Horizontally transmitted symbiont populations in deep-sea mussels are genetically isolated

Devani Romero Picazo[1*], Tal Dagan[1], Rebecca Ansorge[2], Jillian M. Petersen[3], Nicole Dubilier[2], Anne Kupczok[1*]

[1] Genomic Microbiology Group, Institute of General Microbiology, Christian-Albrechts University, Kiel
[2] Max Planck Institute for Marine Microbiology, Bremen
[3] Division of Microbiology and Ecosystem Science, University of Vienna
* Corresponding authors: dpicazo@ifam.uni-kiel.de, akupczok@ifam.uni-kiel.de

Abstract

Eukaryotes are habitats for bacterial organisms where the host colonization and dispersal among individual hosts have consequences for the bacterial ecology and evolution. Vertical symbiont transmission leads to geographic isolation of the microbial population and consequently to genetic isolation of microbiotas from individual hosts. In contrast, the extent of geographic and genetic isolation of horizontally transmitted microbiota is poorly characterized. Here we show that chemosynthetic symbionts of individual *Bathymodiolus brooksi* mussels constitute genetically isolated populations. The reconstruction of core genome-wide strain sequences from high-resolution metagenomes revealed distinct phylogenetic clades. Nucleotide diversity and strain composition vary along the mussel lifespan and individual hosts show a high degree of genetic isolation. Our results suggest that the uptake of environmental bacteria is a restricted process in *B. brooksi*, where self-infection of the gill tissue results in serial founder effects during symbiont evolution. We conclude that bacterial colonization dynamics over the host life-cycle is thus an important determinant of population structure and genome evolution of horizontally transmitted symbionts.

Bacteria inhabit most eukaryotes where their presence has consequences for key aspects of their host biology[1], such as host development[2], nutrition[3], or behavior[4]. From the bacterial perspective, animals constitute an ecological niche, where microbial communities utilize the resources of their host habitat[5]. The microbiota biodiversity over the host life cycle is determined by bacteria colonization dynamics and by host properties, including biotic and abiotic factors. For example, the microbiota can be affected by the host diet[6] or the host physiological state (e.g., hibernation[7] or pregnancy[8]). In addition, changes in the host environmental conditions such as temperature[9] or the availability of reduced compounds[10] can have an effect on the microbiota community composition.

Microbiota dispersal over the host life cycle depends on the level of fidelity between the host and its microbiota; in faithful interactions, vertically transmitted bacteria are transferred from adults to their progeny during early host developmental stages, while in less faithful interactions, horizontally transmitted bacteria are acquired from the environment throughout the host life cycle[11]. Strictly vertically transmitted bacteria are specialized in their host niche and their association with the host imposes an extreme geographic isolation. Bacterial inheritance over host generations imposes a strong bottleneck on the microbiota population and leads to reduced intra-host genetic diversity[12]. Examples are monoclonal or biclonal populations observed in symbiotic bacteria inhabiting grass sharpshooter[13] and pea aphids[14]. Furthermore, the geographic isolation of vertically transmitted bacteria leads to genetic isolation and to symbiont genome reduction over time as a consequence of genetic drift[15]. In contrast, dispersal is expected to be higher for horizontally transmitted bacteria, where host-associated populations are connected to one another through the environmental pool[16]. Nonetheless, the genetic diversity of horizontally transmitted microbial populations may also be reduced due to bottlenecks during symbiont transmission and host colonization. Stochastic effects in the colonization of horizontally transmitted bacteria may manifest themselves in differences in microbiota strain composition among hosts[17,18]. This would lead to subdivided symbiont populations where the geographic isolation of the microbiota depends on the degree of symbiont dispersal among individual hosts. Geographic isolation of individual hosts over the host life span would then lead to genetic isolation of the symbiont populations and to symbiont population structure. Genomic variation and genetic isolation have been observed for horizontally transmitted symbionts of the human gut microbiome[19] and of the honey bee gut microbiome[20]. Moreover, structured symbiont populations can also emerge within an individual host, as observed for *Vibrio fischeri* colonizing the squid light organ, where different light organ

60 crypts are infected by a specific strain[21]. The degree of dispersal of horizontally transmitted
61 symbionts remains understudied; hence, whether populations from different microbiomes are
62 intermixing or are genetically isolated is generally unknown.

63 Here we study the microbiota strain composition of horizontally transmitted
64 endosymbionts across individual *Bathymodiolus brooksi* deep-sea mussels. *Bathymodiolus*
65 mussels live in a nutritional symbiosis with the chemosynthetic sulfur-oxidizing (SOX) and
66 methane-oxidizing (MOX) bacteria. The symbionts are acquired horizontally from the seawater
67 and are harbored in bacteriocytes within the gill epithelium[22,23]. Most Bathymodiolus species
68 harbor only a single 16S rRNA phylotype for each symbiont, including *B. brooksi*[24].
69 Nevertheless, a recent metagenomic analysis of *Bathymodiolus* species from hydrothermal
70 vents in the mid Atlantic ridge showed the presence of different SOX strains with differing
71 metabolic capacity[25]. Mussel gills constantly develop new filaments that are continuously
72 infected[26]. However, whether the new gill filaments in *Bathymodiolus brooksi* are colonized
73 predominantly by environmental bacteria or by symbionts from older filaments of the same host
74 remains unknown. These two alternative scenarios are expected to impose different degrees of
75 geographic isolation on the symbiont population: in continuous environmental acquisition, the
76 level of inter-host dispersal is high while self-infection limits the symbiont dispersal. Here we
77 studied the impact of tissue colonization dynamics of horizontally transmitted intracellular
78 symbionts on the degree of symbiont diversity. Furthermore, we quantified the level of genetic
79 isolation among communities across individual mussels and its impact on symbiont genome
80 evolution. For that, we implemented a high-resolution metagenomics approach that captures
81 genome-wide diversity for both symbionts in multiple *Bathymodiolus brooksi* individuals from a
82 single site.

