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4 Barbara Porro¹, Thamilla Zamoum¹, Cédric Mallien¹, Benjamin C.C. Hume², Christian R.
5 Voolstra³, Eric Röttinger¹, Paola Furla^{1*} & Didier Forcioli^{1*§}

6

7 ¹Université Côte d'Azur, CNRS, INSERM, Institute for Research on Cancer and Aging (IRCAN),
8 28 avenue de Valombrose, 06107 Nice, France

9

10 ²Red Sea Research Center, Division of Biological and Environmental Science and Engineering,
11 King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi
12 Arabia

13

14 ³Department of Biology, University of Konstanz, Konstanz, Germany

15

16 [§]Corresponding author: Didier Forcioli

17 Email: forcioli@unice.fr

18 Phone: + 33 4 93 37 77 39

19

20 *These authors contributed equally: Paola Furla & Didier Forcioli

21

22 **ABSTRACT**

23 All metazoans are in fact holobionts, resulting from the association of several organisms, and
24 organismal adaptation is then due to the composite response of this association to the
25 environment. Deciphering the mechanisms of symbiont acquisition in a holobiont is
26 therefore essential to understanding the extent of its adaptive capacities. In cnidarians, some
27 species acquire their photosynthetic symbionts directly from their parents (vertical
28 transmission) but may also acquire symbionts from the environment (horizontal acquisition)
29 at the adult stage. The Mediterranean snakelocks sea anemone, *Anemonia viridis* (Forskål,
30 1775), passes down symbionts from one generation to the next by vertical transmission, but
31 the capacity for such horizontal acquisition is still unexplored. To unravel the flexibility of the
32 association between the different host lineages identified in *A. viridis* and its
33 Symbiodiniaceae, we genotyped both the animal hosts and their symbiont communities in
34 members of host clones in five different locations in the North Western Mediterranean Sea.
35 The composition of within-host symbiont populations was more dependent on the
36 geographical origin of the hosts than their membership to a given lineage or even to a given
37 clone. Additionally, similarities in host symbiont communities were greater among genets
38 (*i.e.* among different clones) than among ramets (*i.e.* among members of the same given
39 clonal genotype). Taken together, our results demonstrate that *A. viridis* may form
40 associations with a range of symbiotic dinoflagellates and suggest a capacity for horizontal
41 acquisition. A mixed-mode transmission strategy in *A. viridis*, as we posit here, may help
42 explain the large phenotypic plasticity that characterises this anemone.

43 **Keywords:** Symbiosis, Clonality, Cryptic species, RAD-Seq, microsatellite, ITS2

44 **INTRODUCTION**

45 From their earliest development stages, all metazoans live in close association with different
46 microorganisms (symbionts) forming a so-called holobiont (Margulis & Fester, 1991). The
47 evolution of these holobionts is influenced by the transmission mode of symbionts. Two
48 modes of symbiont acquisition have been described: from the parents or from the
49 environment, called vertical transmission and horizontal acquisition, respectively. Vertical
50 transmission is traditionally more associated with mutualism whereas horizontal acquisition
51 corresponds more to parasitism (Moran & Wernegreen, 2000). Vertical transmission indeed
52 ensures the maintenance of beneficial associations at the embryonic stage (Zilber-Rosenberg
53 & Rosenberg, 2008), but with less flexibility in the host-symbiont association. A strong
54 correlation between the host and symbionts phylogenies can therefore be expected (for a
55 review on the topic, see Baumann, 2005). However, this binary vision of a clearly split
56 transmission mode between mutualism and parasitism is not so obvious and many examples
57 of mutualistic symbiosis maintained by horizontal acquisition have been observed and
58 described (Henry et al., 2013; Kaufman, Ikeda, Patton, van Dykhuizen, & Epel, 1998; Nelsen &
59 Gargas, 2008; Rowan & Knowlton, 1995). In such case, the horizontal acquisition, which
60 could be hazardous because of the uncertainty in partner occurrence, is nevertheless an
61 efficient way to obtain new or locally better adapted symbionts.

62 Host symbiont acquisition is not necessarily exclusive by means of vertical or horizontal
63 mechanisms: a situation named by Ebert (2013) as the mixed-mode transmission of
64 symbionts. This mixed-mode has already been observed in cases where host-symbiont
65 phylogenies were not totally coupled due to occasional horizontal acquisition in holobionts
66 alternatively classified as vertical transmitters (Stewart, Young, & Cavanaugh, 2008). The

67 horizontal acquisition and the mixed-mode transmission imply some flexibility in the
68 symbiotic association, an important point for the response to environmental variations.
69 Indeed, if the whole holobiont is the unit of selection in a strict vertical transmission, in a
70 mixed-mode transmission, the selective pressure could act on either hosts or symbionts as
71 well as the holobiont level (Theis et al., 2016).

72 In marine Cnidaria (including hydrozoans, corals, gorgonians, and sea anemones), the animal
73 host can be associated with eukaryotic mutualistic photosynthetic symbionts (see for review
74 Clavijo, Donath, Serôdio, & Christa, 2018). These symbionts play key roles in the physiology
75 and ecology of their hosts. Among Anthozoa, the main photosynthetic symbionts are
76 dinoflagellates belonging to the Symbiodiniaceae family (LaJeunesse et al., 2018). Located
77 intracellularly, these symbionts translocate most of the organic compounds they produce by
78 photosynthesis to their host cells (Davy, Allemand, & Weis, 2012). The cnidarian-
79 dinoflagellate symbiosis is highly diverse with the possible occurrence of multiple
80 dinoflagellate genotypes within a given host. This diversity can be partially explained by
81 diverse ecological factors which can lead to the presence of different dinoflagellate
82 genotypes (see Parkinson & Baums, 2014 for review). In addition, bacterial symbionts are
83 involved in the good working of the cnidarian-dinoflagellate holobiont (*e.g.* for nitrogen
84 cycle) and contribute to the holobiont diversity (Rohwer, Seguritan, Azam, & Knowlton, 2002;
85 Suggett, Warner, & Leggat, 2017). The dinoflagellate symbionts can be acquired by the
86 cnidarian hosts via vertical transmission or horizontal acquisition (at larval or adult stages).
87 Why vertical or horizontal symbiont acquisition modes are associated with a given cnidarian
88 species is still not understood, but in scleractinian corals, only 25% of spawning species (*i.e.*
89 those that emit their gametes directly into the ocean) have symbionts in their eggs (*i.e.*,

