

Phylogeography of *Ophryotrocha labronica* (Polychaeta,  
Dorvilleidae) along the Italian coasts

Running head: Phylogeography of *Ophryotrocha labronica*

**P. Cossu<sup>1,4,\*</sup>, F. Maltagliati<sup>1</sup>, F.G. Pannacciulli<sup>2</sup>, R. Simonini<sup>3</sup>, G. Massamba-  
N'Siala<sup>3</sup>, M. Casu<sup>4</sup>, C. Lardicci<sup>1</sup>, D. Prevedelli<sup>3</sup>, A. Castelli<sup>1</sup>**

<sup>1</sup>Dipartimento di Biologia, Via Derna 1, 56126, Pisa, Italy

<sup>2</sup>ENEA - Marine Environment Research Centre, PO Box 224, 19100, La Spezia,  
Italy

<sup>3</sup>Dipartimento di Scienze della Vita, Via Campi 213/d, 41125, Modena, Italy

<sup>4</sup>Dipartimento di Scienze della Natura e del Territorio, Via Muroni 25, 07100,  
Sassari, Italy

\*Email: picossu@uniss.it

1 ABSTRACT

2 Species of the genus *Ophryotrocha* are a well-studied group of organisms but,  
3 despite the relatively large body of biological studies, little is known about the intra-  
4 specific patterns of genetic diversity. In the present study, we analysed the patterns of  
5 genetic variation in samples of *Ophryotrocha labronica* (Polychaeta, Dorvilleidae)  
6 collected along the Italian coasts within three regions with different thermal regimes:  
7 the Northern Adriatic Sea (NAS), the Ligurian Sea (LS), and the south/south-east  
8 Sicilian Sea (SS). A partial sequence of the cytochrome c oxidase subunit I gene  
9 (COI) was used as a genetic marker. An analysis of molecular variance (AMOVA)  
10 showed significant genetic differentiation between the NAS and the other regions.  
11 Conversely, little or no genetic structuring was found between the LS and the SS or  
12 among locations within a given region. A Bayesian phylogenetic tree and a Median-  
13 joining network provided evidence for the occurrence of two highly divergent  
14 genetic lineages characterised by a high average sequence divergence (17.2%,  
15 Kimura 2-parameter distance). The spatial patterns of genetic variation found in *O.*  
16 *labronica* may reflect the signature of past expansion events of the two genetic  
17 lineages. Though the high sequence divergence suggested that cryptic speciation  
18 within *O. labronica* may have occurred, other traits such as the absence of  
19 reproductive isolation, the pattern of phenotypic variation, and the habitat specificity  
20 prompted us to regard the two groups as distinct COI lineages of *O. labronica*.

KEYWORDS

*Ophryotrocha labronica*, COI, genetic diversity, mtDNA lineages, Mediterranean Sea

## INTRODUCTION

21 The genus *Ophryotrocha* Claparède and Mecznirow (1869) (Annelida, Polychaeta)  
22 is a well-studied group of marine polychaetes inhabiting very diverse environments  
23 that range from polluted harbours to deep-sea sediments (see Thornhill *et al.* 2009 for  
24 a review). Many shallow-water species of *Ophryotrocha* can be easily reared in the  
25 laboratory due to their relatively short generation time and a variety of reproductive  
26 modes. These characteristics make the species of this genus ideal systems for a wide  
27 range of biological investigations, including ecological, behavioural, reproductive,  
28 developmental and toxicological studies (Thornhill *et al.* 2009 and references  
29 therein).

30         However, despite the relatively large body of biological studies on  
31 *Ophryotrocha* species, little is known about the intra-specific patterns of genetic  
32 diversity, though this information is necessary for understanding evolutionary and  
33 ecological processes such as dispersal, local adaptation and, ultimately, speciation.  
34 For instance, phenotypic variation along an environmental gradient or across habitats  
35 with different ecological conditions may reflect phenotypic plasticity or local  
36 adaptation. Investigating the extent to which the genetic and phenotypic variation are  
37 related may reveal whether the phenotypic variation reflect local adaptation or  
38 phenotypic plasticity (Reusch & Wood 2007). In addition, information on the  
39 species' genetic structure and the levels of gene flow among populations may shed  
40 light on the species' ability to cope with environmental changes (Zhakarow &  
41 Hellman 2008). Molecular tools are useful in delimiting species boundaries or  
42 revealing cryptic speciation. In particular, the identification of candidate cryptic  
43 species (Alonso *et al.* 2012 and references therein) may be important for the species  
44 used as models in different research fields. To tackle these issues, we used genetic

45 tools to analyse the patterns of intra-specific genetic diversity in one of the most  
46 well-known species of this genus, *Ophryotrocha labronica* La Greca & Bacci 1962.  
47 The species is actually subdivided into two partially reproductively isolated “sub-  
48 species” (Åkesson & Paxton 2005): *Ophryotrocha labronica pacifica* Paxton &  
49 Åkesson, 2007, which has been reported only in the Pacific Ocean, and  
50 *Ophryotrocha labronica labronica* La Greca & Bacci, 1962, which is typical of the  
51 Mediterranean and Lusitanian provinces, with isolated populations found in the  
52 Northern Red Sea and Southern Pacific. Here, we focus on *O. labronica labronica*  
53 (hereafter referred to as *O. labronica*) collected along the Italian coasts, within three  
54 distinct regions characterised by different thermal regimes (Simonini *et al.* 2010;  
55 Massamba N’Siala *et al.* 2011): a sub-continental temperate climate in the Northern  
56 Adriatic Sea (NAS), warm temperate climate in the Ligurian Sea (LS), and sub-  
57 tropical temperate climate in the south/south-east Sicilian Sea (SS).