83 **Results**

84 *Gene-based metagenomics binning recovers SOX and MOX core genomes*

85 To study the evolution of the SOX and MOX genomes in *Bathymodiolus* mussels we used a
86 high-resolution metagenomics approach. Twenty-three *B. brooksi* individuals of shell sizes
87 ranging between 4.8 cm and 24.3 cm were sampled from a single location at a cold seep site in
88 the northern Gulf of Mexico. Shell size correlates with mussel age[27]; thus, analyzing mussels
89 within a wide shell size range allowed us to study the symbiont population structure across host
90 ages. The mussels were sampled from three separate mussel 'clumps' (small mussel patches
91 residing on the sediment) that were at most 131m apart (Supplementary Fig. 1). Such a 'patchy'

92 distribution has often been observed in deep-sea mussels[28]. To obtain a comprehensive
93 representation of the bacterial population in individual mussels and to accurately infer strain-
94 specific genomes, homogenized gill tissue of each mussel was deeply sequenced (on average,
95 37.8 million paired-end reads of 250bp per sample, Supplementary Table 1). The resulting
96 metagenomic sequencing data was analyzed by a gene-based binning approach[29].

97 The prediction of protein-coding genes from the assembled metagenomes yielded a
98 non-redundant gene catalog of 4.4 million genes that potentially contains every gene present in
99 the samples. This includes genes from the microbial community and from the mussel host. In
100 the metagenomics binning step, genes that covary in their abundance across the different
101 samples were clustered into metagenomic species (MGSs). Our analysis revealed two MGSs
102 that comprise the SOX and MOX core genomes (Supplementary Fig. 2). The distribution of
103 gene coverage in individual samples shows that genes in each core genome have a similar
104 abundance within each mussel. This confirms the classification of the SOX and MOX MGSs as
105 core genomes. The MOX core genome is the largest MGS and it contains 2,518 genes with a
106 total length of 1.97 Mbp. A comparison to Gammaproteobacteria marker genes shows that it is
107 96.2% complete. Furthermore, it contains 1,568 genes (62.3%) that have homologs in MOX-
108 related genomes. The SOX core genome contains 1,439 genes, has a total length of 1.27 Mbp
109 and is considered as 80.2% complete. It contains 1,188 genes (82.6%) with homologs in SOX-
110 related genomes. In addition to the SOX and MOX core genomes, our analysis revealed a third
111 MGS of 1,449 genes (Supplementary Fig. 2) that was found in low abundance in a single
112 mussel and, in addition, 98,944 co-abundant gene groups (CAGs, 3-699 genes). Of the 23
113 metagenomes, four samples were discarded during the metagenomics binning. Two samples
114 were discarded prior to the binning due to high variance in symbiont marker gene coverages
115 and two samples were discarded after binning due to low coverage for both symbionts
116 (Supplementary Figs. 2,3). To gain insight into the SOX and MOX population structure between
117 hosts, we compared the characteristics of the core genomes across the remaining 19 samples.
118 The analysis of the core genome coverages shows that SOX is the dominant member of the
119 mussel microbiota. The differences in the SOX to MOX ratio among the mussel metagenomes
120 are likely explained by differences in the availability of H_2S and CH_4 among clumps, which is a
121 known determinant of SOX and MOX abundance in *Bathymodiolus*[30] (Supplementary
122 Information, Supplementary Fig. 4).

123 To study symbiont diversity below the species level, we analyzed single nucleotide
124 variants (SNVs) that were detected in the core genomes of the two symbionts. In this analysis,

125 we considered SNVs that are fixed in a metagenome as well as polymorphic SNVs, i.e., SNVs,
126 where both the reference and the alternative allele are observed in a single metagenome. We
127 found 18,070 SNVs in SOX (SNV density of 14 SNVs/kbp, 49 multi-state, 0.27%) and 4,652
128 SNVs in MOX (SNV density of 2.4 SNVs/kbp, 5 multi-state, 0.11%). The number of polymorphic
129 SNVs per sample ranges from 162 (0.9%) to 11,064 (61%) for SOX and from 27 (0.58%) to
130 3,026 (65%) for MOX (Supplementary Table 1), thus, most SNVs are polymorphic in at least
131 one sample. It is important to note that the observed difference in strain-level diversity between
132 SOX and MOX cannot be explained by the difference in sequencing depth (Supplementary
133 Information). These results are in agreement with previous reports of SOX genetic diversity in
134 other *Bathymodiolus* species[25]. We further revealed that there is genetic diversity in the MOX
135 symbiont.

136 *Bathymodiolus microbiota is composed of SOX and MOX strains from several clades.*

137 Diversity in natural populations of bacteria is characterized by cohesive associations among
138 genetic loci that contribute to lineage formation and generate distinguishable genetic clusters
139 beyond the species level[31]. The formation of niche-specific genotypes (i.e., ecotypes) has been
140 mainly studied in populations of free-living organisms such as the cyanobacterium
141 *Prochlorococcus* spp.[32]. Here we consider a strain to be a genetic entity that is present in
142 multiple hosts and is characterized by a set of clustered variants in the core genome. To study
143 lineage formation in symbiont populations associated with *Bathymodiolus* mussels, we
144 reconstructed the strain consensus core genomes from strain-specific variants that show similar
145 frequencies in a metagenomic sample.

146 The SNVs found in multiple samples and their covariation across samples were used for
147 strain deconvolution of the core genomes using DESMAN[33]. This revealed that SOX is
148 composed of eleven different strains with a mean strain core genome sequence identity of
149 99.52%. Phylogenetic reconstruction shows that the eleven strains cluster into four clades,
150 which are separated by relatively long internal branches (Fig. 1b). Notably, 849 of the SNVs on
151 the SOX core genome (4.7%) do not differentiate between strains. Thus, the resulting strain
152 alignment is invariant for each of these positions and they are termed invariant SNVs from here
153 on. For MOX, six strains with a mean core genome sequence identity of 99.88% were
154 reconstructed. The phylogenetic network shows that the six strains cluster into two clades
155 comprising three strains each (Fig. 1e). Of the total SNVs, 1,138 (24.4%) are invariant in the
156 strain alignment. The overall MOX branch lengths are shorter than those of SOX. We detected

157 no effect of sequencing coverage on the inference of the strain clades for SOX (Supplementary
158 Information, Supplementary Fig. 5).

