptic species with a bathymetric segregation of a few hundred meters are not rare in polychaetes (*e.g.* Nygren *et al*. 2005, 2010), although this contrasts with the recently reported wide bathymetric range up to 1,000 m for *Ophryotrocha scutellus* (Ravara *et al*. 2015). Importantly, the co-occurrence of *O. puerilis* 'Shallow' and *O. puerilis sensu stricto* in

the same bones challenges previous identifications of *O. puerilis* in the shallow-water Mediterranean.

Cryptic species living in sympatry has been well documented in the past in a plethora of organisms and it is quite common for marine invertebrates in general and for polychaetes in particular (Knowlton 1993; Bickford *et al*. 2007; Nygren 2014). For example within *Ophryotrocha*, *O. japonica* and *Ophryotrocha notoglandulata* Pfannenstiel, 1972 (the first time cryptic speciation was reported for the genus) occur in sympatry at least in Japanese waters and appear to only differ morphologically in their maximum size and the number of rosette glands (the latter character only noticeable using SEM; Paxton & Åkesson 2010). Interestingly, these species showed *ca.* 5 % of K2p *COI* divergence (Wiklund *et al*. 2009), less than five times the average divergence reported for the *O. puerilis* sibling species reported in our study (see Table 2). This divergence threshold reported for *O. japonica* and *O. notoglandulata*, though, does not seem to hold for *O. labronica*. In their recent study, Cossu *et al*. (2015) demonstrated that *O. labronica* populations along the Italian coast can be in fact subdivided in two highly divergent haplogroups (average 17.2 % K2p distance for *COI*), which was suggested to be the result of allopatric divergence followed by secondary contact. Remarkably, cross-breeding experiments showed that individuals from the two haplogroups were inter-fertile (Massamba-N'Siala *et al*. 2011), thus indicating that these haplogroups are in fact two separate lineages of the species *O. labronica* (Cossu *et al*. 2015). However, given that no nuclear markers were sequenced for these haplogroups (neither for the haplogroups nor for the resulting 'hybrids' after cross-breeding), we can not dismiss the possibility that these two lineages in fact correspond to two different species whose reproductive prezygotic isolation is incomplete at least in *in vitro* conditions, as it has been reported for different species of echinoderms (*e.g.* Muths *et al*. 2006, 2010). In our study, the molecular divergences for sympatric lineages of *Ophryotrocha* within the *O. puerilis* clade (Table 2) follow the approximate ratio of interspecific/intraspecific variation of 10x *COI* divergence proposed to delimit species boundaries (Hebert *et al*. 2004; Carr *et al*. 2011) and also exceed the 15 % *COI* distance observed for other cryptic species of polychaetes (Nygren 2014).

Demographic analysis

Our molecular analyses of the two predominant taxa appearing in the experimental bones in the study by Taboada *et al*. (2016), *i.e.*, *O. puerilis* 'Shallow' and *O. alborana*, showed contrasting demographic patterns. For *O. puerilis* 'Shallow', the most common organism in the experimental bones during the first two trimesters (Taboada *et al*. 2016), four of the most common haplotypes found were shared between samples from both trimesters (H1–H4; 54 %), while the rest of individuals were only present at bones from one trimester (Fig. 3B). Based on this, we propose that colonization of the experimental bones by external populations may have been achieved by a combination of (i) a few organisms (the ones presenting the most common haplotypes shared across time) that arrived to the bones and were able to reseed, and (ii) by several individuals arriving to the experimental bones and not persisting across time. Thus, our results are partially in agreement with what was already inferred from size-frequency analyses by Taboada *et al*. (2016): most of the bones they analyzed appeared to harbour various cohorts of individuals presumably from various colonization events, although some of the bones appeared to have a single cohort of individuals. Stable or ephemeral populations developing in the different habitats we considered in our study (*e.g.* Blanes harbour, seagull bones) might be the sources for colonization of experimental bones deployed, although other non-investigated habitats such as sewage pipelines and riverine discharges (quite common in the area) may also play an important role.

Interestingly, the *O. alborana* haplotype network showed that none of the haplotypes present in the three different bones (= three different trimesters) was shared, suggesting that there was not a prevalence of the populations of this species in the bones across time (Fig. 3A). These results corroborate what was observed by Taboada *et al*. (2016), who suggested that only one event of colonization per bone could be inferred from the size-frequency histograms after analyzing four bones and a total of ca. 500 individuals. All this may suggest that, in contrast to *O. puerilis* 'Shallow', colonization of the bones by *O. alborana* occured *via* multiple independent events. In other words, the populations of *O. alborana* arriving to the bones during the second trimester were totally replaced by new individuals during the third and fourth trimesters. This statement fits with the metapopulation scenario invoked for the congeneric *O. labronica*, a species subdivided into many local populations that proliferate under favourable conditions and may rapidly go extinct to be replaced by new populations

(Åkesson & Paxton 2005; Prevedelli *et al*. 2005). However, our results for *O. alborana* must be considered as very preliminary due to the relatively low number of individuals per population analyzed in the study (10–12 individuals per population; Table 1).