90 vertical transmission) and belong to only a few specific genera (*Montipora*, *Acropora*,
91 *Pocillopora*, and *Porites*; Baird, Guest, & Willis, 2009). In contrast, temperate symbiotic sea
92 anemones have mainly vertical “closed acquisition systems” (Muller-Parker & Davy, 2005).
93 Although horizontal acquisition in adult cnidarians has been poorly studied in the field
94 (except after post-bleaching episodes and mainly for scleractinian corals (Boulotte et al.,
95 2016; Silverstein, Cunning, & Baker, 2015)), Byler et al. (2013) demonstrated that some
96 species, such as *Stylophora pistillata*, display mixed-mode transmission. The flexibility that
97 horizontal transmission may offer to the host is particularly important considering the
98 functional diversity of symbionts at genus or species level (Brading et al., 2011; Reynolds,
99 Bruns, Fitt, & Schmidt, 2008; Roberty, Furla, & Plumier, 2016; Suggett et al., 2008; Tchernov
100 et al., 2004), and may also apply to associated bacteria (Neave et al., 2017). Even within
101 Symbiodiniaceae species, functional genetic differentiation has been measured between two
102 symbiont populations (Howells et al., 2020, 2012).

103 Using ITS2 variability, we previously investigated the genetic diversity of the symbionts
104 hosted by *Anemonia viridis*, a species of symbiotic sea anemone widely distributed in
105 Europe, from the Mediterranean Sea to the North Sea and the west coast of Ireland, in which
106 different cryptic species (independent lineages) have been recently identified (Porro et al.,
107 2019). In *A. viridis*, a unique Symbiodiniaceae clade, ‘Temperate A’ (LaJeunesse et al., 2018;
108 or *A’ sensu* Savage et al., 2002; and Visram, Wiedenmann, & Douglas, 2006), has been
109 identified, showing a clear genetic diversity within the clade (Casado-Amezúa, Machordom,
110 Bernardo, & González-Wangüemert, 2014; Porro et al., 2019). This symbiont diversity was
111 observed with respect to the geographical origin and the host lineage (Porro et al., 2019).
112 Specifically, in this latter study, we revealed the occurrence of different assemblages of

113 Temperate A Symbiodiniaceae in at least two lineages of *A. viridis* (English Channel and
114 Mediterranean Sea). However, as the two lineages were found in two far geographically
115 distinct areas, it was unclear whether the symbiotic assemblage was a result of response to
116 the environment vs. a result of genetic drift in a closed system.

117 *A. viridis* can reproduce both sexually, emitting gametes in the water column, and asexually,
118 by longitudinal fission of the gastrovascular column (from the foot to the mouth, Shick,
119 1991). Whatever the reproduction mode, this sea anemone is thought to inherit its
120 symbionts by vertical transmission either by (i) maternal transmission through oocytes
121 (Schäfer, 1984; Utrilla, Castro-Claros, Urra, Navas, & Salas, 2019) with a mean of 300
122 symbionts per oocytes (personal observations) or (ii) by partitioning of the symbiont
123 population of the “mother” between the two “daughter” sea anemones. The vertical
124 transmission guarantees the continuity of this important association, as the *A. viridis*
125 holobiont has the potential to be completely autotrophic (Davy, Lucas, & Turner, 1996;
126 Muller-Parker & Davy, 2005). Even if symbiont diversity has been studied in *A. viridis* (Casado-
127 Amezúa et al., 2014; Porro et al., 2019; Savage et al., 2002; Suggett et al., 2012; Visram et al.,
128 2006), no study of the dynamics of symbiont populations at the adult stage has been
129 conducted so far, although it would fill an important gap in the understanding of the plastic
130 and adaptive capacities of this species. Indeed, many studies have highlighted its strong
131 capacity for resistance to extreme and disturbed environments (Richier, Furla, Plantivaux,
132 Merle, & Allemand, 2005; Suggett et al., 2012; Ventura et al., 2016). In this context, the
133 occasional occurrence of horizontal symbiont acquisition on top of the vertical transmission
134 may provide a mechanism for adaptation, adding to the intergenerational stability of the
135 association the capacity to acquire locally better adapted symbiont genotype (Parkinson &

136 Baums, 2014).

137 To determine whether a closed vertical mode of symbiont transmission occurs in *Anemonia*
138 *viridis* or if concurrent and complementary horizontal symbiont acquisition within a mixed-
139 mode transmission is also possible in this species, we investigated the strength of the link
140 between host genetic diversity and symbiont assemblage. A more extensive sampling of the
141 North Western Mediterranean Sea than in Porro et al. (2019) was used. We first delimited
142 reproductively isolated lineages within the *A. viridis* species complex (using RAD sequencing
143 markers), and then compared their symbiotic contents (based on NGS acquired nuclear ITS2
144 and microsatellite diversity) to establish the host-symbiont genetic specificity. We also used
145 the pervasive asexual reproduction occurring within these *A. viridis* lineages to estimate the
146 similarity of the symbiont communities within and among different sea anemone clonal
147 genotypes (as identified with RAD sequencing markers) sampled in distinct geographical
148 locations. We hypothesized that, in strictly closed vertical transmission, ramets (*i.e.*
149 representatives of the same clonal genotype) should be more similar in their symbiont
150 content than two sea anemones from different genets (different clonal genotypes),
151 irrespective of their geographical location. In contrast, the occurrence of horizontal symbiont
152 acquisition would be revealed by a strong geographical effect independent of the lineage
153 and/or genotype of the host.