159 To study the community assembly at the strain level, we examined the strain distribution
160 across individual mussels. Each SOX strain could be identified in between three and eight
161 samples (frequency ≥5%; Fig. 1a). Only one or two strains were detected with a frequency of at
162 least 5% in small mussels (≤7 cm), two to nine strains in medium-sized mussels (7.2 cm – 14.1
163 cm) and one to two strains in large mussels (14.6 cm – 24.1 cm). Notably, only strains from
164 clades S1 and S2 are present in large mussels (≥14.6 cm). One of the large mussels (S) is an
165 exception as it hosts three SOX strains and contains strains from both clades S1 and S2. Six
166 mussels have one dominant SOX strain (frequency ≥90%). Five of these are large mussels (M,
167 N, P, Q, R) and only one is a small mussel (C). The dominant strain is either S1.4, S2.1, or S2.2
168 (Fig. 1a; Supplementary Table 1). The MOX strain composition across mussels shows that each
169 MOX strain occurs (frequency ≥5%; Fig. 1d) in four to 17 mussels and each mussel contains
170 two to four MOX strains. Additionally, strains of clade M2 are dominant in ten of the mussels.

171 To investigate the degree of genetic cohesion within strain clades in the population, we
172 studied the allele frequency spectrum (AFS) of each mussel. A visual inspection of the derived
173 allele frequency spectra revealed multimodal distributions for both symbiont populations. The
174 modes reach high allele frequencies and are associated with the main phylogenetic clades; this
175 suggests that the clades constitute cohesive genetic units (Fig. 1c,f; Supplementary Fig. 6). The
176 presence of high-frequency modes is especially apparent for SOX in medium-sized mussels
177 that contain multiple strains. To identify sample-specific strain sequences, we reconstructed
178 dominant haplotypes (major allele frequency ≥90%) for the samples that contain a dominant
179 strain (strain frequency ≥90%). By comparing dominant haplotypes among samples containing
180 the same dominant strain, we found that these can contain between 42 and 74 differential SNVs
181 (Supplementary Table 1). This suggests that the fixation of variants within individual mussels
182 contributes to the observed population structure.

183 Overall, our results revealed that the symbiont populations are composed of strains that
184 cluster into few clades, which appear to be maintained by strong cohesive forces. In addition,
185 the strains are shared among multiple mussels and multiple strains are capable of dominating
186 different hosts. This suggests that stochastic processes are governing the symbiont community
187 assembly, as previously proposed for other *Bathymodiolus* species[34].

188 *SOX strains evolve under purifying selection while MOX evolution is characterized by neutral*
189 *processes*

190 To study the evolution of SOX and MOX strains in *Bathymodiolus*, we examined the selection
191 regimes that have been involved in the formation of cohesive genetic SOX and MOX units. The
192 core genome-wide ratio of pN/pS is higher in MOX (pN/pS of 0.425) in comparison to SOX
193 (pN/pS of 0.137), which indicates that the strength of purifying selection is higher for SOX. In
194 addition, we estimated pN/pS for each of the symbiont core genes. This revealed that MOX
195 genes are characterized by large pN/pS and small pS values, while SOX genes have small
196 pN/pS and large pS values (Supplementary Fig. 7). The relative rate of nonsynonymous to
197 synonymous substitutions has been shown to depend on the divergence of the analyzed
198 species[35,36]. For populations of low divergence, SNVs comprise substitutions that have been
199 fixed in the population and mutations that arose recently. The latter include slightly deleterious
200 mutations that were not yet purged by selection, resulting in an elevated ratio of
201 nonsynonymous to synonymous replacements. Thus, this ratio is not suitable for analyzing
202 closely related genomes, which is usually the case when studying variation within bacterial
203 species.

204 To circumvent the bias in pN/pS, we tested for differences in selection regimes in the
205 evolution of SOX and MOX strains using the neutrality index (NI). NI is used to distinguish
206 between divergent and polymorphic SNVs and to quantify the departure of a population from the
207 neutral expectation. An excess of divergent nonsynonymous mutations (NI<1) indicates that the
208 population underwent positive selection or an important demographic change in the past[37]. We
209 estimated NI by considering two different levels of divergence and polymorphism. In the first
210 level, all identified strains are considered as diverged taxonomic units; in the second level, we
211 disregard the small-scale strain classification and consider only the clades as diverged
212 taxonomic units (Table 1). Considering all strains as divergent, we observed a low NI^{MOX} (<1),
213 which suggests that MOX evolved under a neutral (NI~1) or positive selection regime. NI^{MOX}
214 increased when considering the clades as diverged, which suggests that the low NI^{MOX}
215 observed at the strain level is the result of an excess of nonsynonymous SNVs within the strain
216 clades that may constitute transient polymorphisms. Thus, the excess of nonsynonymous
217 mutations observed for MOX is biased by the low level of divergence; hence, similar to the
218 pN/pS ratio, it cannot serve as an indication for positive selection. On the other hand, we found
219 that purifying selection is in action for SOX (NI^{SOX}>1). Similar to MOX, when using the clades as

220 divergent, NI^{SOX} slightly increases. This indicates that the SNVs that differ between clades are
221 more likely to be substitutions in comparison to those that differ among within-clade strains.

222 Altogether, these results suggest differences in the selection regimes during the
223 evolution of the SOX and MOX strains. While the SOX core genome is shaped by purifying
224 selection, we cannot detect deviation from the neutral expectation in the MOX core genome.
225 These differences likely stem from the different divergence levels among the strains of both
226 symbiont populations. The association of SOX with *Bathymodiolus* mussels is considered to be
227 ancient in chemosynthetic deep-sea mussels whereas the MOX association is thought to have
228 evolved secondarily during *Bathymodiolus* diversification[38]. This is in agreement with the larger
229 degree of divergence observed here for SOX. Since we observed no evidence for positive
230 selection on the symbiont core genomes, we suggest that the strains constitute cohesive
231 genetic units within one ecotype[39], where all strains are functionally equivalent at the core
232 genome level. Notwithstanding, the strains might be linked to differences in the accessory gene
233 content, as observed, for example, in the free-living cyanobacterium *Prochlorococcus* spp.[32] and
234 in SOX symbionts of other *Bathymodiolus* species[25].