Ophryotrocha puerilis 'Deep' showed a similar haplotype pattern when compared with the other two sibling species considered in the study. Although it was less clear for the case of *O. puerilis* 'Deep', there were no significant differences among the haplotype patterns among *O. puerilis* species; they all had a few high-frequency haplotypes and either a few (*O. puerilis* 'Deep'*)* or many low-frequency haplotypes (*O. puerilis* 'Shallow' and *O. puerilis sensu stricto*) (Fig. 3B–D). This suggests that in the study area there are diverse and stable populations of the three different species in naturally occurring and/or anthropogenically derived potential habitats that provided recruits to the bones. Further studies should be directed to investigate deeper water habitats (*e.g.* Blanes submarine canyon), where suitable substrates for the development of these organisms might be more sparse and scarce and hence the demographic patterns observed might also be different. In fact, to our knowledge the only examples investigating the genetic structure over time of marine invertebrates colonizing whale-falls come from deep waters of the Pacific Ocean and include one siboglinid polychaete from the genus *Osedax* and two mytilid molluscs (Vrijenhoek *et al*. 2008; Fukasawa *et al*. 2015). The two mytilid mussels colonizing deep-water whale carcasses off the Japanese coast seemed to maintain an unchanged poorly structured genetic composition over the course of years (Fukasawa *et al*. 2015), which is similar to what was observed for the polychaete *Osedax rubiplumus* Rouse, Goffredi & Vrijenhoek, 2004 in deep-water whale remains off the Monterey Bay in California (Vrijenhoek *et al*. 2008). In both studies, large and probably distant common pools of individuals were hypothesized to be responsible for the demographic patterns observed, which reflect recruitment of larvae with high dispersal ability (Vrijenhoek *et al*. 2008; Fukasawa *et al*. 2015). *Ophryotrocha puerilis* 'Shallow' in our study showed a similar demographic pattern to that observed for the two mytilids and the siboglinid but, as explained above, common pools of individuals in *O. puerilis* 'Shallow' are likely to be located nearby.

In conclusion, our study provides a new example on cryptic speciation in annelid polychaetes in well-known taxa from well-studied areas (see Nygren 2014). Importantly, the discovery of a species complex in *Ophryotrocha puerilis* poses some

569 doubts about previous morphologically-based identifications of this species which is
570 commonly occurs in Mediterranean harbours (Simonini et al. 2010) and is used as a
571 bioindicator of organically-enriched habitats. Future studies should be directed to
572 morphologically describe the two cryptic species found in our phylogenetic and species
573 delimitation analyses. As for the colonization processes inferred from *COI* sequences
574 for *O. puerilis* 'Shallow' and *O. alborana*, although contrasting results were observed
575 for the two species, caution should be taken when interpreting these results due to the
576 limited number of specimens and the limited number of locations investigated in our
577 study. Anyway, still several questions remain unsolved related to the colonization
578 patterns of these and other *Ophryotrocha* species occurring in ephemeral habitats such
579 as mammal bones. In our view, further studies should address how deeper and sparser
580 habitats are colonized and which of the dispersal phases of these organisms (males,
581 females –gravid or not– and/or juveniles) are the ones in charge of looking for new
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761

762 **Figure Legends**

763 **Fig. 1.** Map of the study area indicating the location of the Bone Experiments and the
764 Controls (Harbour Control-14, Harbour Control-15, Seagull Control and Blanes
765 submarine canyon Control). Adapted from Vila & Serra (2015).
766
767 **Fig. 2.** Phylogenetic tree of *Ophryotrocha* based on the concatenated analyses of *COI*,
768 *16S* and *H3* from Bayesian inference analysis (BI). Left circles on the nodes refer to BI
769 while right circles refer to Maximum Likelihood analysis (ML). Red circles indicate
770 posterior probability values (PP) > 0.95 or bootstrap support (BS) > 75. Blue circles
771 indicate PP < 0.95 or BS < 75. White circles indicate that this topology was not
772 recovered in ML. Three major clades ('*labronica*', '*lobifera*' and '*hartmanni*') are
773 highlighted (see Taboada *et al*. 2013) and the new individuals included in our analyses
774 are in bold. For *O. puerilis* and *O. alborana* collected in this study only specimens
775 displaying the most divergent *COI* haplotypes were included.
776
777 **Fig. 3.** *COI* haplotype networks for **A** *Ophryotrocha alborana*, **B** *O. puerilis* 'Shallow',
778 **C** *O. puerilis sensu stricto*, and **D** *O. puerilis* 'Deep'. Circles are proportional to the
779 number of individuals for each haplotype. Number of mutations between haplotypes is
780 indicated with crossed lines. Color coding is indicated for every species; in black
781 missing inferred haplotypes.