58 *Ophryotrocha labronica* is a small-sized, gonochoristic polychaete (4 mm of  
59 maximum length) with semi-continuous reproduction and direct development,  
60 typically found in harbours and brackish-water environments (Paxton & Åkesson  
61 2007). Despite its very limited dispersal capabilities, the species displays a  
62 worldwide, patchy geographical distribution (Simonini *et al.* 2009; Prevedelli *et al.*  
63 2005). Long-distance dispersal due to human-mediated transport or other natural  
64 vectors (e.g., drifting and/or rafting) may be responsible for this distribution  
65 (Åkesson & Paxton 2005), though the overall causative factors are still debated  
66 (Simonini *et al.* 2010). *Ophryotrocha labronica* is by far the most common species of  
67 this genus along the Italian coasts, where its distribution is not affected by the  
68 climate regime and/or the type of fouling community in which it occurs (Simonini *et*  
69 *al.* 2010). Moreover, the species shows a complex pattern of phenotypic variation

70 that varies at both the local and regional scale (Massamba N'Siala *et al.* 2011).  
71 According to this study the heterogeneity of abiotic conditions within harbour  
72 habitats may have promoted the differentiation of isolated patches through local  
73 adaptation or random genetic drift. On the other hand, the environmental factors  
74 acting at a larger spatial scale, such as the thermal regime, may have shaped the  
75 observed pattern of phenotypic variation on the regional scale. Indeed, the life-  
76 history traits varied among the three regions (Massamba N'Siala *et al.* 2011).

77 The aim of this study was to analyse the pattern of spatial genetic structure  
78 within and among the aforementioned regions. In particular, we aimed to assess  
79 whether local adaptation, random genetic drift or population history were the main  
80 drivers of the species' genetic structure, if any was discovered. The patterns of  
81 genetic variation of *O. labronica* were examined using a partial sequence of the  
82 mitochondrial cytochrome c oxidase subunit I (COI) gene. This marker has proven  
83 effective in elucidating the phylogeographical structure and the underlying  
84 evolutionary history in polychaetes (e.g., Virgilio *et al.* 2009), as well as highlighting  
85 cryptic speciation (e.g., Audzjionyte *et al.* 2008; Carr *et al.* 2011).

86

## 87 MATERIALS AND METHODS

### 88 Sampling design

89 Samples of *Ophryotrocha labronica* were collected along the Italian coast  
90 following the procedures reported in Prevedelli *et al.* (2005). We adopted the  
91 sampling design described in Massamba N'Siala *et al.* (2011); four harbours within  
92 each region, and two locations within each harbour to check for small- scale genetic  
93 heterogeneity. However, only samples collected in 2007 were used in this study to  
94 avoid the potential confounding effects due to temporal genetic variability. Hence, it

95 was possible sampling two locations only at one harbour per region. The locations  
96 used in this study were: Grado site 3 (GRC), Chioggia site 1 (CHA), Ravenna site 1  
97 (RVA) and site 2 (RVB) and Rimini site 1 (RIA) for NAS; Genova site 1 (GEA) and  
98 site 2 (GEB), La Spezia site 1 (LSA), Viareggio site 2 (VIB) and Livorno site 1  
99 (LIA) for LS; Catania site 3 (CTC), Siracusa site 1 (SIA), Porto Palo di Capo Passero  
100 site 1 (PPA), Gela site 1 (GLA) and site 2 (GLB) for SS (Fig. 1).

101

#### 102 DNA extraction

103 The specimens were stored in absolute ethanol at -20 °C until DNA  
104 extraction. Whole genomic DNA was extracted from 150 specimens (10 individuals  
105 per sample) using the following procedure: Specimens were washed with sterile  
106 water to remove residual ethanol and placed in 1.5 ml tubes containing 60 µl of  
107 deionised sterile water (Sigma). After this osmotic shock, the specimens were boiled  
108 for 3 min at 100 °C in a thermal block. The tubes were then vortexed for 30 s and  
109 successively centrifuged at 3000 rpm for 2 min. The genomic DNA was stored at -20  
110 °C until the genetic analyses were performed.

111

#### 112 DNA sequencing and alignment

113 A partial sequence of the cytochrome c oxidase subunit 1 gene (COI) was  
114 amplified by Polymerase Chain Reaction (PCR) using the universal primers  
115 LCO1490 (5'-ggtaacaatacataaagatattgg-3') and HCO2198 (5'-  
116 taaacttcagggtgaccaaataatca-3) (Folmer *et al.* 1994). The amplifications were carried  
117 out in a 20 µl reaction containing ~30 ng of genomic DNA, 0.5 U of Euroclone *Taq*  
118 DNA polymerase, 1× *Taq* Buffer, 2.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP and 0.2 µM  
119 of each primer. The PCR reactions were performed on a MJ PTC 200 thermal cycler

120 with an initial denaturing step at 94 °C for 2 min, 35 amplification cycles (94 °C for  
121 30 s, 50 °C for 60 s, 72 °C for 90 s) and a final elongation step at 72 °C for 7 min.

122 The PCR products were directly sequenced by the BMR Genomics sequencing  
123 service (Padova, Italy).

124

125 Data analysis

126 The sequences were aligned using the algorithm CLUSTAL W implemented  
127 in MEGA 5 (Tamura *et al.* 2011) with the default settings and were checked  
128 manually. The levels of genetic polymorphism for each location and region were  
129 estimated by the number of haplotypes ( $N_h$ ), haplotype diversity ( $h$ ) and nucleotide  
130 diversity ( $\pi$ ) using DNAsp 5.10.01 (Librado & Rozas 2009). The Mann-Whitney  $U$ -  
131 test was used to test for differences in the haplotype and nucleotide diversity values  
132 between the three regions.

