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Spatial and temporal genetic structure of *Velella velella* (Hydrozoa, Porpitidae) and its predator *Janthina pallida* (Gastropoda, Epitoniidae)

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Morello B., Bo M., Betti F., Bavestrello G., Abbiati M., Costantini F. (2023). Spatial and temporal genetic structure of *Velella velella* (Hydrozoa, Porpitidae) and its predator *Janthina pallida* (Gastropoda, Epitoniidae). *HYDROBIOLOGIA*, 850(8), 1-1751 [10.1007/s10750-022-05089-z].

Availability:

This version is available at: <https://hdl.handle.net/11585/911458> since: 2023-05-17

Published:

DOI: <http://doi.org/10.1007/s10750-022-05089-z>

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1 **Spatial and temporal genetic structure of *Veleva veleva* (Hydrozoa, Porpitiidae) and its predator *Janthina pallida***
2 **(Gastropoda, Epitoniidae)**

3
4
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16
17 **ABSTRACT**

18
19 The by-the-wind sailor *Veleva veleva*, is a cosmopolitan, pleustonic colonial hydrozoan, commonly in the Mediterranean
20 Sea. It forms offshore aggregations that occasionally strand on the coasts, following strong wind conditions. Colonies are
21 often found associated with *Janthina pallida*, a pleustonic gastropod that floats thanks to the secretion of a bubble raft,
22 and which prey on them. Because of the massive occurrence of these species along the Italian coasts, and their often
23 neglected ecological roles, it is pivotal to increase the knowledge of their genetic structure to improve monitoring
24 activities and to provide elements to further understand the functioning of Mediterranean pleustonic zooplankton.
25 Analyses of the genetic variability of *V. veleva* and *J. pallida*, stranded on the beach in springs 2016 and 2017 at ten
26 locations along the Ligurian and Tyrrhenian seas, show the existence of panmictic Mediterranean populations.
27 Nevertheless, *V. veleva* showed high genetic variability with a high number of singleton haplotypes, while *J. pallida*
28 showed a recent population expansion. Considering the ecological and socio-economic importance of gelatinous
29 zooplankton in today’s changing marine ecosystems, these results lay the basis for better understanding the ecological
30 role and population dynamics of pleustonic species.

31
32
33 **KEYWORDS:** Hydrozoa, Mollusca, stranding, molecular markers, Mediterranean Sea
34

35 1. INTRODUCTION

36 Many marine planktonic species are subjected to periodic blooms that usually depend on their peculiar explosive
37 reproductive strategies, often coupled with the insurgence of specific environmental conditions (including temperature,
38 pH, presence of upwellings, ...) and variations in biological constraints (e.g., changes in the abundance of preys and
39 predators) (e.g., Evans & Parslow 1985, Graham et al. 2001, Findlay et al. 2006, Suthers et al. 2019, Helm 2021). Such
40 copious and rapid growth in population size plays an important role in the functioning of marine ecosystems and it is now
41 understood that the occurrence of these phenomena is an essential component of a good and healthy ecosystem (Boero et
42 al. 2008, Boero 2013, Suther et al. 2019, Jones et al. 2021).

43 Pleustonic zooplankton include many species that float on the water-air interface, dragged by wind and superficial
44 currents. Widespread distribution and seasonal bloom events commonly characterize them. One of the most typical
45 pleustonic species is the by-the-wind sailor *Velevella velevella* (Linnaeus, 1758), a circumtropical hydrozoan belonging to the
46 family Porpitidae (Schuchert 2010), characterized by a floating polymorphic colony (Figure 1a) (Larson 1980, Ricketts
47 & Calvin 1997, Bouillon et al. 2004, Schuchert 2010). The life cycle of *V. velevella* is little known. It includes the release
48 of many tiny, zooxanthellate, epipelagic medusae, which supposedly generate planula larvae (never observed to date)
49 (Woltereck 1904, Schuchert 2010, Helm 2021).

50 Large-scale offshore blooms of *V. velevella* (Figure 1b) occur seasonally in the Western Mediterranean Sea, reaching a peak
51 of abundance in spring; this mass occurrence, combined with specific environmental factors, often result in pulse
52 strandings along the coasts from April to June (Boero et al. 2016, Pires et al. 2018), with average densities ranging from
53 about 600 to 11400 colonies m⁻² (Betti et al. 2019).

54 *V. velevella* strandings are often associated with the beaching of other pleustonic organisms, like the violet sea-snails
55 *Janthina* spp. These gastropods are holoplanktonic organisms living upside down on the lower side of a raft created by
56 enveloping air bubbles in a mucus layer (Lalli & Gilmer 1989) (Figure 1b). The reproduction is gonochoric, with aphyllid
57 males that produce sperms that swim into the female's genital tract, where fertilization occurs (Graham 1954). Fertilized
58 eggs are subsequently glued to the raft; embryos develop into planktonic trochophore larvae and later into juvenile veligers
59 before becoming fully grown adults (Beu 2017). A massive exceptional stranding of *Janthina pallida* (Thompson 1841),
60 with average densities ranging from 13 to 2400 individuals m⁻², was recently reported along the Ligurian coasts (Betti et
61 al. 2017). *Velevella velevella* and *J. pallida* are linked by a prey-predator interaction (Lepoint et al. 2016, Helm 2021),
62 therefore the feeding activity of the gastropod may play a role in controlling the impact that dense aggregations of *V.*
63 *velevella* have on fish recruitment and macroplanktonic assemblages (Purcell et al. 2015).

64 Considering the lack of empirical data on the genetic structure and diversity of these two pleustonic species, mitochondrial
65 markers (COI and 16S) were used to 1) evaluate the genetic variability and structure of these species along the Ligurian
66 and Tyrrhenian coasts and in two different years, and 2) evaluate the phylogenetic relationship of the two species with
67 their close relatives.