235 *Intra-sample diversity is higher for SOX than for MOX.*

236 The association with the host limits the dispersal of bacterial populations where the association
237 across generations is likely maintained by symbiont dispersal between host individuals. If
238 symbionts are not continuously taken up from the environment, each individual host constitutes
239 an isolated habitat over its lifetime[5]. Geographic isolation between habitats results in genetic
240 isolation and contributes to the formation of cohesive associations of genetic loci[31]. Previous
241 studies showed that geographic isolation during vertical transmission can lead to the reduction
242 of intra-host genetic diversity in the bacterial populations[12], nonetheless, the degree of isolation
243 remains understudied for horizontally transmitted microbes. To characterize the contribution of
244 geographic isolation to strain formation in the *Bathymodiolous* symbiosis, we next studied the
245 degree of genetic isolation. Our sample collection of mussels covering a range of sizes (and
246 thus ages) enabled us to compare symbiont genome diversity among individual hosts of
247 different age within a single sampling site, thus minimizing the putative effect of biogeography
248 on population structure. The host species *B. brooksi* is ideal for such an analysis as it grows to
249 unusally large sizes and possibly lives longer than many other *Bathymodiolus* species. To study
250 differences in genome diversity of the two symbionts across individual mussels, we estimated

251 the intra-sample nucleotide diversity (π) and the ecological measure α-diversity at the resolution
252 of the SOX and MOX strains.

253 We found a high variability of π^{SOX} among different mussels (intra-sample π^{SOX} between
254 $5.2x10^{-5}$ and $3.6x10^{-3}$, Table 2, Fig. 2). Furthermore, π^{SOX} and the SOX α-diversity are
255 significantly positively correlated (ρ^2=0.98, $p<10^{-6}$, Spearman correlation, Fig. 2a); hence, the
256 intra-sample strain diversity is well explained by the nucleotide diversity. The variability in π^{SOX}
257 agrees with the three age-related groups observed before for the number of SOX strains across
258 mussel size. Small mussels (≤7cm) and large mussels (14.6cm – 24.1cm) have a low π^{SOX} and
259 harbor one to two strains. Medium-sized mussels (7.2cm – 14.1cm) have a high π^{SOX} and harbor
260 two to nine strains. The community in the largest mussel is an exception, as it has a high π^{SOX},
261 similar to medium-sized mussels, which can be explained by the presence of three strains from
262 two clades.

263 The MOX nucleotide diversity is significantly lower in comparison to SOX (intra-sample
264 π^{MOX} between $5.6x10^{-6}$ and $7.0x10^{-4}$, Table 2, Wilcoxon signed rank test, p=0.015, Fig. 2).
265 Similar to SOX, the MOX α-diversity is significantly positively correlated with π^{MOX} (ρ^2=0.89,
266 $p<10^{-6}$, Spearman correlation) (Fig. 2b). One group of mussels harbors only MOX strains from
267 clade 2 and is characterized by low MOX nucleotide diversity (A, C, J, L, M, P, Q, R, S, π^{MOX}
268 between $5.6x10^{-6}$ and $2.1x10^{-5}$), while the other group habors MOX strains from both clades and
269 is characterized by high MOX nucleotide diversity (B, D, E, F, G, H, I, K, N, O, π^{MOX} between
270 $1.4x10^{-4}$ and $7.0x10^{-4}$). These groups are not associated with mussel size. Taken together, we
271 observed a strong correlation between the nucleotide diversity π and α-diversity for both
272 symbionts. Notably, π is based on all the detected SNVs whereas the α-diversity is based only
273 on the strain composition and relatedness. Thus, the strong correlation demonstrates that the
274 strain diversity captures most of the core genome-wide nucleotide diversity.

275 A comparison of the π values estimated here to other microbiome studies shows that
276 higher π^{SOX} have been observed in other *Bathymodiolus* species (mean between 2.2 $x10^{-3}$ and
277 $3.9x10^{-3}$)[25]. The average SOX and MOX nucleotide diversity estimated here is within the range
278 of π values observed in the clam *Solemya velum* microbiome where the symbiont transmission
279 mode is thought to be a mixture of vertical and horizontal transmission[40]. Furthermore, our π
280 estimates are lower than those observed for most bacterial species in the human gut
281 microbiome that are considered horizontally transmitted[19].

Geographic isolation of bacterial communities associated with individual mussels.

Symbiont transmission mode is an important determinant of the community assembly dynamics[11]. For horizontally transmitted microbiota, similar community composition among hosts may develop depending on factors that affect the community assembly such as the environmental bacterial biodiversity or the order of colonization[41]. To study the degree of geographic isolation between mussel hosts, we calculated genome-wide fixation index F_{ST} and the ecological measure β-diversity at the strain resolution across the metagenomic samples for the two symbionts. Small F_{ST} indicates that the samples stem from the same population whereas large F_{ST} indicates that the samples constitute subpopulations.

Our results revealed generally high pairwise F_{ST} values, indicating a strong genetic isolation between individual mussels (mean pairwise F_{ST}^{SOX} of 0.618, mean pairwise F_{ST}^{MOX} of 0.495, Fig. 2); hence, most mussels in our sample harbor an isolated symbiont subpopulation of SOX and MOX. In addition, the SOX β-diversity is significantly positively correlated with F_{ST}^{SOX}(ρ^2=0.7, p <10^{-6}, Spearman correlation). We observed subpopulations of mussels that are characterized by a low pairwise F_{ST}^{SOX} within the subpopulation and a high pairwise F_{ST}^{SOX} with other mussels. This subpopulation structure is also represented in the distribution of β-diversity (Fig. 2). Thus, mussels from the same subpopulation harbor genetically similar SOX communities and similar strain composition. Examples are one group of mussels including L, O, P, and Q that contains only strains of clade S2 and another group including the mussels M, N, and R that contains only strains of clade S1 (Fig. 2a). Notably, the two subpopulations contain only large mussels that are characterized by a low π^{SOX}.

The distribution of pairwise F_{ST}^{MOX} revealed two main groups: one mussel group is characterized by high pairwise F_{ST}^{MOX} and low π^{MOX} while the other group is characterized by lower F_{ST}^{MOX} and high π^{MOX} (Fig. 2b). These correspond to the previously described groups, where one contains mussels with a low π^{MOX} and strains from clade M2 and the other group contains mussels with a high π^{MOX} and strains from both clades. We did not observe an association between MOX β-diversity and F_{ST}^{MOX} (p>0.05, Spearman correlation), which can be explained by the high proportion of invariant SNVs in MOX. Although the analysis of F_{ST}^{MOX} did not reveal MOX subpopulations, the pattern of β-diversity uncovered subpopulations that show a high pairwise F_{ST}^{MOX}. These subpopulations have a low β-diversity and a low nucleotide diversity. One subpopulation consisting of large mussels (P, Q, S) is characterized by the

313 presence of strain M2.3 and the absence of clade M1. Another subpopulation (A, C, J, L, M, R)
314 containing mussels of different sizes is characterized by the dominance of strains M2.1 and
315 M2.2 and the absence of clade M1. Thus, the comparison of strain composition across mussels
316 revealed that the population of MOX is substructured similarly to SOX. However, unlike SOX,
317 the MOX subpopulations are not associated with specific mussel shell sizes.