133 Hierarchical analysis of molecular variance (AMOVA) was used to quantify  
134 the partitioning of genetic variance within and among the localities and among the  
135 three regions. We used the approach implemented in the software Arlequin 3.5.1.3  
136 (Excoffier & Lischer 2010). The Tamura & Nei (1993) genetic distances with gamma  
137 distribution ( $\alpha = 0.346$ ) were used according to the most likely model of sequence  
138 evolution (TPM3uf+G) estimated with the J-ModelTest 0.1 (Posada 2008) using the  
139 default options. Likelihood scores were computed for each of the 88 models under  
140 the maximum likelihood framework and then the best fitting model was selected by  
141 the Akaike information criterion (AIC). We also computed pairwise  $\Phi_{ST}$  values  
142 among all location pairs. The significance of the variance components and pairwise  
143  $\Phi_{ST}$  values were assessed by a permutation test with 10,000 replicates; where

144 necessary, the false discovery rate method (FDR) was used to correct for multiple  
145 testing (Benjamini & Yekutieli 2001).

146         The phylogenetic relationships among the observed haplotypes were inferred  
147 using a Bayesian approach. Two published COI sequences of *Ophryotrocha japonica*  
148 were used as outgroups (GenBank accession number EF464541 and GQ415478). We  
149 chose this species as 1) it is not too phylogenetically divergent from *O. labronica*,  
150 and 2) being the second most common species along Italian coasts (Simonini et al.  
151 2010), we want to rule out even the most little chance of mtDNA introgression  
152 between the two species. The COI dataset was partitioned according to codon  
153 position, and for each of the resulting datasets, the most likely model of sequence  
154 evolution was estimated using J-ModelTest 0.1 (Posada 2008). Bayesian  
155 phylogenetic inference was carried out using MrBayes 3.1.2 (Ronquist &  
156 Huelsenbeck 2003). We used a partitioned model to estimate the parameters  
157 independently for each codon position with the following settings: nst = 1, rates =  
158 gamma for the first codon position, according to the model F81+G; nst = 2, rates =  
159 equal for the second codon position, according to the selected HKY model; nst = 6,  
160 rates = equal for the third codon position, according to selected TIM3ef model. The  
161 option UNLINK was used to estimate the parameters independently for each  
162 partition. With the other options set to the default values, two independent runs, each  
163 of four Metropolis-coupled MCMC chains (one cold and three heated chains), were  
164 run simultaneously to optimise the search in tree-space. The convergence of runs was  
165 assessed by checking that the average standard deviation of the split frequencies  
166 reached and stabilised at values less than 0.01 (Huelsenbeck & Ronquist 2001). Trees  
167 were sampled every 500 generations in each of the two independent runs, resulting in



168 a sample of 10,000 trees for each run (5,000,000 generations). The first 25% of  
169 sampled trees were discarded for each run yielding a total of 15,000 trees.

170 The genetic relationships among haplotypes were also analysed by a Median-  
171 Joining (MJ) network using the software Network 4.6.1.1 (Bandelt et al. 1999). We  
172 used the default options, as changing parameters did not improve the network.  
173 Finally, we used the Kimura 2-parameter (K2P) model to estimate the average  
174 sequence divergence within and among inferred clades. The analyses were carried  
175 out in Mega 5 (Tamura *et al.* 2011).

176 Historical population dynamics, such as past demographic expansions and/or  
177 bottlenecks, were inferred by comparing the observed mismatch distribution of the  
178 DNA substitution pairwise differences to a model of sudden population expansion  
179 (Rogers & Harpending 1992). Such distributions are unimodal when populations  
180 have experienced a recent expansion and multimodal at demographic equilibrium or  
181 when populations are significantly subdivided. Departures from the demographic  
182 expansion model were tested by comparing the sum of squared deviations (SSD)  
183 between the observed and the expected mismatch distribution under such a model.  
184 These analyses were carried out using Arlequin 3.5.1.3, and the significance of was  
185 assessed with 10,000 bootstrap replicates.

186 Tajima's (1989)  $D$  neutrality test was used to infer departures from  
187 equilibrium. The significance was assessed by 10,000 permutations in Arlequin  
188 3.5.1.3. Significant negative values are expected in cases of recent population  
189 expansion, population contraction or selective sweeps, whereas positive values are  
190 expected under balancing selection, population subdivision or recent bottlenecks  
191 (Soriano *et al.* 2008 and references therein). Moreover, to account for bias due to  
192 small sample size, Ramos-Onsins & Rozas' (2002)  $R_2$  statistic, which is considered

193 more powerful in such cases, was performed using DNAsp 5.10.01 (Librado &  
194 Rozas 2009) to test for selective neutrality and/or population expansion. The  
195 significance was tested by coalescent simulations with 10,000 permutations.

196

## 197 RESULTS

### 198 Patterns of genetic variation within and among regions

199         The successful sequencing of the 619 base pair fragment of the COI gene in  
200 135 out of 150 specimens of *Ophryotrocha labronica* distinguished 40 haplotypes  
201 (GenBank Accession numbers: KF305775 to KF305814). These haplotypes derived  
202 from 123 polymorphic sites (19.9%), of which 105 were parsimony informative.  
203 Most polymorphisms were due to single nucleotide changes (114 of the 123 sites)  
204 and 14 mutations produced amino acid replacements (non-synonymous changes).  
205 Thirty-three of the 40 haplotypes were location-private, 29 of which were  
206 represented by a single individual. By contrast, the three most common haplotypes,  
207 which were shared between different sampling sites, were present in 81 of the 135  
208 specimens (Supporting information, Table S1). These haplotypes showed a disjointed  
209 geographical distribution; haplotypes 1 and 2 were present only in the NAS, whereas  
210 haplotype 13 (observed in 53 of the 135 specimens) occurred in all localities of the  
211 SS and the LS, with the exception of Viareggio (VIB).