68 Recognizing the evolutionary history and genetic patterns of connectivity of the populations of the investigated species
69 is pivotal to better understand the processes that drive their very complex reproductive strategies (especially in Epitoniidae
70 gastropods) and their prey-predator specificity. Although herein we did not analyze nuclear markers, the results of this
71 work can be used as a first attempt to monitor changes in the parameters of population genetic and to better understand
72 the processes driving spatio-temporal dynamics of blooms in the Mediterranean Sea (Abboud et al. 2018).

73

74 **2. MATERIAL AND METHODS**

75 We collected specimens of both species along the Ligurian and Tyrrhenian coasts during April and May 2016 and 2017.
76 Sixty-nine individuals of *Verella verella* were collected (38 in 2016 and 31 in 2017), 50 along the Ligurian coasts and 19
77 along the Tyrrhenian ones. Contextually, 76 specimens of *Janthina pallida* (60 in 2016 and 16 in 2017) were collected in
78 the Ligurian area (Table 1, Figure 1c). Specimens from the Ligurian Sea were collected from the beaches of Arenzano,
79 Santa Margherita Ligure and Livorno, and from a bank one nautical mile (NM) off the Genoa harbor; the sampling sites
80 are stretched along about 150 km long NW Italian coasts. The Tyrrhenian sites included the beaches of Passoscuro (Lazio)
81 and Cagliari (Sardinia Island) (Table 1), around 250 km and 500 km distant from the closest Ligurian site (Livorno),
82 respectively (Figure 1c). All the collected organisms were preserved in 80% ethanol alcohol.

83 Genomic DNA was extracted from preserved samples using the CetylTrimethylAmmonium Bromide (CTAB) protocol
84 (Winnepennickx et al. 1993), with minor modifications to optimize DNA extraction quality and yield.

85 Before choosing the markers to use in this paper, we tested several universal primers amplifying both mtDNA genes for
86 both species and we found that COI successfully could be amplified in all *V. verella* individuals and 16S in *J. pallida*
87 individuals. In fact, like all non-model organisms, there were very few markers and sequences available in the literature
88 for *V.verella* (10 sequences available on GenBank) and *J. pallida* (no sequences available, only 14 for *Janthina* genus).
89 Additionally, for both species, no previous studies were performed to test the population genetic variability of 16S and
90 COI markers. Moreover, previous studies on several scyphozoans used COI sequences to detect population genetic
91 structure at various spatial scales (e.g. Lee et al. 2013; Dawson et al. 2015).

92 Here, a portion of the mitochondrial region codifying for the Cytochrome Oxidase subunit I (COI) was amplified for *V.*
93 *verella* using the universal COI primers LCO1490 and HCO2198 (Folmer et al. 1994) and thermocycle conditions outlined

94 in Ortman et al. (2010). For *J. pallida*, a portion of the mitochondrial 16S ribosomal DNA was amplified using universal
95 16S primers developed by Palumbi (1996). For both species, PCR was performed in a total volume of 25 μ l, including
96 2.5 μ l buffer 10X, 2.5 μ l $MgCl_2$ 25 mM, 0.5 μ l dNTPs 10 mM, 1.2 μ l of each primer 10 μ M, 0.1 μ l of Taq polymerase 5
97 U/ μ l and 1 μ l of DNA. The PCR products were purified, and both strands were sequenced by MacroGen Inc. (Seoul,
98 Korea) using the same PCR primers for the sequencing reaction. Chromatograms were manually checked, edited and
99 trimmed with MEGA7 (Kumar et al. 2016) and aligned with CLUSTALW.

100 To assess genetic variability within and between populations, DnaSP v6 (Rozas et al. 2017) was used to calculate the
101 number of haplotypes, haplotype diversity and nucleotide diversity for each sample. Departures from selective neutrality
102 and population equilibrium were further tested with Tajima's D (Tajima 1989) and Fu's F_s tests (Fu 1997) using DnaSP.
103 Pairwise mismatch distribution (Roger & Harpending 1992) were compared to those expected under Rogers' (1995)
104 population expansion model and Excoffier's (2004) range expansion model (Excoffier & Lisher 2010).
105 Hapview (Barrett et al. 2005) was used to graphically represent the relationship between haplotypes and their frequency.
106 Estimation of pairwise Φ_{ST} values and their significance was performed using Permutation tests (1000 permutations) in
107 Arlequin v3.5 (Excoffier & Lischer 2010) to detect genetic variability among samples and between years. For pairwise
108 Φ_{ST} estimates, false discovery rate corrections (Benjamini & Hochberg 1995) were applied in order to adjust significance
109 levels for multiple comparisons.

110 COI and 16S sequences of the target species and close-related species available in Genbank and Bold were included in
111 the analysis (Table S1, Online Resource 1). All the obtained sequences were available in the Online Resource 2 and
112 deposited in NCBI GenBank (Accession numbers: from OM491246 to OM491314 for *Verella vellella* and from
113 OM522756 to OM522831 for *Janthina pallida*).

114 For phylogenetic analyses, jModelTest (Posada 2008) was used to select the best nucleotide substitution model for both
115 species: General Time Reversible model (GTR) for *V. vellella* and Tamura 3-Parameter model with gamma distribution
116 of variable substitution rates among sites (T92 + G) for *J. pallida* (Rodriguez et al. 1990, Tamura 1992). Phylogenetic
117 trees were generated using MEGA7, with a maximum likelihood approach and 1000 bootstrap permutations. For Bayesian
118 inference, MrBayes v.3.2.6 software (Ronquist et al. 2012) was used to calculate posterior probabilities of branch nodes
119 and infer tree topologies. The MCMC chain length was set at 100000 generations, with sampling every 1000 generations
120 and a burn-in value of 250. Reconstructed trees were visualized in FigTree v.1.4.3.