318 The high F_{ST} values and the population structure we observed here reveal population
319 stratification, that is especially pronounced for SOX. One possible factor that influences
320 symbiont population structure is host genetics, whose impact on the composition of horizontally
321 transmitted microbiota has been debated in the literature. Studies of the mammal gut
322 microbiome showed that the host genotype had a contribution to the microbiome composition in
323 mice[42], whereas the association with host genetics was reported to be weak in humans[43].
324 Analyzing 175 SNVs in 12 mitochondrial genes, we detected no association between mussel
325 F_{ST} and symbiont F_{ST} for any of the two symbionts (Supplementary Information, Supplementary
326 Fig. 8). Consequently, we conclude that the strong subpopulation structure observed for SOX
327 and MOX cannot be explained by mussel relatedness (i.e., host genetics) or location.

328 Our results provide evidence for a strong genetic isolation between the symbiont
329 populations associated with individual mussels. This finding is consistent with the observed
330 individual-specific symbiont strain composition. In contrast, much lower F_{ST} values were found
331 for SOX populations in other *Bathymodiolus* species sampled from hydrothermal vents (mean
332 F_{ST} per site between 0.05 and 0.17), which implies a weaker genetic isolation in these vents[25].
333 Our analysis of cold seep *B. brooksi* data revealed SOX subpopulations with low genetic
334 isolation that are observed using both F_{ST}, which takes all SNVs into account, and β-diversity at
335 the level of strains. In contrast, only β-diversity disclosed subpopulations for MOX. Thus, strain-
336 resolved metagenomics resolves similarities between individual mussel microbiomes below the
337 species level.

338 **Discussion**

339 Our analysis revealed strong genetic isolation of symbiotic bacterial populations in individual
340 mussel hosts, indicating geographic isolation between mussels. We hypothesize that this
341 geographic isolation occurs through restricted uptake of SOX and MOX symbionts from the
342 environment over time. The lack of evidence for strong adaptive selection in SOX and MOX
343 strains suggests that the inter-host population structure is the result of neutral processes rather
344 than host discrimination against different strains. Here, we propose a neutral model for symbiont

community assembly that explains how restricted symbiont uptake and colonization impose barriers to the symbiont dispersal, which can, over time, lead to inter-host population structure and contribute to the formation of cohesive genetic units within the symbiont population (Fig. 3). In our model, bacteria are acquired from the environmental symbiont pool in juveniles[44]. The presence of a symbiont environmental pool was suggested before based on the detection of symbiont genes in adjacent seawater[45,46]. Nevertheless, the loss of central metabolic enzymes suggests that bacteria disperse in a dormant state[47]. We hypothesize that the dormancy of free-living symbionts and the preservation of few symbiont cells inside bacteriocytes[23] contribute to the isolation of bacterial populations inside the host cells from the rest of the population, which can lead to recombination barriers. Our results support the self-infection hypothesis[26], according to which, once the gill is first colonized, bacteria present in ontogenically older tissue infect newly formed gill filaments; thus, the uptake of symbionts from the environment is limited. In addition, decreased growth rate in older mussels may also lead to decreased symbiont uptake. This model provides a plausible explanation for the observed pattern of strong symbiont genetic isolation between mussels and of reduced SOX strain diversity in large mussels. Possible later infections of the gill tissue from the environmental pool may occur due to symbiont loss and replacement driven by environmental changes or increased gill growth rate. Notably, our results are in contrast to a recent study on other *Bathymodiolus* species from hydrothermal vents, that concluded that SOX populations from individual mussels of the same site intermix[25]. This contrast may be explained by differences in the symbiont abundance in the seawater, which is expected to play a role in the colonization process. Our samples originate from a cold seep site with low mussel density (Supplementary Fig. 1); thus, the concentration of symbionts in the surrounding seawater may be correspondingly low. The low symbiont abundance would result in a low probability of later infections and a prevalence of self-infection. In contrast, the symbiont abundance in the seawater at large and densely populated mussel beds at hydrothermal vents is expected to be higher, resulting in a higher probability of later infections.

The colonization of new filaments over the mussel lifespan via self-infection entails serial founder events on the bacterial population. Throughout this process, new mutations arising in the symbiont population during the lifetime of the mussel can reach fixation due to genetic drift following population bottelnecks. This process is expected to lead to a reduction of symbiont genetic diversity over the mussel life time. Thus, individual mussels develop into independent habitats that harbor individual populations, which are genetically isolated from other mussel-associated symbiont populations and from the environmental pool. The evolution of vertically

transmitted endosymbiont populations is similarly affected by serial founder effects[48], as we suggest here for horizontally transmitted bacteria. However, migration between host-associated populations and the environmental pool results in an increased effective population size for horizontally transmitted bacteria; thus, the population is not subject to the fate of genome degradation as commonly observed in vertically transmitted symbionts[15]. Serial founder effects and recombination barriers due to geographic isolation are important drivers of lineage formation in bacteria[39]. Reduction of genetic diversity due to transmission bottlenecks is considered a hallmark of pathogen genome evolution[49]; examples are *Yersinia pestis*[50] and *Listeria monocytogenes*[51]. Our model demonstrates that, similar to pathogenic bacteria, genome evolution of bacteria with a symbiotic lifestyle can be affected by serial founder effects due to self-infection.