154

155 **MATERIAL AND METHOD**

156 **Mediterranean Sea anemones**

157 We sampled 237 individuals of *Anemonia viridis* (Forskål, 1775) var *smaragdina*

158 (characterised by pink tips and a green fluorescence, Mallien et al., 2017; J. Wiedenmann,
159 Röcker, & Funke, 1999) from five locations in the Mediterranean eastern basin: Banyuls [Ban,
160 50 sea anemones sampled], Barcarès [Bar, 44], Sète [Tha, 48], Marseille [Mar, 49] and
161 Scandola [Sca, 46] (see figure 1 for GPS coordinates). For each sea anemone, a dozen
162 tentacles were cut, fixed in 70% ethanol and preserved at -80°C until DNA extraction. Total
163 DNA was extracted as in Mallien et al (2017) following a modified “salting out” protocol
164 (Miller, Dykes, & Polesky, 1988).

165 **Host genotyping**

166 *RAD sequencing*

167 The *ad hoc* libraries were prepared following Porro et al. (2019). Sequencing was performed
168 on an Illumina HiSeq 2000 (100-bp single read format) at the Montpellier-GenomiX platform
169 (MGX, Montpellier, France). Raw sequence reads were demultiplexed, filtered and clustered
170 with iPyRAD v0.3041 (Eaton, 2014) with a clustering threshold set to 90%.

171 *Host datasets*

172 From the iPyRAD outputs, we derived a *base dataset* by filtering on missing data: the
173 individuals with a low proportion of shared loci were identified using the R package RADami
174 v1.1-2 (Hipp, 2014) and removed using VCFTools v0.1.15 (Danecek et al., 2011); the loci were
175 then filtered to only keep loci present in at least 95% of individuals (197 individuals and
176 25603 SNPs) (figure 2).

177 We then filtered the *base dataset* to keep only biallelic loci with a minimum allele frequency
178 (maf) of 1% This new dataset was called *N197 dataset* with 197 individuals and 5299 SNPs.

179 This dataset was used to detect clonality. By selecting only the 148 individuals for which the
180 symbionts could be genotyped (see below), we defined the *N148 dataset*. This dataset was
181 used for host/symbiont differentiations comparison.

182 After clonality detection, we were able to filter the individuals from the base dataset by
183 keeping only one ramet per genet, and we only kept the biallelic loci with a maf of 1%. This
184 constituted the *N65 dataset* with 65 individuals and 6498 SNPs that was used for host
185 genetic clustering and lineages identification.

186 The *N65 dataset* was further filtered to keep 14 individuals only (one individual per location
187 and genetic cluster), and for the SNPs without missing data, with a maf of 10% and a linkage
188 disequilibrium pruning threshold (LD) of 0.2 (package SNPRelate v1.11.2, Zheng, 2012) which
189 constituted the *BFD dataset* of 1197 SNPs. This dataset was used for the test of species
190 hypotheses by BFD* (see below).

191 *Detection of clonality*

192 Even if no anemones did share the same multi locus genotypes, we repeatedly detected
193 similar genotypes in the sampling. These genotypes were likely a consequence of asexual
194 reproduction events and have been grouped into multilocus lineages called genets (see
195 Harper, 1977) in this paper (the representatives of each genet being referred to as ramets).
196 To formally delineate these genets and associate individual samples to them, we computed
197 between individual pairwise distances for all individuals in the *N197 dataset* (figure 2) with
198 the R package RClone v1.0.2 (Bailleul, Stoeckel, & Arnaud-Haond, 2016). The observed
199 distributions of pairwise genetic distances thus obtained were compared to simulated
200 distributions of the expected genetic distances under the assumption of purely sexual

201 reproduction (with or without selfing) computed with RClone (using the functions `genet_dist`
202 and `genet_dist_sim`) (Bailleul et al., 2016).) This allowed us to identify a gap in distance
203 distribution and define a threshold distance value separating distances within genets from
204 distances among genets. We could thus associate individuals to genets using the function
205 `mlg.filter` (with the “`farthest_neighbor`” algorithm) from the R package `poppr` v2.5.0 (Kamvar,
206 Tabima, & Grünwald, 2014). We then calculated the clonal richness (R) considering the
207 number of genets as the number of different genotypes over the total number of sampled
208 sea anemones (globally, per location and per host genetic cluster).

209 *Structure of host genetic diversity*

210 To identify putative species within *A.viridis* individuals from the Western Mediterranean
211 basin, we used the *N65 dataset* (figure 2) and we computed a species tree using SVDquartets
212 (Chifman & Kubatko, 2014) implemented in PAUP* v4.0A165 (Swofford, 1998; Swofford,
213 2001) with an exhaustive quartet sampling and performed a sNMF quantitative hierarchical
214 genetic clustering with the LEA (v2.2.0) R package (Frichot & François, 2015), based on
215 individual ancestry coefficients. The optimal number of ancestral clusters, from which the
216 individuals in the dataset could be traced back, was determined using the entropy criterion.
217 The results were visualized using a STRUCTURE-like representation showing, for each
218 individual, the relative contribution of each ancestral cluster to its multilocus genotype. The
219 ramets not kept in the N65 dataset were attributed by extension to the main cluster of their
220 genet.