121 MEGA7 was also used to estimate mean net nucleotide divergences among clades based on uncorrected pairwise
122 distances.

123 In the case of *V. velella*, the distance-based Automatic Barcode Gap Discovery (ABGD) software (Puillandre *et al.*, 2012)
124 was used for species delimitation. Aligned sequences of both datasets were uploaded on ABGD webserver
125 (<https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>) to sort them into hypothetical species based on pairwise distances
126 by detecting differences between intraspecific and interspecific genetic distances using a range of priors ($P_{min} = 0.001$;
127 $P_{max} = 1$), while the proxy X for the minimum gap width was set to 1.5, with 10 recursive steps and 20 bins.

128

129 3. RESULTS

130 3.1. Genetic structure and evolutionary traits of *Velevella velella*

131 A 519 bp fragment of the mitochondrial Cytochrome Oxidase subunit I (COI) gene was sequenced for all 69 specimens
132 of *Velevella velella*. The fragments showed 59 variable sites, of which 26 are ascribed as parsimony-informative. The
133 alignment of the sequences revealed 56 haplotypes, with the Are16 location exhibiting the highest number of haplotypes
134 (Figure 2a). Samples generally displayed high haplotype diversity, but low nucleotide diversity (Table 1). The haplotype
135 network highlighted the presence of one main haplotype, shared among five samples. Most of the haplotypes were private
136 and only three haplotypes were shared among two or three samples. The haplotype network did not show any clear
137 geographical and temporal pattern (Figure 2a).

138 Tajima's (1989) D was negative (-2.158) and significant ($p < 0.05$) such as Fu's (1997) ($F_s = -3.79$, $p < 0.02$). The
139 nucleotide mismatch distribution among individuals is unimodal evidencing a recent demographic expansion (data not
140 shown).

141 Pairwise Φ_{ST} values were very low, ranging from -0.141 (between "SMa16" and "Laz17") to 0.083 (between "Off16"
142 and "Laz17"). All pairwise comparisons between samples were not statistically significant ($p > 0.05$) (Table S2, Online
143 Resource 3).

144 Phylogenetic trees, estimated from maximum likelihood and, Bayesian inference, showed concordant topologies. *Velevella*
145 *velevella* sequences from Ligurian and Tyrrhenian seas clustered together and were differentiated from the single
146 representative sequence retrieved from the eastern Pacific Ocean (bootstrap and posterior probability values $> 90\%$).
147 Moreover, this clade (Clade I) was well separated (bootstrap and posterior probability values $> 90\%$) from a monophyletic
148 clade, including individuals from Australia and French Polynesia (Clade II) (Figure 3). Species delimitation analyses
149 (ABGCD) consistently suggested that the two phylogenetic clades correspond to two putative cryptic species. The two
150 clades have a net nucleotide divergence of 0.099 ± 0.012 , while the net divergence between *V. velella* clades and *Porpita*
151 *porpita* (Linnaeus, 1758) clade were 0.15 ± 0.015 and 0.130 ± 0.014 respectively.

152

153 3.2. Genetic structure and evolutionary traits of *Janthina pallida*

154 A 363 bp fragment of the mitochondrial 16S ribosomal RNA gene was sequenced for all 76 specimens of *Janthina pallida*.

155 Sequence alignment showed 20 variable sites, with 3 of them as parsimony-informative.

156 The sequence dataset showed 20 haplotypes, with the SMA16 sample having the most haplotypes but the lowest haplotype
157 diversity. By contrast, SMA17 sample showed the highest haplotype and nucleotide diversity (Table 1). The haplotype
158 network was star-shaped, with a central haplotype shared by all populations and many private haplotypes differing from
159 the central one for a maximum of three substitutions (Figure 2b).

160 Tajima's (1989) D was negative (-2.578) and significant ($p < 0.001$) such as Fu's (1997) ($F_s = -5.252$, $p < 0.02$). The
161 nucleotide mismatch distribution among individuals is unimodal evidencing a recent demographic expansion (data not
162 shown).

163 Pairwise Φ_{ST} index values ranged from -0.057 (between Are17 and Nol17) to 0.077 (between SMA17 and SMA16). All
164 pairwise comparisons between samples were not statistically significant ($p > 0.05$) (Table S3, Online Resource 3).

165 Maximum likelihood and Bayesian inference phylogenetic trees showed similar topologies. Sequences from the Ligurian
166 *J. pallida* clustered together, but differentiation from the congenierics, *Janthina cf. prolongata* (Swainson, 1822) (accepted
167 as *Janthina globosa* Swainson, 1822) and *Janthina umbilicata* (D'Orbigny, 1841) was not highly supported. Conversely,
168 these three species were clearly differentiated from a cluster made by *Janthina janthina* (Linnaeus, 1758) and *Janthina*
169 *exigua* (Lamarck, 1816) (Bootstrap values $> 90\%$) (Figure 4). Phylogenetic results were supported by the net nucleotide
170 divergence. In fact, the pairwise differences between *J. pallida*, *J. cf. prolongata* and *J. umbilicata* were equal to 0.033
171 ± 0.009 and 0.05 ± 0.01 , respectively; while between *J. pallida*, *J. janthina* and *J. exigua* were equal to 0.025 ± 0.008 for
172 both pairwise comparison.

173

174 **4. DISCUSSION**

175 This study is the first to analyze the genetic variability and structure of two pleustonic species in the Mediterranean Sea:
176 the hydrozoan *Velella velella* and its predator, the gastropod *Janthina pallida*. The results highlight 1) the absence of any
177 significant spatial and temporal effects on the genetic structure of either species and 2) the need to deepen the phylogenetic
178 relationship within the two genera.