Methods

Collection and sequencing

Twenty-three individuals of *Bathymodiolus brooksi* mussels were collected during a research cruise with the E/V *Nautilus* from the cold seep location GC853 at the northern Gulf of Mexico in May 2015. The mussel distribution at the cold seep was patchy and mussel individuals were collected from three distinct clumps within a radius of 131 meters (coordinates clump a: 28.1237, -89.1404 depth: -1073m, clump b: 28.1241, -89.1401 + depth: -1073m, clump c: 28.1237, -89.1404 + depth: -1073 to 1078m). The gills from each mussel individual were dissected immediately after retrieval and homogenized with sterilized stainless steal beads, 3.2 mm in diameter (biostep, Germany). A subsample of the homogenate for sequencing analyses was preserved in RNA later (Sigma, Germany) and stored at -80°C. DNA was extracted from these subsamples as described by[52]. TruSeq library preparation and sequencing using Illumina HiSeq2500 was performed by the Max Planck Genome Centre in Cologne, Germany, resulting in 250 bp paired-end reads with a median insert size of 400 bp. The raw reads have been deposited in NCBI under BioProject PRJNA508280.

Construction of the non-redundant gene catalog

Illumina paired-end raw reads from the samples were trimmed for adapters and filtered by quality using BBMap tools[53]. Only reads with more than 30bp and quality above 10 were kept. This results in 37.7 million paired-end reads per sample on average (Supplementary Table 1).

408 We assembled each of the metagenomic samples individually using metaSPAdes[54].
409 Genes were predicted *ab initio* on contigs with metaProdigal[55]. These predicted genes were
410 clustered by single-linkage according to sequence similarity using BLAT[56] (at least 95% of
411 sequence identity in at least 90% of the length of the shortest protein and e-value < 10^{-6}). To
412 reduce the potential inflation caused by the single-linkage clustering, we applied two additional
413 filters to discard hits: the maximum ratio allowed between the two compared sequence lengths
414 must be 4 and hits between partial and non-partial genes are discarded. These filters are meant
415 to remove spurious links between sequences due to the presence of commonly spread protein
416 domains. This clustering was performed in two successive steps; first, we obtained sample-
417 specific gene catalogs by performing intra-sample clustering. This is meant to reduce sequence
418 redundancy, resulting in an average of ~676,000 non-redundant genes per sample
419 (Supplementary Table 1). Second, one-sided similarity search was performed across all pairs of
420 sample catalogs. This resulted in 1,156,207 clusters (26.5%) and 3,207,869 (73.5%) singletons,
421 which make up a catalog of 4,364,076 million non-redundant genes. For each of the clusters,
422 we reconstructed a consensus sequence as cluster representative. To this end, we took the
423 majority nucleotide at each position (ties were resolved randomly).

424 *Taxonomic annotation of gene catalog*

425 Taxonomic annotation of the gene catalog was performed by aligning the translated genes to
426 the non-redundant protein NCBI database (date: 24/05/18) using diamond[57] (e-value<10^{-3},
427 sequence identity ≥ 30%) and obtaining the best hit. Genes were annotated as MOX-related if
428 their best hit is *Bathymodiolus platifrons* methanotrophic gill symbiont (NCBI Taxonomy ID
429 113268) or *Methyloprofundus sedimenti* (NCBI Taxonomy ID 1420851). For SOX, the genomes
430 of thioautotrophic symbionts belonging to four different Bathymodiolus species were used for
431 annotation (NCBI Taxonomy IDs: 2360, 174145, 113267 and 235205). In addition, the gene
432 catalog was screened for mitochondrial genes using best blastp hits against the *Bathymodiolus*
433 *platifrons* mitochondrial protein sequences (NC_035421.1)[58] (all e-values <10^{-40}). The gene
434 catalog was also screened for symbiont marker genes by best blastp hits to a published protein
435 database for *Bathymodiolus azoricus* symbionts[47] (80% of protein identity and 100% of query
436 coverage). This allowed to identify 86 SOX and 39 MOX marker genes. The marker gene
437 coverages are generally uniform across a sample, however a high variance in coverage is
438 present in two of the samples (Supplementary Fig. 3). Since the binning method relies on the
439 covariation of coverage across samples, the presence of a high variance in coverage can

interfere with the proper clustering of genes, thus, two samples were discarded from further analysis (Dsc1, Dsc2).

Estimation of the gene catalog coverages

To estimate the gene abundances, we mapped the reads of each metagenomic sample to the gene catalog using bwa mem[59]. Reads below 95% of sequence identity or mapping quality of 20, as well as not primary alignments were discarded. Coverage per position for each gene in the catalog across samples was calculated using samtools depth[60] and the gene coverage is given by the mean coverage across positions. We first downsampled the reads in each sample to the minimum number of reads found (33M, Supplementary Table 1) and calculated mean coverage per gene to perform the binning and the analyses of coverage variance across symbiont marker genes (see above).

Genome binning and symbiont core genome identification

Next, we performed co-abundance gene segregation by using a canopy clustering algorithm[29], which clusters genes into bins that covary in their abundances across the different samples. This approach allows to recover from chimeric associations obtained in the assembly process and to automatically separate core from accessory genes. Gene coverages across samples were used as the abundance profiles for binning. First, genes with a Pearson correlation coefficient (PCC) > 0.9 to the cluster abundance profile were clustered. Then, clusters with PCC > 0.97 between their median abundance profiles were merged and outlier clusters for which the coverage signal originates from less than three samples were removed. In addition, we removed a gene from a cluster if Spearman correlation coefficient to the median canopy coverage profile is lower than 0.7. Finally, overlaps among the clusters were removed by keeping a gene in the largest of the clusters in which it has been found.

This allowed us to cluster 900,310 genes into 98,944 co-abundant gene groups (3 to 699 genes) and three MetaGenomic Species (MGSs, ≥700 genes). An additional filter was applied to the MGSs to obtain final bins by removing outlier genes based on their coverage (Supplementary Fig. 2). To this end, we used the Median Absolute Deviations (MAD) statistic as a cutoff to discard highly or lowly covered genes. We removed genes that are at least 24 times MAD far from the median in at least one of the samples. The bins after outlier gene removal constitute the core genomes of the MGSs. We checked for the completeness of the symbiont

470 bins with CheckM, by screening for the presence of Gammaproteobacteria universal single copy
471 marker genes[61].