221 To test whether the sNMF clusters and SVD clades were independent species, we compared
222 the likelihood of the species hypothesis they constitute, to the likelihood of a primary species
223 hypothesis based on the geographical locations of the individuals using Bayes factor

224 delimitation with genomic data (BFD*; Leaché, Fujita, Minin, & Bouckaert, 2014). In order to
225 manage computing times, these analyses were performed using the *BFD dataset* (figure 2).
226 The BFD* analyses were performed in BEAST2 (Bouckaert et al., 2014) with 48 path sampler
227 sets of 100000 MCMC repetitions, to sample in 800000 MCMC iterations of SNAPP (Bryant,
228 Bouckaert, Felsenstein, Rosenberg, & RoyChoudhury, 2012). The number of SNAPP iterations
229 was chosen to ensure proper convergence of the species tree inference. To ensure the
230 convergence of the computations while maintaining amenable computing times, the BFD*
231 analyses were repeated on two partitions of the 1197 SNPs of the *BFD dataset*. The first
232 analysis was run on the 598 first SNPs and the second one the other 599 SNPs of the dataset.

233 Wright's F-statistics among and within populations were computed using the R packages
234 StAMPP v1.5.1 (Pembleton, Cogan, & Forster, 2013) and hierfstat v0.04-22 (Goudet &
235 Jombart, 2015).

236 **Symbiont genotyping**

237 *Amplicon sequencing*

238 The symbiont populations were genotyped using the nuclear ribosomal DNA internal
239 transcribed spacer 2 (ITS2). We used the primers from Stat et al. (2009): the forward itsD (5'-
240 GTGAATTGCAGAACTCCGTG -3') and the reverse its2rev2 (5'- CCTCCGCTTACTTATATGCTT -3').

241 The symbiont populations were also genotyped using four microsatellites: three designed
242 from an Expressed Sequence Tag library of *A. viridis* holobionts (Sabourault, Ganot, Deleury,
243 Allemand, & Furla, 2009) and one from the study of Catanéo et al (2009) (see also
244 Abdoullaye et al., 2010) about the Temperate A symbionts from the Mediterranean
245 gorgonian *Eunicella singularis* (with the NCBI accession number FJ917549.1, table 1). These

246 markers are particularly useful to genotype populations instead of species (Coffroth &
247 Santos, 2005) as they are more informative of recent evolutionary events (Cornuet, Ravigué,
248 & Estoup, 2010).

249 PCR amplifications of the symbiont markers were performed by Access Array (FLUIDIGM) PCR
250 and next generation sequencing of the amplicons were obtained by MiSeq Illumina
251 Technology (2x250bp paired-end) at the Brain and Spine Institute (ICM, Paris, France).

252 *Genotyping methods*

253 Because a given sea anemone can host 3 to 20 million symbiont cells per milligram of protein
254 (values from field and lab experiments, Ventura et al., 2016), the sequences obtained from
255 each anemone were in fact obtained from a pooled sample of symbionts. Their genotyping
256 was therefore not straightforward and required additional analysis steps.

257 In this context, we performed an analysis of the symbiont diversity within the *A. viridis* using
258 ITS2 and microsatellite sequence variants. The analysis of ITS2 sequence variant assemblages
259 is an effective method for comparing Symbiodiniaceae populations despite the marker's
260 multicopy nature, which complicates data interpretation (see Hume et al., 2019). The ITS2
261 sequences were processed and identified using the SymPortal pipeline (Hume, 2019; Hume
262 et al., 2019) based on a minimum entropy decomposition algorithm, as in Porro et al. (2019).

263 For the microsatellite markers, the forward and reverse reads were assembled, then trimmed
264 and tagged by microsatellite locus using OBITools scripts (Boyer et al., 2016). To filter
265 background noise and to identify reads with actual microsatellite repeats, we used the
266 MicNeSs python script (Suez et al., 2016) with a tolerance of one substitution in the
267 microsatellite motif. Because MicNeSs was designed for diploid organisms, and because we

268 did not want to bias the genetic diversity by forcing a maximum of two microsatellite alleles
269 per within host symbiont population, we chose to consider for each individual, instead of the
270 genotype, the raw observed distribution of the reads containing a given microsatellite locus.
271 We kept for further analysis only the sea anemones with amplification for at least three out
272 of the 4 symbiont microsatellite markers used. Therefore, as we did not attempt to ascertain
273 any of the microsatellite alleles present in the read collections, this raw observed distribution
274 of the reads is more a fingerprint of the symbiont community composition than a proper
275 collection of individual genotypes. Nevertheless, it can still be used to measure the level of
276 differentiation in symbiont content among different sea anemones.

277 The symbiont populations were not successfully amplified from all the sampled individuals,
278 and only 148 sea anemones could be genotyped for both host and symbiont microsatellite
279 markers. These individuals composed the *N148 dataset* (table S1).

280 *Symbiont differentiation*

281 We compared the different compositions of symbiont populations using over the N148
282 dataset, for the ITS2, the presence/absence of sequence variants among the hosts, and for
283 the microsatellite markers, the observed relative frequency distributions of the reads. For
284 these reasons, the differentiation between hosts in terms of symbiont community
285 composition was expressed both as a Jaccard's distance (for ITS2 sequence variant
286 assemblage considering the presence and absence of the variants) and a Manhattan's
287 distance (for the distributions of microsatellite markers). The distances among
288 hosts/symbiont community compositions were visualized with Neighbour Joining trees with
289 R package ape v5.3 (Paradis, Claude, & Strimmer, 2004) with 1000 bootstraps. We measured
290 the correlation between symbiont population distance and host genetic distance by Mantel's

291 tests. We also tested by PERMANOVA with the R package vegan v2.5-5 (Oksanen et al., 2016)
292 the differences in symbiont community composition between sea anemones depending on
293 (i) the host genetic clusters, or (ii) the sampling location. When these factors had significant
294 effects on the distribution of the genetic diversity of the symbionts, we then computed
295 pairwise PERMANOVAs between each level of the given factor with an FDR correction to
296 avoid false positives. The Manhattan's distances were also used to compare the
297 differentiation within multi-location genets. Following the approach of Baums et al. (2014) in
298 a coral species, we took advantage of asexual reproduction in *A. viridis* to test for the
299 tightness of the closed vertical symbiont transmission in *A. viridis*, by comparing the
300 symbiont diversity among ramets. If symbiont diversity is only the result of mother to
301 offspring transfer, we expected that ramets from the same genet should have a similar
302 symbiont community composition and hence the distances should be shorter compared to
303 genetically distant hosts. The differences were statistically tested with a Kruskal-Wallis test
304 implemented in R (R Core Team, 2018).