179

180 *Genetic variability and connectivity of pleustonic taxa*

181 In our study area, *V. verella* is characterized by a high number of singleton haplotypes (i.e. haplotype found only in one
182 individual), with low nucleotide variability and a signature of demographic expansion. Despite the low sample size that
183 could explain the high number of singleton, the haplotype network and the genetic pairwise differentiation suggest that
184 this species represents a local panmictic population in the Ligurian and the Tyrrhenian Sea. No significant genetic
185 structure was, in fact, observed among the stranded samples recorded in sites up to 500 kilometers far from each other.
186 These results are consistent with its pleustonic life style (Pires et al. 2018). Partitioning in different banks of specimens
187 belonging to a single offshore population, resulting in multiple strandings, is weather-induced (Betti et al. 2019) with the
188 colonies transported from one location to another through wind and superficial currents. To our knowledge, few studies
189 were performed on the phylogeography of Hydrozoa (Govindarajan et al. 2005, Churchill 2012, Boissin et al. 2018) and
190 all of them analyze the pattern of population structure at a spatial scale greater than our in the Atlantic, Pacific and Indian
191 oceans. Churchill (2012), using mitochondrial cytochrome oxidase I, observed global panmixis in *V. verella* compared to
192 *Porpita porpita*, which showed regional and within-gyre panmixis. In the Mediterranean Sea, meroplanktonic cnidarians
193 (Scyphozoa) showed different phylogeographic patterns with panmixia in *Pelagia noctiluca* (Forsskål, 1775) (Stopar et
194 al. 2010, but see Aglieri et al. 2014) and regional genetic differentiation in *Aurelia aurita* (Linnaeus, 1758) (Ramsak et
195 al. 2012) and *Rhizostoma pulmo* (Macri, 1778) (Ben Faleh et al. 2017).

196 These contrasting genetic structures partially agree with the life cycles of the species (e.g. holoplanktonic (*V. verella* and
197 *P. noctiluca*) or meroplanktonic (*A. aurita* and *R. pulmo*) and suggest that these categories may predict population genetic
198 structure in macromedusae (Abboud et al. 2018).

199 In *J. pallida* we observed a low haplotype number and low nucleotide diversity. Moreover, we noted a star-shaped
200 haplotype network (i.e., a common haplotype surrounded by several private haplotypes) and a presence of a single
201 panmictic population, suggesting a recent entrance of this species in the Mediterranean Sea (see below) and in the Ligurian
202 Sea. Indeed, Betti et al. (2017) suggested that the Ligurian massive banks of this species recently beached on Ligurian
203 shores possibly originated from the Atlantic Ocean, as shown by the simultaneous presence of the rare Atlantic buoy
204 barnacle, *Dosima fascicularis* (Ellis & Solander 1786). In this regard, Atlantic surface currents are known to facilitate the
205 transport of adult and larval species through the Strait of Gibraltar (e.g.: Sciberras & Schembri 2007, 2008, Bianchi et al.
206 2012). Thus, a combination of present-day and historical processes (high gene flow and the homogenizing effect of a
207 recent expansion) may both contribute to the lack of genetic differentiation detected between populations within the
208 North-western Mediterranean Sea. Finally, like *V. verella*, also *J. pallida* has a very slow movement and cannot oppose
209 the sea currents. It is, therefore, possible that currents contribute in uniting these two organisms (Helm 2021) confirming
210 the connectivity observed in both species at the analyzed spatial scale.

211 The genetic structure of the studied *V. velella* populations did not show any significant difference also among sampling
212 years (i.e. individuals stranded in Arenzano in 2016 and 2017). This supports the inter-annual genetic continuity of the
213 populations, and indicates that the two observed blooms originated from the same pool, and were not caused by
214 ingressions of new banks in the area. Part of the larvae released during the 2016 bloom probably remained in the region,
215 representing the inoculum for the following year's bloom in the same area. This behavior, suggested by the study of the
216 *conaria* larval stage (Woltereck 1904), does not exclude the existence of a yet unknown benthic resting stage; indeed,
217 cysts are known to control seasonal planktonic blooms of many marine species (Boero 1994, Belmonte et al. 1997;
218 Ellegaard et al. 2018). Nevertheless, also based on the same results obtained for *J. janthina*, the genetic composition of
219 the populations may change over time, but, likely, at temporal scales much larger than the one studied here. As observed
220 in Aglieri et al. (2014) a spatial-temporal chaotic genetic patchiness can be observed also in highly dispersive
221 holoplanktonic species suggesting that a genetic monitoring at longer scales is needed. Since blooms of the two pleustonic
222 species occur occasionally all along the Italian coasts (Boero et al. 2016; Betti et al. 2017), a more widespread sampling
223 effort and more variable molecular markers will be needed to evaluate which are the sink and source populations so to
224 better understand the processes driving spatio-temporal dynamics of these blooms.

225

226 *Phylogenetic relationships of pleustonic taxa*

227 The presence of two monophyletic clades supports the existence of two cryptic species in *V. velella*. Mediterranean and
228 Californian specimens of *V. velella* clustered in a separated clade (Clade I) compared to the specimens retrieved from
229 Australia and South Polinesia (Clade II). ABGD analysis strongly supported this result that is in accordance with Churchill
230 (2012), who also evidences different structures in *V. velella* corresponding to northern and southern hemisphere. Previous
231 studies in other Hydrozoa (see for example Dawson & Martin (2001) and Boissin et al. (2018)) have already observed
232 the presence of genetically distinct temperate and tropical subpopulations suggesting the existence of geographically
233 putative cryptic species in this group. Although we did not time-calibrated phylogenetic trees, the short length of the
234 branches between the two clades suggests that the distribution of the two putative cryptic species reflects past geological
235 processes and the spreading of ancestor species to the new environments after the last glaciations period (Dawson &
236 Jacobs, 2001, Schroth et al., 2002). Moreover, Schroth et al. (2002), using ecological data, evidenced that, in *Aurelia*
237 *aurita*, climatic adaptation may have forced ecological and phylogenetic divergence during evolutionary history. In the
238 case of *V. velella*, the presence of cryptic species could be related to morphological differences inducing ecological
239 divergence between hemispheres. In the Mediterranean Sea, *V. velella* has a left-handed sail while in the other seas or
240 oceans it can also have a right-hand sail if the coast of the continent that bathes those seas is on the western side or vice