212         The mean nucleotide diversity across all locations was  $\pi = 0.008 \pm 0.001$ ,  
213 ranging from  $\pi = 0.000$  to  $0.082 \pm 0.014$  (Table 1). The samples from the NAS  
214 showed a mean nucleotide diversity ( $\pi = 0.007 \pm 0.001$ ) that was lower than the  
215 values from the SS and LS ( $\pi = 0.033 \pm 0.012$  and  $\pi = 0.056 \pm 0.010$ , respectively);  
216 however, these values were not significantly different (Mann-Whitney *U*-test; NAS  
217 vs. SS,  $p = 0.600$ ; NAS vs. LS,  $p = 0.600$ ; LS vs. SS,  $p = 0.917$ ). The higher mean

218 nucleotide diversities found in the LS and SS were due to some outlier samples that  
219 showed nucleotide diversities remarkably higher than those observed elsewhere:  
220 Genova 1 (GEA), Viareggio (VIB) and Livorno (LIA) in the LS and Porto Palo di  
221 Capo Passero (PPA) in the SS.

222 Similarly, the values of haplotype diversity were not significantly different  
223 between the three regions (Mann-Whitney *U*-test; NAS vs. SS,  $p = 0.151$ ; NAS vs.  
224 LS,  $p = 0.690$ ; LS vs. SS,  $p = 0.841$ ). The lowest value was observed in the SS and  
225 was mainly due to the presence of a single haplotype at Gela site 1 (GLA). The  
226 highest haplotype diversity was observed in the VIB sample from the LS ( $h = 0.933$   
227  $\pm 0.077$ ).

228 Significant genetic differentiation, which accounted for 63.1% of the total  
229 variation (Table 2), was observed among the three regions (AMOVA,  $\Phi_{CT} = 0.631$ ,  $p$   
230  $< 0.001$ ). Such genetic structure was almost entirely due to genetic divergence  
231 between the NAS and the other regions, as evidenced by pairwise  $\Phi_{ST}$  values (Table  
232 3). Indeed, 39 of the 44 comparisons that showed significant genetic differentiation  
233 after correction for multiple testing involved comparisons between the Adriatic and  
234 non-Adriatic samples. Two samples, PPA (IS) and VIB (LS), were not genetically  
235 divergent from any of the Adriatic samples. Furthermore, the LIA sample (LS) was  
236 not genetically divergent from the northernmost sample from the NAS (GRC). No  
237 other clear patterns of genetic structure emerged (Table 3). Non-significant genetic  
238 divergence was found in most comparisons between the LS and SS, as well as within  
239 each region (50 of the 55 location pairwise  $F_{ST}$  values). Exceptions to this  
240 observation involved the VIB and PPA samples (Table 3).

241

242 Phylogenetic analyses

243           The Bayesian phylogenetic tree evidenced two well-supported, reciprocally  
244 monophyletic clades (Fig. 2), hereafter referred to as haplogroups A (HG-A) and B  
245 (HG-B). The deep genetic divergence between HG-A and HG-B, as compared to  
246 genetic variation within haplogroups, is highlighted by the MJ network (supporting  
247 information, Fig. S1). The two haplogroups showed a different, though partially  
248 overlapping, geographical distribution (Fig. 1 and Fig. S1). HG-A haplotypes were  
249 present in all three of the regions, whereas HG-B haplotypes were absent in the NAS.  
250 Conversely, HG-B haplotypes were dominant at most sampling sites in the LS and  
251 SS regions. At localities where both types occurred sympatrically, HG-B  
252 predominated at the GEA and LIA sites (LS), whereas HG-A was the most prominent  
253 haplogroup at the VIB (LS) and PPA (IS) sites.

254           A total of 90 mutations (83 of which were fixed) distinguished the two  
255 haplogroups, resulting in an average Kimura 2-Parameter (K2P) distance of 17.2%.  
256 The divergence between the haplogroups was 18.3 and 41.9 times larger than the  
257 average sequence divergence within HG-A (K2P = 0.94%) or HG-B (K2P = 0.41%),  
258 respectively. HG-A displayed a deeper, more structured phylogeny than HG-B (Fig. 2  
259 and Fig. S1). Most haplotypes were restricted to a single region, and only 3 of the 18  
260 haplotypes were shared between two regions. Nonetheless, HG-A did not show a  
261 clear-cut geographical structuring; the haplotypes did not cluster into sub-clades  
262 corresponding sharply to the three regions. Instead, haplotypes from different regions  
263 may be as genealogically related as haplotypes from the same region. Conversely, no  
264 genetic structuring was found within the HG-B haplogroup, for which the network  
265 revealed a star-like phylogeny (Fig. S1).

266

267 Demographic history

268           The two haplogroups showed different mismatch distributions of pairwise  
269 DNA differences (supporting information, Fig. S2); both of which, however, fit the  
270 model of demographic expansion (goodness-of-fit test, Table 4). Conversely, HG-A  
271 and HG-B evidenced discordant signatures of departure from equilibrium models  
272 according to the neutrality tests (Table 4). Only the HG-B haplotypes departed from  
273 an equilibrium model; the outcome of the neutrality tests were consistent with the  
274 results of the mismatch distributions, as significant and negative values of Tajima's  
275  $D$  and low values of the  $R_2$  statistic were found (Table 4).