305

306 **RESULTS**

307 **Clonality in *A. viridis* var. *smaragdina* populations**

308 We detected clonality in the dataset with a gap in the distribution of pairwise genetic
309 distances among *A. viridis* individuals (figure S1), and hence identified 65 genets over the 197
310 sea anemones sequenced, with 37 genets considered as unique (one ramet) and 28 genets
311 (here called clonal genets) with at least two representatives (*i.e.* at least two ramets
312 produced from asexual reproduction events, see table S1). These 28 clonal genets could be

313 found in different distant sampling locations (table S1). We hence estimated a global clonal
314 richness of 0.33.

315 **Host genetic structure**

316 To identify independent genetic pools, we performed a sNMF genetic clustering. Based on
317 minimum cross-entropy value (figure S2), we determined K=4 as the optimal number of
318 ancestral genetic clusters among the 65 genets (N65 dataset). Among these four different
319 genetic clusters, we detected admixture, more specifically two of them seemed to be more
320 admixed (K1 and K2, figure 1.A): more than a third of the sea anemones in K2 were not
321 purely descended from this cluster, and had at least 15% of their loci from K1 and,
322 reciprocally, more than half of the sea anemones in K1 admixed with the K2 cluster (figure
323 1.A and table S1). All these clusters were found in at least two different sampling locations
324 (figure 1.A and 1.B) with K2 being the most common cluster among the Mediterranean
325 sampled populations. The admixture K1/K2 was correlated with the lowest F_{ST} value of 0.119
326 compared with the other pairwise differentiations (table S2). The K4 cluster appeared as the
327 most differentiated genetic cluster in the Mediterranean Sea with F_{ST} values to other clusters
328 ranging from 0.242 to 0.350

329 We also used SVDquartets to produce a coalescent taxonomic partitioning from the N65
330 dataset (figure S3). The SVDquartets analyses showed three main clades (clades 1, 3 and 4)
331 and an unresolved group (clade 2). These clades are partially congruent with the clusters
332 identified by the sNMF analysis: the clades 3 and 4 corresponded to the clusters K3 and K4
333 respectively, and clade 1 corresponded to a part of the cluster K1 (table S1).

334 To know which one of the partitions given by sNMF clustering (hypothesis 2 in table 2) or

335 SVDquartet clades (hypothesis 3 in table 2) was the most likely secondary species hypothesis
336 (SSH), we compared them to a null hypothesis (a primary species hypothesis, hypothesis 1)
337 based on geographical origin, using BFD*. In addition to these species hypotheses we
338 considered another one bringing K1 and K2 together (hypothesis 4) because of the
339 admixture between K1 and K2 (figure 1.A). In addition, using two different sets of host SNPs
340 (see Material and Method section), the best SSH, using Bayes Factor comparisons, was the
341 sNMF clusters partition (table 2). We hence identified four different host genetic lineages in
342 *A. viridis* Mediterranean populations.

343 **Symbiont differentiation**

344 The two types of genetic markers (ITS2 and microsatellites) used to genotype the symbiont
345 populations from 148 *A. viridis* individuals (N148 dataset) revealed an overall similar picture
346 of the symbiotic composition, with no clear partitioning of the dataset based on either the
347 obtained 122 ITS2 variants (figure 3.A) or the microsatellite markers (figure 3.B).
348 Independently of the host genetic cluster, we further tested the correlation between host
349 genetic distances and the differences in symbiont content. The analysis did not show global
350 correlation either with the ITS2 assemblages (Mantel's $r = -0.06$ and a p-value of 0.839,
351 figure 4.A) or with the microsatellite markers (with a Mantel's $r = 0.06$ and a p-value of 0.086,
352 figure 4.B).

353 However, we detected with both types of markers a global effect of the sampling location on
354 the symbiont composition with PERMANOVA analyses (table 3, table S3 and table S4) with
355 similar symbiont content among host clusters at the same location (table S4). Nevertheless,
356 with microsatellite markers, a weak effect of the host genetic cluster was revealed (table 3)
357 mainly due to a differentiation between K2 and K4 occurring at Scandola (table S4).

358 Symbiont community composition was more similar among sea anemones from the same
359 location (intra- and inter-lineages Manhattan's average distances of $D = 2.186$ and 2.502
360 respectively, figure 5.A), than within the same host lineage at different locations (intra-
361 lineage inter-location, $D = 2.951$) and finally non-related individuals (inter-lineage inter-
362 location, $D = 3.063$). At a finer genetic scale, we used the clonal host genets found in
363 different locations to evaluate the differentiation of the symbiont community composition
364 within a genet. Six of the identified genets were distributed in at least two locations with at
365 least two ramets within a location (table S5). If vertical transmission was the only mode of
366 transmission of symbionts, we expected that the weakest differentiation would be observed
367 within a genet independently of the geographical origin. However, the differences in the
368 symbiont community composition among individuals from the same location were
369 significantly lower (intra- and inter-genets: $D = 1.331, 2.782$ resp.) than the -differences
370 among individuals from different locations (intra- and inter-genets: $D = 3.223, 3.027$ resp.,
371 figure 5.B). Hence the genets from the same location had similar symbiont populations.

372

373 **DISCUSSION**

374 In this study, we questioned whether the symbiont diversity observed in *A. viridis* adult
375 individuals was the result of closed vertical transmission only or whether secondary
376 horizontal acquisition and/or local selection could also occur and modify the symbiont
377 population in respect to the environment. We showed that sea anemones belonging to the
378 same host lineage, but sampled from different locations, harbour different symbiont
379 communities, revealing a mixed-mode symbiont transmission. However, how different are *A.*
380 *viridis* host lineages?