241 versa (Edwards 1966). Moreover, a complex flexibility in the zooxanthellae–jellyfish association was observed in this
242 species (Djeghri et al. 2019). *V. verella* can form symbiotic associations with different dinoflagellate genera
243 (*Brandtodinium* and *Scrippsiella* (or *Ensiculifera*)), possibly with a biogeographical pattern (*Brandtodinium* in the
244 Atlantic Ocean and possibly the Mediterranean Sea, *Scrippsiella* (or *Ensiculifera*) in the Pacific Ocean) (Probert et
245 al. 2014, Djeghri et al. 2019). However, a broader geographic sampling effort coupled with additional observational data
246 collection, molecular analysis, and biophysical global ocean modeling (Dawson et al. 2005) is necessary to corroborate
247 this hypothesis.

248 The phylogeny of *Janthina* spp. generated from mitochondrial 16S sequences is congruent with the molecular phylogeny
249 of Curchill et al. (2011) but partially incongruent with recent morphology-based phylogenies proposed by Beu (2017).
250 The two approaches evidenced a close relationship between *J. pallida* and *Janthina cf. prolongata* (now accepted as
251 *Janthina globosa*), but it remains unclear if *J. pallida* is a daughter species that originated from *J. globosa* (Beu 2017) or
252 vice versa. In fact, *Janthina umbilicata* and *J. globosa* were more similar to each other compared to *J. pallida*, while in
253 Beu (2017) *J. umbilicata* was more distant from *J. globosa* and morphologically more similar to *Janthina exigua*. Fossil
254 data place *J. globosa* in the late Piacenzian or Gelasian (c. 3–2 Ma) to Holocene; while all the other species were placed
255 in the Holocene (last 11,700 years of Earth's history) (Beu 2017). Because of their morphological similarity, an integrative
256 revision of the genus *Janthina* is needed, including not only additional molecular markers but also new three-dimensional
257 (3D) anatomical reconstruction. Conversely, a concordance between morphological and molecular data was observed in
258 the high evolutive distance between *J. pallida* and *J. janthina*. Differences between these two species are evidenced also
259 in their biological traits being *J. pallida* oviparous, while *J. janthina* ovoviviparous (Calabrò et al. 2019). In addition,
260 the two species seem to prefer different preys: while *J. janthina* feeds on *Physalia* spp. and *V. verella*, *J. pallida* seems
261 adapted to prey only on *V. verella* (Helm 2021).

262 In conclusion, our study reports the persistence, in two seasonally abundant pleustonic species, of single populations, with
263 minimal geographical structure, at least in the North-western Mediterranean Sea. Natural changes through time and space,
264 commonly affecting these organisms, seem not to influence their genetic structure. The obtained information represents
265 the basis for better understanding the ecological role and population dynamics of these important and often neglected
266 pleustonic species in the Mediterranean basin.

267

268

269 **DATA AVAILABILITY STATEMENT:** All relevant data are within the paper and its Supporting Information files.

270 **ACKNOWLEDGEMENTS:** We would like to thank V. Bertuccio and A. Ferrari for the help provided in the data
271 analysis. We would also like to thank Prof. Angelo Cau (UniCa), Dr. Michela Giusti (ISPRA), and Dr. Fabrizio Serena
272 (IUCN) for their help in samples collection. We are grateful to Dr. Barbara Mikac for improving the English of this
273 manuscript.

274

275 **DECLARATIONS:**

276 **Funding:** No funding was received for conducting this study.

277 **Conflicts of interest/Competing interests:** The authors have no conflicts of interest to declare that are relevant to the
278 content of this article

279 **Availability of data and material:** All relevant data are within the paper and its Supporting Information files

280 **Code availability:** Not applicable

281 **Authors' contributions:** Federico Betti, Marzia Bo, Federica Costantini contributed to the study conception and design.
282 Material preparation, data collection and analysis were performed by Bruno Morello, Federico Betti, Marzia Bo,
283 Federica Costantini. The first draft of the manuscript was written by Bruno Morello, Federica Costantini, Federico Betti
284 and all authors commented on previous versions of the manuscript, drafted the work and revised it critically. All authors
285 read and approved the final manuscript. All the Authors agree to be accountable for all aspects of the work in ensuring
286 that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

287 **Ethics approval:** Organisms used in the manuscript were found dead on the beach