276

## 277 DISCUSSION

278           In this study, we analysed the patterns of intra-specific genetic variation in  
279 samples of *Ophryotrocha labronica* from three distinct regions of the Mediterranean  
280 Sea: the Northern Adriatic Sea (NAS), Ligurian Sea (LS) and south/south-east  
281 Sicilian Sea (SS). We found significant genetic differentiation between the NAS and  
282 the other two regions, whilst shallow or no genetic structuring was found within each  
283 region or between the LS and SS. This genetic pattern is strongly characterised by  
284 the occurrence of two highly divergent COI lineages, here referred to as haplogroups  
285 A (HG-A) and B (HG-B), which display different frequencies and geographical  
286 distributions across the three regions.

287

### 288 Patterns of genetic diversity

289           Estimates of within-location genetic diversity (Table 1) were comparable to  
290 those found in other polychaete species (Virgilio *et al.* 2009 and references therein).  
291 Additionally, following Grant & Bowen's (1998) considerations about historical  
292 demographic inference, only six of the 15 locations of *O. labronica* in the present

293 study displayed values of haplotype and nucleotide diversity that were consistent  
294 with the occurrence of a recent bottleneck (GLA location) or a rapid expansion after  
295 a genetic bottleneck (GEB, LSA, CTC, SIA and GLB locations). The marked  
296 temporal and spatial fluctuations in the density of *O. labronica* populations  
297 (Prevedelli *et al.* 2005; Åkesson & Paxton 2005) may account for these results. At  
298 larger spatial scales, the absence of a significant variation in haplotype and  
299 nucleotide diversity across the regions suggests that different environmental  
300 conditions have little influence on the amount of genetic variation within these  
301 locations. This result may reflect the adaptive plasticity of *O. labronica* to the  
302 different environmental conditions that characterise each region. Indeed, populations  
303 of species with a limited adaptive potential may undergo a depletion of genetic  
304 variability if they are not well adapted to environmental conditions, as suggested in  
305 the copepod *Tigriopus californicus* along a thermal gradient (Kelly *et al.* 2012).

306         Alternatively, genetic variation may be maintained by local adaptation driven  
307 by evolution under spatially divergent selection, which requires a set of phenotypes  
308 with maximised fitness in a given habitat type and limited gene flow (Kawecki &  
309 Ebert 2004). In *O. labronica*, life-history traits related to fitness highlighted a  
310 significant differentiation between the LS and the other two regions, the SS and NAS  
311 (Massamba N'Siala *et al.* 2011). Conversely, hierarchical AMOVA (Table 2) and  
312 pairwise  $\Phi_{ST}$  values (Table 4) evidenced a genetic architecture that was partially  
313 different from the pattern of phenotypic variation, showing a remarkable genetic  
314 divergence between the NAS and the other regions. Overall, these results suggest that  
315 either phenotypic plasticity is driving the adaptation to different environmental  
316 regimes or the patterns of COI variation and the life-history traits (Massamba  
317 N'Siala *et al.* 2011) are not related. Indeed, the patterns of genetic variation may

318 reflect genetic drift and/or historical vicariant events, rather than local adaptation  
319 (Kawecki & Ebert 2004).

320

321 Phylogeographical patterns

322         The Adriatic Sea represents a region of phylogeographical discontinuity  
323 within the Mediterranean Sea (Patarnello *et al.* 2007). Here, historical and present-  
324 day barriers to dispersal might have promoted genetic divergence in the Adriatic  
325 populations of many invertebrate species (Virgilio *et al.* 2009 and references therein).  
326 However, the picture may be more complex in *O. labronica*. First, the two main  
327 genetic lineages (HG-A and HG-B) evidenced by the phylogenetic tree (Fig. 2) and  
328 network (Fig. S1) did not show a completely separate geographical distribution, but  
329 were sympatric at some locations in the LS and SS regions (Fig. 1). However,  
330 sympatric divergence is unlikely to occur in *O. labronica*: cross-breeding  
331 experiments showed that individuals collected from the same locations used in this  
332 study, including those in which HG-A and HG-B co-occur, were interfertile  
333 (Massamba N'Siala *et al.* 2011).

334         Taking into account the high level of average sequence divergence between  
335 HG-A and HG-B, allopatric divergence followed by secondary contact is a more  
336 likely explanation than sympatric divergence. Indeed, in geographically isolated  
337 populations, mutations may accumulate for a long evolutionary time before  
338 reproductive barriers arise, if they ever arise (Norris & Hull 2011). As suggested by  
339 Virgilio *et al.* (2009), concerning the Adriatic and non-Adriatic haplotypes of the  
340 polychaete *Hediste diversicolor* in the Mediterranean, the divergence may be older  
341 than the re-flooding of the northern and central Adriatic basins subsequent to the end  
342 of the last ice age (ca. 18,000 years ago). Such a scenario is consistent with our

343 results on *O. labronica*, as *Ophryotrocha* species may easily survive in ballast waters  
344 and in fouling communities on ship hulls (Åkesson & Paxton 2005); hence, vessel-  
345 mediated dispersal may promote the secondary contacts of geographically isolated  
346 lineages. The chance of dispersal by ship transport, even over long distances, is  
347 supported by Simonini *et al.* (2009) observations of *Ophryotrocha japonica* and  
348 *Ophryotrocha diadema*, which were recently introduced into the Mediterranean  
349 through vessel-mediated dispersal, and by Åkesson & Paxton's (2005) findings on  
350 the population of *O. labronica* recorded in the Sydney harbour (Australia), which  
351 may have reached the Pacific Ocean through the same mechanism.