472 *SNV discovery on the core genomes*

473 To perform single nucleotide variant (SNV) discovery, we mapped the downsampled reads
474 individually for each sample to the gene catalog. Because sample size has been shown to have
475 an effect on variant detection[62], we normalized the data across samples. To this end, we
476 normalized each sample to the smallest median coverage found in a sample (482x coverage for
477 SOX, 36x coverage for MOX and 568x for mitochdonrial genes). LoFreq was used for
478 probabilistic realignment and variant calling of each sample independently[63]. SNVs detected
479 with LoFreq have been hard filtered using the parameters suggested by GATK best practices[64].
480 Briefly, SNVs with quality by depth below 2, Fisher's exact test Phred-scaled probability for
481 strand bias above 60, root mean square of mapping quality below 40, root mean square of base
482 quality above 30, mapping quality rank sum test below -12.5 and read position rank sum test
483 below -8 are kept for further analyses.

484 The resulting SNVs can be fixed or polymorphic in a sample. Polymorphic SNVs are
485 characterized by the allele frequency of the alternative allele whereas fixed SNVs have an allele
486 frequency of 1. Here, we define SNVs as polymorphic in a metagenomic sample if their
487 frequency is between 0.05 and 0.95 in the sample.

488 *Population structure analyses*

489 SNV data is used for calculating intra-sample and inter-sample nucleotide diversity (π) as
490 applied before to human gut microbiome species[19]. Intra-sample nucleotide diversity (π) is given
491 as:

492 $$\pi(H,G) = \frac{1}{|G|}\sum_{i=1}^{|G|} \sum_{B_1 \in \{ACTG\}} \sum_{B_2 \in \{ACTG\} \setminus B_1} \frac{X_{i,B_1}}{C_i}\frac{X_{i,B_2}}{C_i - 1}$$

493 where H corresponds to the sample, G to the bacterial genome, $|G|$ is the length of the analyzed
494 genome and $X_{i,Bj}$ is the count of a specific nucleotide B_j at a specific locus i with coverage C_i.
495 Inter-sample nucleotide diversity (π) is then given as follows, where H_1 and H_2 correspond to the
496 two samples compared:

497 $$\pi(H_1, H_2, G) = \frac{1}{|G|}\sum_{i=1}^{|G|} \sum_{B_1 \in \{ACTG\}} \sum_{B_2 \in \{ACTG\} \setminus B_1} \frac{X_{i,B_1,S_1}}{C_{i,S_1}} \frac{X_{i,B_2,S_2}}{C_{i,S_2}}$$

498 Finally, these diversity measures are used to estimate the fixation index (F_{ST}), which measures
499 genetic differentiation based on the nucleotide diversity present within and between populations.

500 $$F_{ST}(S_1, S_2, G) = 1 - \frac{\pi_{within}}{\pi_{between}} = 1 - \frac{\pi(S_1,G)+\pi(S_2,G)}{2\,\pi(S_1,S_2,G)}$$

501 The scripts to calculate genome-wide inter and intra-sample nucleotide diversity (π) and fixation
502 index (F_{ST}) across all inter-sample comparisons from pooled SNV data have been deposited at
503 https://github.com/deropi/BathyBrooksiSymbionts.

504 *Strain deconvolution*

505 We reconstructed the strains for the core genomes with DESMAN[33]. The SNVs with two states
506 and their frequencies in each sample are used by DESMAN to identify strains in the core
507 genomes that are present over multiple samples. Thereby, the program uses the SNV
508 frequency covariation across samples to assign the SNV states to a specific genotype. For
509 SOX, we ran the strain deconvolution five times using different seed numbers and 500
510 iterations. Due to computational limitations, a subset of 5,000 SNVs was used and the
511 haplotypes considering the whole SNV dataset were inferred *a posteriori*. The five replicates
512 were run for an increasing number of strains from seven to twelve. The program uses posterior
513 mean deviance as a proxy for model fit. A posterior mean deviance lower than 5% was reached
514 in the transition from eleven to twelve strains, therefore the number of inferred SOX strains is
515 eleven. We did not run fewer numbers of strains due to the presence of large posterior mean
516 deviances between runs with a small strain number. Additionally, we ran DESMAN for the SOX
517 dataset that was subsampled to the MOX coverage with no replicates and eleven strains were
518 found using posterior mean deviance. For MOX, we ran four replicates using the whole SNV
519 dataset and 500 iterations. The runs were performed by using an increasing number of strains
520 from two to seven, reaching the optimal number of six strains. The consensus gene sequences
521 of each strain were concatenated to generate the strain core genomes, which were used for
522 further analyses. Splits network of the strain genome sequences were reconstructed using
523 SplitsTree[65] and uncorrected distances. The position of the root in the splits network was
524 estimated by the minimum ancestral deviation (MAD) method[66], which uses maximum likelihood
525 phylogenetic trees inferred with IQ-TREE[67].

526 *α- and β-diversity*

527 To study the microbial community composition, we estimated α- and β-diversity accounting for
528 strain relatedeness in addition to species richness and eveness. α-diversity was estimated using
529 phylogeny species eveness (PSE)[68] implemented in the R package 'Picante'[69]. β-diversity was
530 estimated using the weighted Unifrac distance, which is implemented in the R package
531 'GUniFrac'[70]. This measure quantifies differences in strain community composition between two
532 samples and accounts for phylogenetic relationships.

533 *Allele frequency spectra estimation*

534 The unfolded allele frequency spectra were calculated from biallelic SNVs for each of the
535 bacterial species within individual samples. The unfolded allele frequency spectrum estimation
536 relies on the presence of ancestral states in the population. Because we have no information
537 about the ancestry relationship among the strains present in the samples, we made one main
538 assumption in this regard: the ancestral SNV state in the population corresponds to the one
539 which is present in the higher number of strains. Ties are resolved by arbitrarily assigning one
540 tip of the tree as ancestral state: M2.2 for MOX and S4 for SOX.

541 *pN/pS and Neutrality Index estimation*

542 We estimated pN/pS for both bacterial populations, which is a variant of dN/dS that can be used
543 based on intra-species SNVs. To this end we first calculated the expected ratio of
544 nonsynonymous and synonymous mutations for each gene by accounting for each possible
545 mutation occurring in each of the codons. Then, we estimated the observed nonsynonymous to
546 synonymous ratio by using the biallelic SNVs. These two measures are later compared,
547 resulting in the pN/pS ratio. pN/pS was estimated genome-wide as well as individually for each
548 of the genes in the two symbiont species. The per-gene pN/pS calculation results into
549 undefinded estimates for genes with no synonymous mutations. To circumvent this limitation,
550 we added 1 to the number of observed synonymous mutations in each gene, which is a
551 standard correction for dN/dS ratios[71].