381 **Multiple *A. viridis* lineages in the Mediterranean Sea**

382 As we previously identified cryptic species in *A. viridis*, it was critical to first identify to which
383 host lineage the sampled individuals belonged. After taking clonality into account, we could
384 group the sampled individuals into 4 genetic clusters, recognized by BFD* as four
385 differentiated lineages. As a subset of samples used in this study had already been analysed
386 in Porro et al. (2019), we could recognize that the K1 and K2 lineages from this study
387 corresponded respectively to the Med1 and Med2 lineages from Porro et al. (2019).
388 Moreover, having increased the Mediterranean sampling density, we were now able to
389 redefine the distribution of Med1 and Med2, which are no longer single-location lineages,
390 and identify two new lineages, named Med3 and Med4, that corresponded to the clusters K3
391 and K4, respectively (figure 1 and table 2).

392 At all locations, lineages were found in sympatry with different degrees of admixture among
393 them. These admixture signals probably explained the incongruence between quantitative
394 genetic clustering (sNMF) and coalescent analysis (SVDquartet), with for example, seven
395 admixed individuals of the cluster K1 belonging to the clade 2 (table S1). As previously found,
396 Banyuls was the most genetically diverse location (with the presence of the four lineages),
397 supporting our supposition of the presence of a strong contact zone between genetic pools
398 in this Mediterranean zone (Porro et al., 2019). In addition, we now describe an ubiquitous
399 presence of the Med2 (K2) lineage at all sites, suggesting a strong ability for this lineage to
400 occupy different habitats and maybe great dispersal capacities as well. In all lineages and
401 sites, we detected an important clonal richness, which confirmed that *A. viridis* can deploy
402 genets with a great number of ramets (Mallien et al., 2017; Porro et al., 2019; Sauer, 1986;
403 Sauer, Müller, & Weber, 1986; Jörg Wiedenmann, Kraus, Funke, & Vogel, 2000), even if,

404 within this sampling, the Med4 (K4) lineage exhibited a much lower clonality. It is interesting
405 to note that the Med2 (K2) lineage showed varying levels of clonality from one site to the
406 other (R varied from 0.05 in Scandola to 0.9 in Sète), in a similar way to what had been
407 observed in another sea anemone, *Metridium senile*, with different clonality rates in
408 different locations along the N W Atlantic coast (from New York to Nova Scotia) (Hoffmann,
409 1986). More surprisingly, the ramets of the Med1 (K1), Med2 (K2) or Med3 (K3) genets were
410 not always located at the same site and some genets were comprised of individuals present
411 in distant sites (tables 4 and S1), suggesting again a potentially high capacity of
412 migration/dispersion in this species, as already alluded to in Porro et al. (2019). To disperse,
413 adult sea anemones may detach and move up to several meters away (Ayre, 1984; Mackie,
414 1974; Shick, 1991), but long-distance migration could likely occur by rafting attached to
415 freely drifting natural (as wood) or artificial (as ships' bottoms) substrates (Jackson, 1986).
416 The dispersal of asexual larvae as it occurs in some corals could also explain the distribution
417 of clonal genets (Gélin et al., 2017) but it was not observed yet in *A. viridis* (Utrilla et al.,
418 2019). *In fine*, even if established sea anemone clones have been considered as highly locally
419 adapted (Ayre, 1985), migration and successful new establishment of ramets are not rare in
420 *A. viridis*.

421 **Hosts from the same genetic lineage harbour different symbiont communities**

422 With both marker types (ITS2 and microsatellites), no correlation was revealed between the
423 genetic distances among symbionts and the genetic distances among their hosts (figure 3
424 and 4) suggesting that the closed vertical transmission is not the only mechanism of
425 symbiont acquisition in adult hosts. Our results also showed that the symbiont ITS2 diversity
426 was only, and strongly, linked to the geographical site of sampling. Microsatellite diversity

427 was strongly linked to the sampling location as well (table 3). The microsatellite markers also
428 revealed a weak correlation of symbiont content with the host genetic lineage (table 3),
429 which would be expected considering that symbionts are transferred through the eggs in this
430 species (Schäfer, 1984; Utrilla et al., 2019). This finding was summarized in the figure 6. It
431 should also be noted that only one of the within site comparisons of symbiont microsatellite
432 size distribution between host lineages was significant; at Scandola, where a strong
433 differentiation between the lineages of Med2 (K2) and Med4 (K4) occurred (table S4). This
434 was likely due to a large effect of host clonality on symbiont differentiation (preponderance
435 of the genet R155 at this site, figure 3B and table S5). The presence of this large genet is
436 surprising and could reflect a sampling artefact but could also be the consequence of the
437 dominance of a highly aggressive clone, a phenomenon already identified in sea anemones
438 by Ayre and Grosberg (1995).

439 **Hosts from the same genet harbour different symbiont communities**

440 If symbionts are vertically transmitted, the individuals belonging to the same genet, present
441 or not at the same site, should be dominated by the same symbiont community. We
442 observed however that individuals from the same genet, but from different locations, were
443 not more similar than individuals from different genets (figure 5.B, intra-genet inter-location
444 vs. inter-genet intra- or inter-location). Considering the high probability that the revealed
445 microsatellite diversity is neutral, this result is strongly in favour of the occurrence of
446 horizontal acquisition of symbionts at the adult stage in *A. viridis*, and thus against the
447 hypothesis of an entirely closed vertical transmission of symbionts.