352

### 353 Demographic history

354         The lack of genetic structure observed within each lineage of *O. labronica*  
355 and within each region, as well as the non-significant genetic divergence between the  
356 LS and SS, contradicts the expectation of deep genetic structuring in species without  
357 pelagic larvae. Along with the contribution to gene flow provided by human-  
358 mediated dispersal, drifting, or rafting (e.g., Simonini *et al.* 2010 and references  
359 therein), other processes such as bottlenecks, expansions and recent colonisations  
360 may have contributed to the observed patterns. Patarnello *et al.* (2007) showed that  
361 recent expansions and/or bottlenecks may explain the lack of genetic structure in  
362 several Mediterranean species.

363         The demographic history of the HG-B haplotypes is consistent with a recent  
364 bottleneck/expansion scenario, with a clear unimodal mismatch distribution (Fig.  
365 S2e, f and g). Furthermore, the concordant results of all neutrality tests (Table 4)  
366 point to population growth rather than other alternative scenarios (Ramos-Onsin &  
367 Rozas 2002; Soriano *et al.* 2008). The picture is more complex for the HG-A



368 haplotypes; notwithstanding a multimodal pattern (Fig. S2a, b, c and d), the observed  
369 mismatch distributions still fit a model of demographic expansion according to the  
370 goodness-of-fit test, whereas none of the neutrality tests showed significant  
371 departures from an equilibrium model (Table 4). However, the observed pattern does  
372 not necessarily imply a picture of demographic stability; multimodal mismatch  
373 distributions combined with slightly negative, but non-significant, Tajima's  $D$  values  
374 may reflect mild population expansions combined with heterogeneity of mutation  
375 rates (Aris-Brosou & Excoffier 1996).

376         Alternatively, these results fit the outcome observed in the range expansion  
377 model, in which genetically structured populations exchange genes (Ray *et al.* 2003).  
378 Under these conditions, the mismatch distributions display multimodal patterns and  
379 the neutrality tests may fail to reject departure from equilibrium. Furthermore, as  
380 expected under this model for moderate migration rates (Excoffier 2004), the  
381 multimodal mismatch distribution of the HG-A haplotypes showed a peak close to  
382 the origin. Patarnello *et al.* (2007) suggested that similar patterns of mismatch  
383 distributions reflect multiple expansion events. Moreover, expansions after a  
384 population size reduction may determine the lack of genetic structure observed in  
385 some species, even in the absence of gene flow. Indeed, fluctuations in abundance  
386 over time and variance in reproductive success may lead to shallow genetic  
387 structuring in marine species (Grant & Bowen 1998). This picture fits with the  
388 metapopulation scenario invoked for *O. labronica*, an opportunistic species  
389 subdivided into many local populations that proliferate under favourable conditions  
390 and may rapidly go extinct to be replaced by new populations (Åkesson & Paxton  
391 2005; Prevedelli *et al.* 2005).

392

393 Genetic differentiation between haplogroups

394           Our results raise the question whether the two haplogroups may be the result  
395 of cryptic speciation. The occurrence of cryptic species is rather common within the  
396 polychaetes (Carr *et al.* 2011 and references therein). The average sequence  
397 divergence between the two lineages detected in the present study (K2P distance =  
398 17.2%) was comparable to the values of COI divergence found among polychaete  
399 cryptic species. For instance, the COI sequencing of 1876 specimens from 142  
400 morphologically identified species of Canadian polychaetes yielded a total of 333  
401 divergent lineages, with a mean K2P distance between the genetic clusters that  
402 averaged 16.5% (Carr *et al.* 2011). In the ragworm *Hediste diversicolor*, the mtDNA  
403 sequence divergence among distinct lineages and/or across different geographical  
404 regions ranged between 4.5 and 8% (Virgilio *et al.* 2009). At least two of these  
405 lineages are likely to be cryptic species, consistent with the results of studies that  
406 used nuclear molecular markers (Audzijonyte *et al.* 2008). Moreover, the mean  
407 sequence divergence between the haplogroups of *O. labronica* was 18 and 43 times  
408 higher than within-haplogroup variation for HG-A and HG-B (K2P distance = 0.94%  
409 and 0.41%), respectively. These ratios exceeded the threshold used for the COI  
410 barcoding gap, by which inter-specific variation is expected to be at least ten times  
411 larger than intra-specific variation (Hebert *et al.* 2004).

412 Although COI proved its effectiveness for DNA barcoding and species identification  
413 in polychaetes (Carr *et al.* 2011), caution is needed when inferring cryptic speciation  
414 in *O. labronica*. Cryptic polychaete species inferred by COI barcoding usually  
415 showed differences in other traits, such as those related to life- history, ecology,  
416 habitat specificity and/or the presence of reproductive isolation (Carr *et al.* 2011 and  
417 references therein). The individuals of the two haplogroups found in *O. labronica*

418 were neither reproductively isolated, nor did they show habitat specificity. Moreover,  
419 the patterns of mtDNA and life-history trait variation were poorly correlated, and the  
420 geographical distribution of the haplogroups could not be straightforwardly linked to  
421 ecological differences among the three regions. Hence, given the absence of such  
422 lines of evidence and the comparison of mtDNA data with nuclear markers,  
423 following the recommendations of Bickford *et al.* (2007), HG-A and HG-B should be  
424 regarded as distinct mtDNA lineages of the species *O. labronica*.

425

#### 426 ACKNOWLEDGEMENTS

427 This research was supported by the national PRIN 2005 project (prot. 2005050952),  
428 funded by the Italian *Ministero dell'Istruzione, dell'Universita e della Ricerca*.

429 Genetic analyses were carried out at the Molecular Ecology laboratory of the ENEA,  
430 Marine Environment Research Centre Santa Teresa, la Spezia, Italy.