288

289 **REFERENCES**

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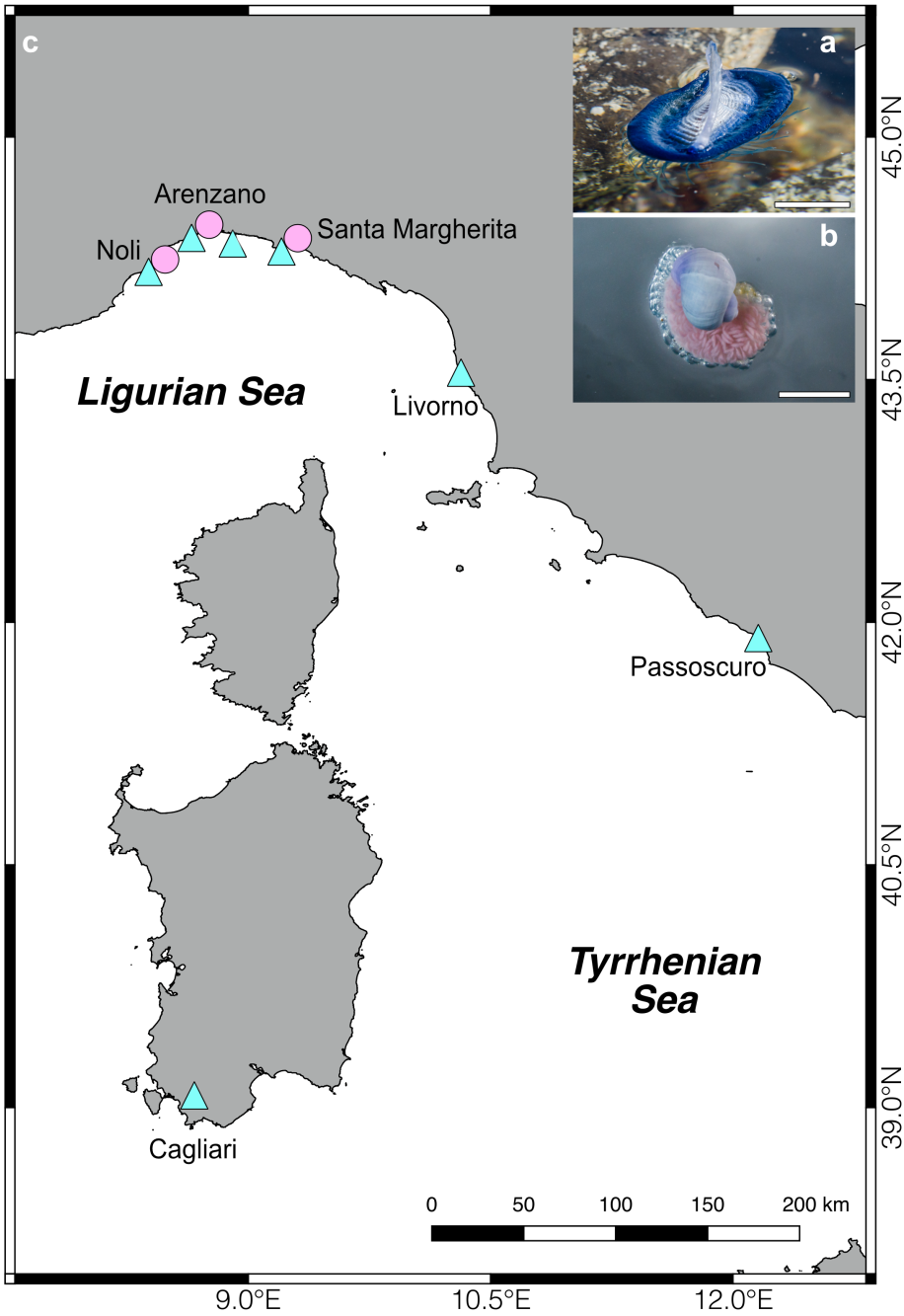
Table 1: Name of the locations, codes, sampling dates, GPS coordinates, number of sampled individuals, and the genetic variability of *Verella verella* and *Janthina pallida*. Genetic variability is reported as number of individuals (n), number of haplotypes (h), haplotype diversity (hd) with standard deviation (Dev. St.) and nucleotide diversity (Pi).

Species	Location	Code	Date	Coordinates	N° of individuals	h	hd (Dev. St)	Pi
<i>Verella verella</i>	Noli-Liguria	Nol16	13/04/16	44°12'14"N 08°25'01"E	13	12	0.987 (0.035)	0.00887
	Arenzano-Liguria	Are16	12/04/16	44°24'28"N 08°41'32"E	13	13	1 (0.030)	0.00892
	S. Margherita-Liguria	SMa16	15/04/16	44°20'09"N 09°13'27"E	4	4	1 (0.177)	0.01509
	Offshore-Liguria	Off16	12/04/16	44°22'0.12"N 8°52'21.72"E	8	8	1 (0.063)	0.00860
	Arenzano-Liguria	Are17	01/04/17	44°24'28"N 08°41'32"E	12	10	0.955 (0.057)	0.00928
	Livorno-Toscana	Liv17	07/05/17	43°32'36"N 10°19'1" E	8	8	1 (0.063)	0.01060
	Cagliari-Sardegna	Sar17	08/05/17	39°04'28"N 08°39'57"E	10	9	0.978 (0.054)	0.00818
	Passoscuro-Lazio	Laz17	09/05/17	41°54'08"N 12°09'26"E	1	1	0	0
TOTAL					69	64	0.986 (0.007)	0.00948
<i>Janthina pallida</i>	Noli-Liguria	Nol16	13/04/16	44°12'14"N 08°25'01"E	11	5	0.618 (0.164)	0.00200
	Arenzano-Liguria	Are16	12/04/16	44°24'28"N 08°41'32"E	19	4	0.380 (0.134)	0.00113
	S. Margherita-Liguria	SMa16	15/04/16	44°20'09"N 09°13'27"E	21	6	0.043 (0.134)	0.00131
	Arenzano-Liguria	Are17	01/04/16	44°24'28"N 08°41'32"E	9	3	0.417 (0.191)	0.00122
	Noli-Liguria	Nol17	01/05/17	44°12'14"N 08°25'01"E	8	4	0.643 (0.184)	0.00207
	S. Margherita-Liguria	SMa17	02/05/17	44°20'09"N 09°13'27"E	8	4	0.750 (0.139)	0.00394
TOTAL					76	20	0.497 (0.072)	0.00173