448 **A mixed-mode symbiont transmission in *Anemonia viridis***

449 This result highlights the potential for a plastic response of *A. viridis* lineages to the
450 environment by modifying their symbiont community composition. Previous studies stated
451 that no genetic changes in the symbiont community composition occurred in *A. viridis* in
452 response to environmental change between subtidal and intertidal habitats (Bythell,
453 Douglas, Sharp, Searle, & Brown, 1997) or different pH conditions (Borell, Steinke, Horwitz, &
454 Fine, 2014; Suggett et al., 2012); or after living for a decade in proximity to cnidarians
455 containing mostly clade C (genus *Cladocopium* now) symbionts (Hartle-Mougiou et al., 2012).
456 These studies used ribosomal sequences as a genetic marker, but the flexibility has never
457 been investigated at the symbiont diversity scale using marker analysis by NGS (ITS2 or
458 microsatellite) or sampling geographically distant *A. viridis* populations. Such flexibility would
459 be beneficial for an organism living in a diverse and changing environment such as this
460 temperate sea anemone (see for example Secord & Muller-Parker, 2005).

461 However, the mechanism involved in this symbiont flexibility cannot be unambiguously
462 resolved yet as it could still be putatively due to i) a renewal of symbiont content by
463 horizontal acquisition from the environment at the adult stage or; ii) convergent changes in
464 symbiont frequency within large and diverse inherited symbiont populations. In the coral
465 *Montipora capitata*, comparing ITS2 sequence assemblages in eggs and their parent colonies,
466 Padilla-Gamiño et al. (2012) demonstrated that the eggs harboured a large range of ITS2
467 sequences and that symbiont assemblages in the eggs were strongly influenced by symbiont
468 composition of the parent colony, which in turn differed and reflected characteristics of their
469 physical environment. In *M. capitata*, an environmental selection of the inherited symbiont
470 diversity is then more plausible, as was also strongly suggested by Quigley et al. (2019) in
471 another species of the same genus. However, these *Montipora* species harbour different

472 genera (or species) of Symbiodiniaceae, known to have different ecophysiological profiles.
473 Therefore, the relative proportions of these different symbiont strains within a host could
474 very likely be influenced by local selection.

475 In our case, considering that *A. viridis* harbours only Temperate A Symbiodiniaceae, to
476 explain the observed pattern of local similarity in symbiont composition among host genets
477 and even lineages by convergent selection without horizontal acquisition, one would have to
478 imagine that each host larva, independently of its mother's affiliation to a clone or to a
479 reproductively isolated lineage, should inherit the whole complement of symbiont genetic
480 diversity that would allow such local differentiation. This would mean that adult sea
481 anemones belonging to genetically differentiated lineages still manage to transfer a wide
482 array of microsatellite diversity to their offspring, despite generations of local adaptation and
483 successive bottlenecks (at the production of the ovule). A mixed-mode of symbiont
484 acquisition with episodes of horizontal acquisition from local environment is the most
485 parsimonious explanation of the distribution of symbiotic diversity we observed. Indeed, a
486 very similar pattern of variability in symbiont population among host ramets had also been
487 observed in *Acropora palmata*, a coral species that only acquires its symbiont from the
488 environment at each generation (Baums et al., 2014).

489 Additional horizontal symbiont acquisition in hosts with maternal vertical symbiont
490 transmission has already been suggested in some corals such as *Pocillopora meandrina*,
491 *Pocillopora damicornis* *Stylophora pistillata* and *Seriatopora hystrix* (Boulotte et al., 2016;
492 Byler et al., 2013; Magalon, Baudry, Husté, Adjeroud, & Veuille, 2006; Quigley, Strader, &
493 Matz, 2018). In addition, analysing specifically within-colony symbiont diversity in *Pocillopora*
494 sp., Pettay et al. (2011), also strongly suggested that switching may occur between clonal

495 host lineages and that this species can mix vertical and horizontal symbiont acquisition.
496 Mixed-mode symbiont acquisition is frequent in symbiotic cnidarians and supports the
497 conclusions of Ebert (2013) that symbionts with mixed-mode transmission may be the most
498 common type of symbionts in animals.

499

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512

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803

804 **DATA ACCESSIBILITY**

805 The data will be available on Dryad

806 **CONFLICT OF INTEREST**

807 The authors declare that they have no conflict of interest

808 **AUTHOR CONTRIBUTION**

809 B.P. and C.M. prepared the samples from field sample to DNA extraction, B.P. and T.Z.
810 developed and set up molecular marker design, C.M. prepared the RAD-seq libraries, B.P.
811 and B.C.C.H. did post-sequencing *in silico* treatments and analyses. B.P., P.F. and D.F. designed
812 the research and participated to the writing. Finally, B.C.C.H., C.R.V., E.R. and C.M. reviewed
813 the paper.

814 **ORCID**

815 Barbara Porro <https://orcid.org/0000-0003-0294-1886>

816 Thamilla Zamoum <https://orcid.org/0000-0002-2649-5772>

817 Cédric Mallien <http://orcid.org/0000-0001-8701-6520>

818 Benjamin C.C. Hume <https://orcid.org/0000-0001-7753-3903>

819 Christian R. Voolstra <https://orcid.org/0000-0003-4555-3795>

820 Eric Rottinger <https://orcid.org/0000-0002-2938-6774>

821 Paola Furla <https://orcid.org/0000-0001-9899-942X>

822 Didier Forcioli <https://orcid.org/0000-0002-5505-0932>

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Loci	Symbiodiniaceae ex	Motif	Primers (forward reverse)	References
CAES-67	<i>Eunicella singularis</i>	GT	GTTTCGGGTCTCGGGATAGG TTAGCCCCAAAAACATAAACC	Catanéo et al. 2009 (FJ917549.1)
64238		CA	AGGGGTCTAGTTTCCTGAC GGACCGGATTCCTCTTGC	
71379	<i>Anemonia viridis</i>	CT	CAGCAGTTAGTCAATTTAAACGGC TCATGGTCGGTACTACTGGG	present study
72276		AG	GGGAGTTATCAAGCTGTTGGC TGTTTACATGCATAAACATCAGGAC	

826 Table 1: Detail of the symbiont microsatellite markers sequenced by NGS

827

828 Table 2: Test of *A. viridis* species hypotheses by BFD*. The four species hypotheses tested
829 were (1) a geographic partitioning (the primary species hypothesis), (2) the sNMF clustering,
830 (3) the SVDquartets partitioning and (4) an intermediate SVDquartets partitioning
831 considering the K1 and K2 admixture. (MLE = maximum likelihood estimator, BF = bayes
832 factor). In bold the secondary species hypothesis retained with the lowest MLE and the
833 degree of significance when compared with the other species hypotheses.