431

#### 432 LITERATURE CITED

433 Åkesson B., Paxton H. (2005) Biogeography and incipient speciation in *O. labronica*  
434 (Polychaeta, Dorvilleidae). *Marine Biology Research*, **1**, 27–139.

435 Alonso R., Crawford A.J., Bermingham E. (2012) Molecular phylogeny of an  
436 endemic radiation of Cuban toads (Bufonidae: Peltophryne) based on  
437 mitochondrial and nuclear genes. *Journal of Biogeography*, **39**, 434–451.

438 Aris-Brosou S., Excoffier L. (1996) The impact of population expansion and  
439 mutation rate heterogeneity on DNA sequence polymorphism. *Molecular Biology*  
440 *and Evolution*, **13**, 494–504.

441 Audzijonyte A., Ovcarenko I., Bastrop R., Väinölä R. (2008) Two cryptic species of  
442 the *Hediste diversicolor* group (Polychaeta, Nereididae) in the Baltic Sea, with

443 mitochondrial signatures of different population histories. *Marine Biology*, **155**,  
444 599–612.

445 Bandelt H.J., Forster P., Rohl A. (1999) Median-joining networks for inferring  
446 intraspecific phylogenies. *Molecular Biology and Evolution*, **16**: 37–48.

447 Benjamini Y., Yekutieli D. (2001) The control of the false discovery rate in multiple  
448 testing under dependency. *The Annals of Statistics*, **29**, 1165–1188.

449 Bickford D., Lohman D.J., Sodhi N.S., Ng P.K.L., Meier R., Winker K., Ingram  
450 K.K., Das I. (2007) Cryptic species as window on diversity and conservation.  
451 *Trends in Ecology and Evolution*, **22**, 148–155.

452

453 Carr C.M., Hardy S.M., Brown T.M., Macdonald T.A., Hebert P.D. (2011) A Tri-  
454 Oceanic perspective: DNA barcoding reveals geographic structure and cryptic  
455 diversity in Canadian polychaetes. *PLoS One*, **6**, e22232.

456

457 Excoffier L. (2004) Patterns of DNA sequence diversity and genetic structure after a  
458 range expansion: lessons from the infinite-island model. *Molecular Ecology*, **13**,  
459 853–864.

460 Excoffier L., Lischer H.E.L. (2010) Arlequin suite ver 3.5: A new series of programs  
461 to perform population genetics analyses under Linux and Windows. *Molecular*  
462 *Ecology Resources*, **10**, 564–567.

463 Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R. (1994) DNA primers for  
464 amplification of mitochondrial cytochrome c oxidase subunit I from diverse  
465 metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–  
466 296.

467 Grant W.A.S., Bowen B.W. (1998) Shallow population histories in deep evolutionary  
468 lineages of marine fishes: insights from sardines and anchovies and lessons for  
469 conservation. *Journal of Heredity*, **89**, 415–426.

470 Hebert P.N., Stoeckle M.Y., Zemplak T.S., Francis C.M. (2004) Identification of birds  
471 through DNA barcodes. *PLoS Biology*, **2**, 1657–1663.

472 Huelsenbeck J.P., Ronquist F. (2001) MRBAYES: Bayesian inference of phylogeny.  
473 *Bioinformatics*, **17**, 754–755.

474

475 Kawecki T.D., Ebert D. (2004) Conceptual issues in local adaptation. *Ecology*  
476 *Letters*, **7**, 1225–1241.

477 Kelly M.W., Sanford E., Grosberg R.K. (2012) Limited potential for adaptation to  
478 climate change in a broadly distributed marine crustacean. *Proceeding of the*  
479 *Royal Society B*, **279**, 349–356.

480 Librado P., Rozas J. (2009) DnaSP v5: A software for comprehensive analysis of  
481 DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.

482 Massamba-N’Siala G., Simonini R., Cossu P., Maltagliati F., Castelli A., Prevedelli  
483 D. (2011) Life-history and demographic spatial variation in Mediterranean  
484 populations of the opportunistic polychaete *Ophryotrocha labronica* (Polychaeta,  
485 Dorvilleidae). *Marine Biology*, **158**, 1523–1535.

486 Norris R.D., Hull P.M. (2012) The temporal dimension of marine speciation.  
487 *Evolutionary Ecology*, **26**, 393–415.

488 Patarnello T., Volckaert F.A.M.J., Castilho R. (2007) Pillars of hercules: is the  
489 Atlantic-Mediterranean transition a phylogeographical break? *Molecular*  
490 *Ecology*, **16**, 4426–4444.

- 491 Paxton H., Åkesson B. (2007) Redescription of *Ophryotrocha puerilis* and *O.*  
492 *labronica* (Annelida, Dorvilleidae). *Marine Biology Research*, **3**, 3–19.
- 493
- 494 Posada D. (2008) jModelTest: Phylogenetic Model Averaging. *Molecular Biology*  
495 *and Evolution*, **25**, 1253–1256.
- 496
- 497 Prevedelli D., Massamba-N'Siala G., Simonini R. (2005) The seasonal dynamics of  
498 six species of Dorvilleidae (Polychaeta) in the harbour of La Spezia (Italy).  
499 *Marine Ecology*, **26**, 286–293.
- 500 Ramos-Onsins S.E., Rozas J.E. (2002) Statistical properties of new neutrality tests  
501 against population growth. *Molecular Biology and Evolution*, **19**, 2092–2100.
- 502 Ray N., Currat M., Excoffier L. (2003) Intra-deme molecular diversity in spatially  
503 expanding populations. *Molecular Biology and Evolution*, **20**, 76–86.
- 504 Reusch T.B.H., Wood T.E. (2007) Molecular ecology of global change. *Molecular*  
505 *Ecology*, **16**, 3973–3992.
- 506 Rogers A.R., Harpending H. (1992) population growth makes waves in the  
507 distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9**,  
508 552–569.
- 509 Ronquist F., Huelsenbeck J.P. (2003) mrbayes 3: Bayesian phylogenetic inference  
510 under mixed models. *Bioinformatics*, **19**, 1572–1574.
- 511 Simonini, R., Massamba-N'Siala, G., Prandi, V., Prevedelli, D. (2009) Distribution of  
512 the genus *Ophryotrocha* (Polychaeta) in Italy: new records and comments on the  
513 biogeography of Mediterranean species. *Vie et Milieu*, **59**, 79–88.
- 514 Simonini R., Grandi V., Massamba-N'Siala G., Martino M.P., Castelli A., Prevedelli  
515 D. (2010) Diversity, habitat affinities and diet of *Ophryotrocha* species