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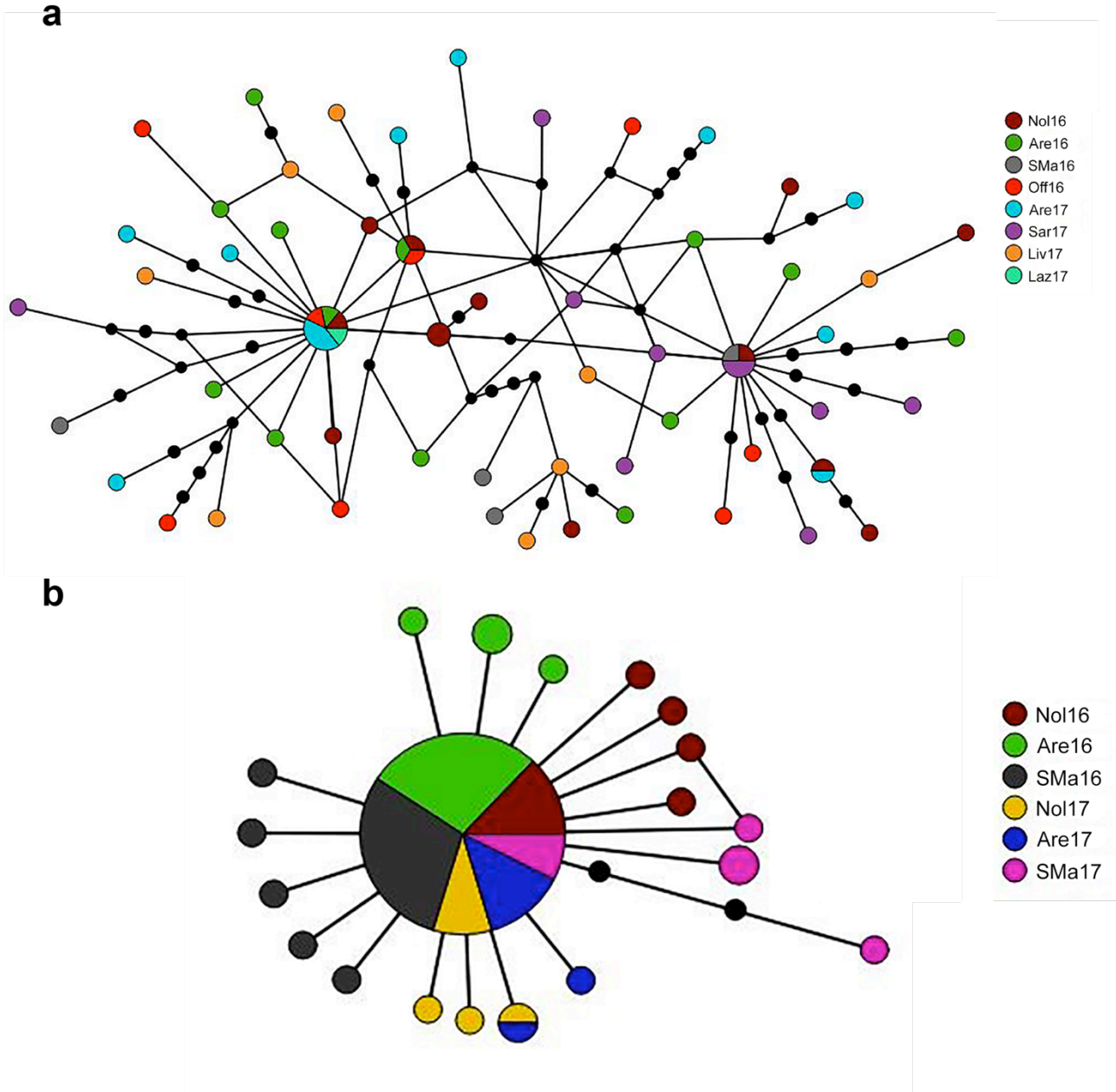
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Figure 1. a) *Veella veella* colony floating on the surface. b) *Janthina pallida* as seen from below the surface, with the bubble raft and the pink eggs. c) Map of the sampling sites. Purple dots indicate *Janthina pallida* sampling locations, while blue triangles refer to the sampling sites of *Veella veella*. White scale bar = 1 cm.