Species hypotheses	First BFD* run (598 SNPs)				Second BFD* run (599 SNPs)			
	MLE	2 ln BF			MLE	2 ln BF		
		1 vs	2 vs	3 vs		1 vs	2 vs	3 vs
1 Ban/Bar/Mar/Sca/Tha	-8844.82				-9010.55			
2 K1/K2/K3/K4	-8478.39	-732.85			-8694.89	-631.32		
3 Clade1/Clade2/Clade3/Clade4	-8506.24	-677.16	55.69		-8713.98	-593.15	38.17	
4 (Clade1+Clade2)/Clade3/Clade4	-8584.71	-520.22	212.63	156.94	-8793.75	-433.60	197.72	159.54

834

835 Table 3: PERMANOVA tests of differentiation of symbiont community compositions based on
836 ITS2 sequences (upper part) or microsatellite distributions (lower part).

markers	factors	Df	Sums of squares	Mean Sqs	F.Model	R2	Pr(>F)
ITS2	Location	4	2.4868	0.6217	2.92662	0.08028	1.00E-05
	Lineage	3	0.6705	0.2235	1.05212	0.02165	0.3437
	LocationxLineage	7	1.2646	0.18066	0.85043	0.04083	0.857
	Residuals	125	26.5536	0.21243		0.85725	
	Total	139	30.9755			1	
microsatellite	Location	4	196.64	49.16	15.152	0.2829	1.00E-05
	Lineage	3	25.66	8.552	2.636	0.03691	0.00221
	LocationxLineage	7	41.29	5.898	1.818	0.0594	0.00566
	Residuals	133	431.5	3.244		0.62079	
	Total	147	695.09			1	

837 PERMANOVAs were realised over 99999 permutations. The bold values indicate factors with statistically

838 significant effect on the symbiont differentiation.

839 FIGURES

840 Figure 1: sNMF genetic clusters of *A. viridis* and their geographic distribution in the
841 Mediterranean Sea.

842 A: Bar plot representation of the sNMF analysis of the animal host (N65 dataset). The genetic
843 diversity could originate from 4 ancestral genetic clusters (named K1 to K4, respectively in
844 purple, red, blue and green). The bar plot represents the contribution of each of these
845 clusters to the genetic constitution of each individual in this dataset. The sampling location of
846 each individual is mentioned above the barplot. B: Proportion of each cluster in each
847 sampling location (N148 dataset, table S1), along with local sample size and clonal richness R.
848 (Banyuls: 42°28'50"N 03°07'50"E; Barcarès: 42°47'15.828"N 3°2'11.633"E;
849 Sète: 43°23'32.82"N 3°36'1.43"E; Marseille: 43°17'44.337"N 5°20'34.348"E;
850 Scandola: 42°21'29.571"N 8°33'19.166"E)

851 *Host genetic lineages correspond to genetic lineages either identified in Porro et al (2019)
852 (Med1 and Med2) and in the present study (Med3 and Med4), see discussion section.

853 Figure 2: Host datasets. Description (number of individuals and SNP markers) of the different
854 datasets used for each analysis.

855 BFD is for Bayes Factor Delimitation and refers to the BFD* analysis to test species
856 hypotheses.

857 Figure 3: Differentiation of *A. viridis* individuals based on algal symbiont composition (N148
858 dataset): A. NJ tree based on Jaccard's distances of ITS2 assemblages among the sea
859 anemones; B. NJ tree based on Manhattan's distances of microsatellite distributions among
860 sea anemones. The bootstraps higher than 40 were displayed on the nodes. Colours
861 correspond to the host genetic cluster from the figure 1 (K1: purple, K2: red, K3: blue and K4:
862 green); the symbols correspond to the host genets: the ramets of the same genet shared the
863 same coloured symbol, full circles correspond to the unique genets.

864 Figure 4: Scatterplot and Mantel's tests of pairwise host genetic distances against pairwise
865 symbiont distances among *A. viridis* (N148 dataset): considering, A: the symbiont ITS2
866 sequences assemblage (Jaccard's distance - with a Mantel's $r = -0.06$ and $p\text{-value} = 0.839$); B:
867 the symbiont microsatellite distributions (Manhattan's distance - with a Mantel's $r = 0.06$ and
868 $p\text{-value} = 0.086$). The dashed line indicates the threshold host distance between intra-genet
869 and inter-genet distances (see figure S1).

870 Figure 5: Intra- and inter-locations differentiation in symbiont microsatellite contents for the
871 different host genetic structures (N148 dataset). A: Comparison of distance distributions
872 within lineages (intra-) or between lineages (inter-) from the same sampling location (intra-
873 locations) or between different locations (inter-locations). B: Comparison of distance
874 distributions within genet (intra-) or between genet (inter-) from the same sampling location
875 (intra-locations) or between different locations (inter-locations). The global $p\text{-values}$ for the
876 both comparisons (A and B) is $< 2.2 \times 10^{-16}$ and tested with Kruskal-Wallis tests. The lowercase
877 letters indicate the statistical differences. The boxes are the two first quartiles of
878 distributions, the whiskers are the third and the fourth quartiles. The black horizontal bars (in
879 the boxes) are the medians (with IC95 figured with the notches). The dots correspond to the
880 averages of Manhattan's distances of the distributions the standard deviation is given by the

881 crosses. Blank points are outlier values.

882 Figure 6: Schematic illustration of the host and symbiont differentiation among sites
883 according to our results. The colours of the sea anemones correspond to the host lineages or
884 host lineages, the symbols inside are the symbiont communities.