516 (Polychaeta, Dorvilleidae) living in Mediterranean harbour habitats. *Vie et Milieu*,  
517 **60**, 27–38.

518 Soriano A.R., Ramos-Onsins S.E., Rozas J., Calafell F., Navarro A. (2008) Statistical  
519 power analysis of neutrality tests under demographic expansions, contractions  
520 and bottlenecks with recombination. *Genetics*, **179**, 555–567.

521 Tajima F. (1989) Statistical method for testing the neutral mutation hypothesis by  
522 DNA polymorphism. *Genetics*, **123**, 585–589.

523 Tamura K., Nei M. (1993) Estimations of the number of nucleotide substitutions in  
524 the control region of mitochondrial DNA in humans and chimpanzees. *Molecular*  
525 *Biology and Evolution*, **10**, 512–526.

526 Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. (2011) MEGA5:  
527 Molecular Evolutionary Genetics Analysis using Maximum Likelihood,  
528 Evolutionary Distance, and Maximum Parsimony methods. *Molecular Biology*  
529 *and Evolution*, **28**, 2731–2739.

530 Thornhill D.J., Dahlgren T.G., Halanych K. (2009) The evolution and ecology of  
531 Ophryotrocha (Dorvilleidae, Eunicida). In: D.H. Shain (Eds). *Annelids as Model*  
532 *Systems in the Biological Sciences*. John Wiley and Sons: 242–256.

533 Virgilio M., Fauvelot C., Costantini F., Abbiati M., Backeljau T. (2009)  
534 Phylogeography of the common ragworm *Hediste diversicolor* (Polychaeta:  
535 Nereididae) reveals cryptic diversity and multiple colonization events across its  
536 distribution. *Molecular Ecology*, **18**, 1980–1994.

537 Zakharov E.V., Hellmann J.J. (2008) Genetic differentiation across a latitudinal  
538 gradient in two co-occurring butterfly species: revealing population differences in  
539 a context of climate change. *Molecular Ecology*, **17**, 189–208.

540 TABLE LEGENDS

541

542 Table 1: Estimates of genetic diversity.  $N$ : sample size;  $N_h$ : number of haplotypes;

543  $N_p$ : number of polymorphic sites;  $h$ : haplotype diversity ( $\pm$  standard deviation);  $\delta$ :

544 nucleotide diversity ( $\pm$  standard deviation)

545

546 Table 2: Results of the hierarchical analysis of molecular variance (AMOVA) with

547 groups corresponding to: Northern Adriatic Sea (NAS); Ligurian Sea (LS);

548 south/south-east Sicilian Sea (SS).

549

550 Table 3: Pairwise  $\Phi_{ST}$  values between samples. Values that were significant at  $P <$

551 0.05 after the correction for multiple testing are in bold. The false discovery rate

552 method (FDR) according to Benjamini and Yekutieli (2001) was applied to detect the

553 false positives (Type I error). Label codes are reported in Table 1

554

555 Table 4: Goodness-of-fit tests for mismatch distributions and neutrality tests for the

556 two main lineages and the three regions considered in this study. Goodness-of-fit

557 test: SSD, sums of squared deviations. Neutrality tests:  $D$ , Tajima's (1989) neutrality

558 test;  $R_2$ , Ramos-Onsins & Rozas' (2002) statistics. Significant values are outlined in

559 bold





## FIGURE CAPTIONS

560 Figure 1: a) Geographical location of sampling sites. GRC: Grado (45°40'N,  
561 13°23'E); CHA: Chioggia (45°13', 12°16'E); RVA and RVB: Ravenna (44°28'N,  
562 12°15'E); RIA: Rimini (44°05'N, 12°33'E); GEA and GEB: Genova (44°24'N,  
563 8°54'E); LSA: La Spezia (44°04'N, 9°49'E); VIB: Viareggio (43°51'N, 10°14'E);  
564 LIA: Livorno (43°33'N, 10°18'E); CTC: Catania (37°29'N, 15°05'E); SIA: Siracusa  
565 (37°03'N, 15°17'E); PPA: Porto Palo di Capo Passero (36°40'N, 15°07'E); GLA and  
566 GLB: Gela (37°04'N, 14°03'E). The piecharts depict the Geographical distribution  
567 of the two COI lineages (HG-A and HG-B) at each sampling location

568 Figure 2: Bayesian tree showing the phylogenetic relationships among COI  
569 haplotypes. The two COI lineages are denoted with HG-A (Haplogroup A) and HG-  
570 B (Haplogroup B). Tree was rooted using two sequences of *Ophryotrocha japonica*  
571 as outgroups. Only nodes supported by posterior probabilities  $\geq 95\%$  are reported.  
572 Localities in which were found haplotypes and their frequency are reported in Table  
573 S1 (supplementary material)

574

575

576

577 SSSS