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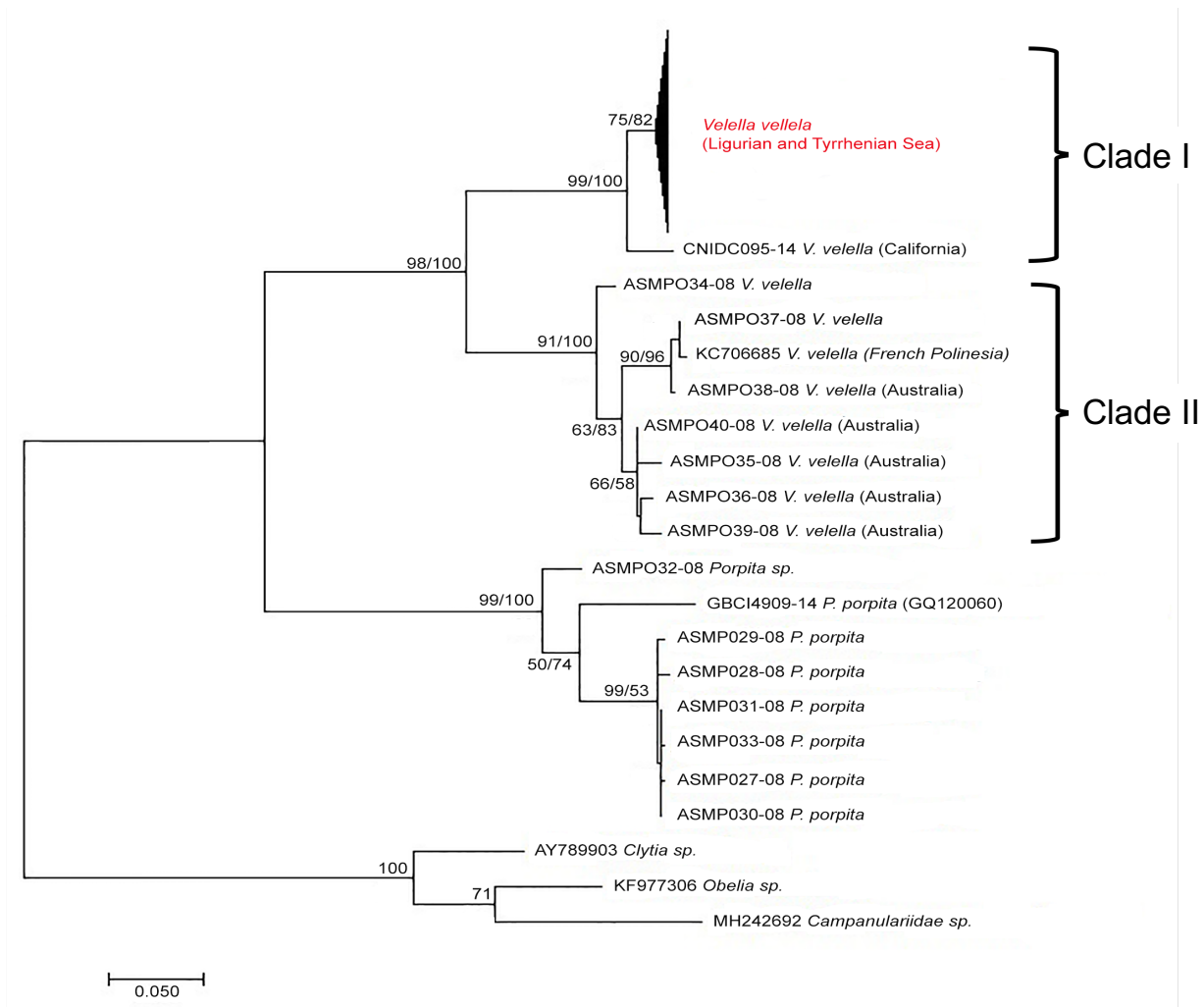
452 **Figure 2.** a) Median joining haplotype networks of the individuals of *Verella verella*; b) Median joining haplotype
 453 networks of the individuals of the Ligurian populations of *Janthina pallida*. The network is standardized in such way
 454 that small circles represent haplotypes present in one individual. Each branch represents 1 mutation between the 2
 455 adjacent sequences and black small circles represent unsampled haplotypes.



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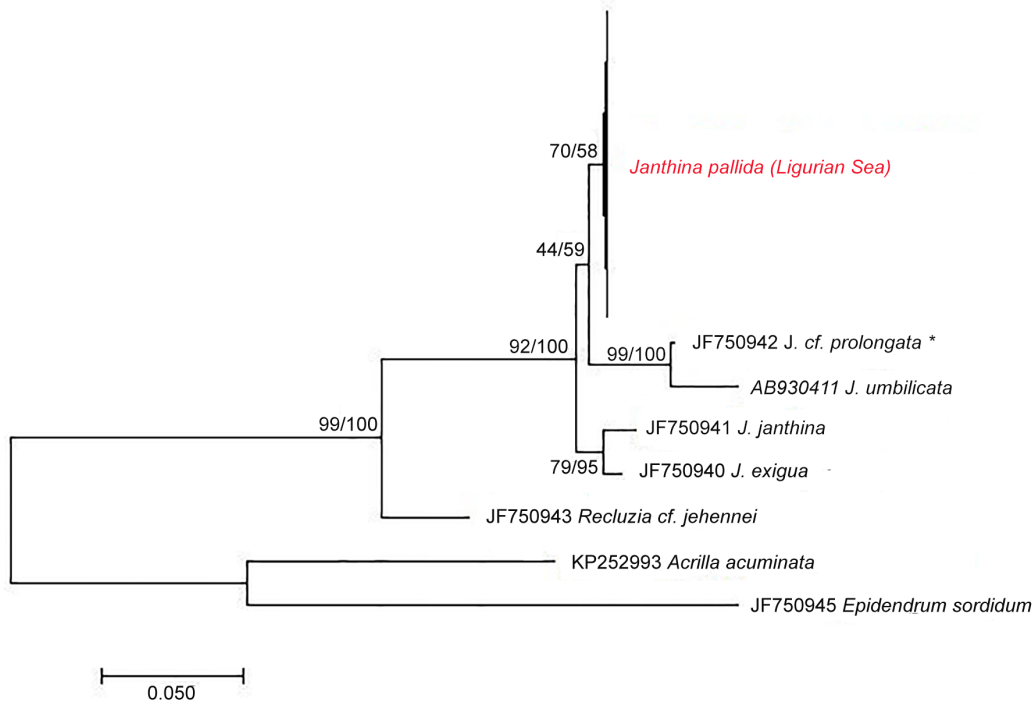
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Figure 3. Maximum-likelihood (ML) tree for the COI sequences of *Verella vellela* and close-related species. Bayesian inference (BI) tree showed the same topology (data not shown). Posterior probability and bootstrap support values are indicated when > 50% (BI/ML). The tree is rooted using *Obelia* sp., *Campanulariidae* and *Clytia* sp. as outgroups.



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465 **Figure 4.** Maximum-likelihood (ML) haplotype tree for the 16S sequences of *Janthina pallida* and close-related
 466 species. Bayesian inference (BI) tree showed the same topology (data not shown). Posterior probability and bootstrap
 467 support values are indicated when > 50% (BI/ML). The tree is rooted using *Recluzia jehennei*, *Acrilla acuminata* and
 468 *Epidendrium sordidum* as outgroups. * Accepted as *Janthina globosa*.
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