552 The neutrality index (NI) accounts for differences in the ratio of nonsynonymous to
553 synonymous variants between divergent and polymorphic SNVs in order to quantify the
554 departure of a population from neutral evolution[37]. $NI = \frac{pN/pS}{dN/dS}$, where *pN*, and *pS* are the
555 number of polymorphic synonymous and nonsynonymous sites, respectively, and *dN* and *dS*

are the number of divergent synonymous and nonsynonymous sites, respectively. For a coalescent population that evolves neutrally, the nature of fixed mutations that are involved in the divergence of the strains should not be different from that of the polymorphic mutations. An excess of divergent nonsynonymous mutations (NI<1) indicates that the population underwent positive selection or a large demographic change in the past[37].

Here we used the NI to analyze if differences in selection have been involved in the evolution of SOX and MOX strains. Different strains are typically found in more than one sample and this supports the notion that SNVs that characterize the strains constitute substitutions. We estimated NI by considering two different levels of divergence and polymorphism. First, we defined as divergent all those SNVs that have two possible states among the strains and as polymorphic all the invariant SNVs. Second, we used a more restrictive level of divergence. To this end, we excluded putative recently acquired SNVs from the set of divergent SNVs, by discarding those that have multiple states among strains from the same group. Polymorphic SNVs are all the remaining. The scripts to calculate the allele frequency spectra, pN/pS and NI have been deposited at https://github.com/deropi/BathyBrooksiSymbionts. Statistics and plotting were done in R[72].

Acknowledgements

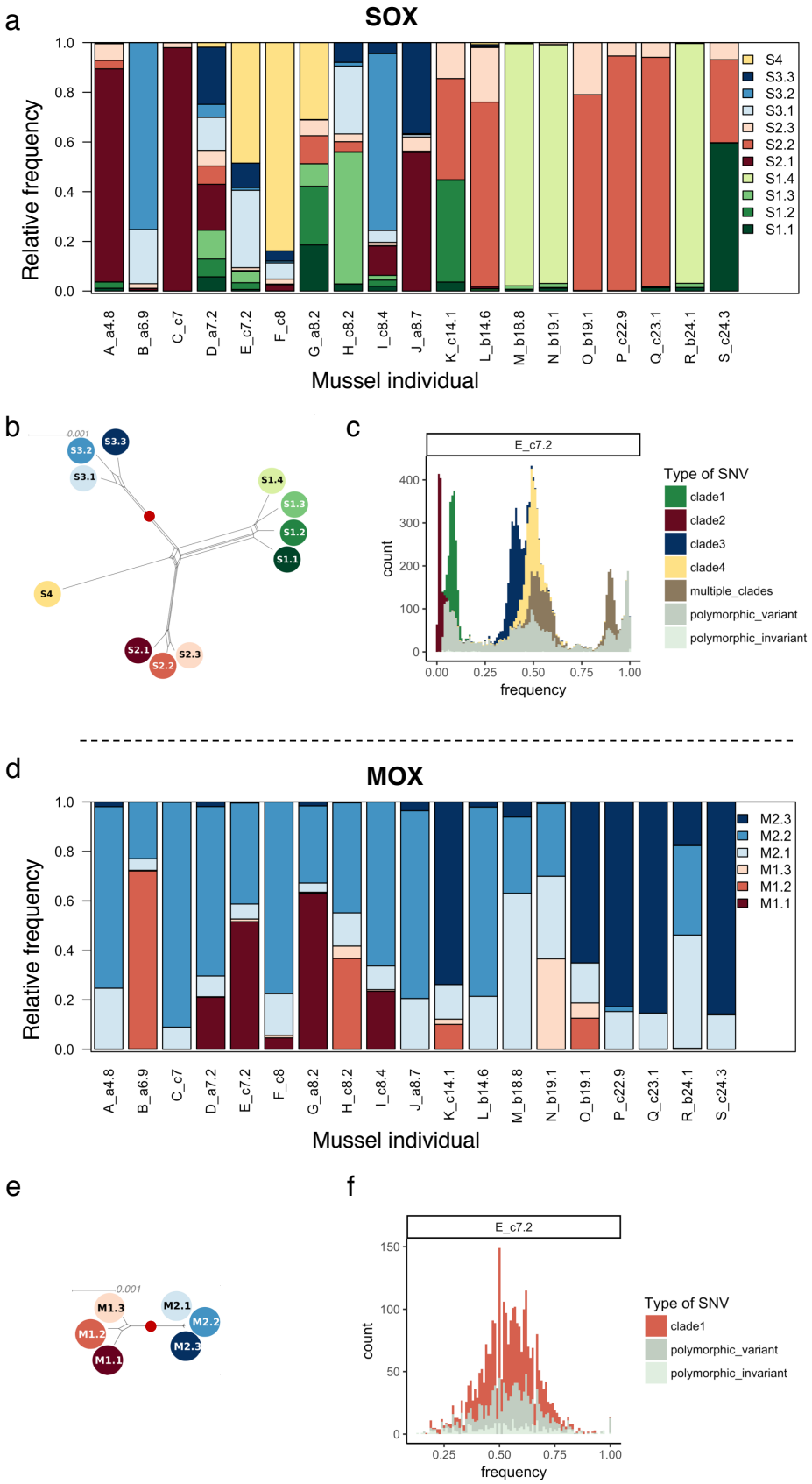
We thank the captain and crew of the E/V *Nautilus* 2015 Expedition, the team of the ROV *Hercules*, the chief scientist on this expedition leg, Eric Cordes, and the expedition leader, Mike Brennan. Additionally, we thank Elie Jami, Robin Koch, Tanita Wein, and Christian Wöhle for critical comments on the manuscript. This work was supported by the CRC1182 *Origin and Function of Metaorganisms*, the Bioinformatics Network at Kiel University, and the North-German Supercomputing Alliance (HLRN, project shb00002). Additional funding support for ND, JMP and RA came from the Max Planck Society, the Gordon and Betty Moore Foundation Marine Microbiology Initiative Investigator Award through grant GBMF3811 to ND and a European Research Council Advanced Grant (BathyBiome, Grant 340535 to ND).

Author contributions

AK, TD, JMP, and ND designed the study, RA, JMP, and ND collected the data, DRP analyzed

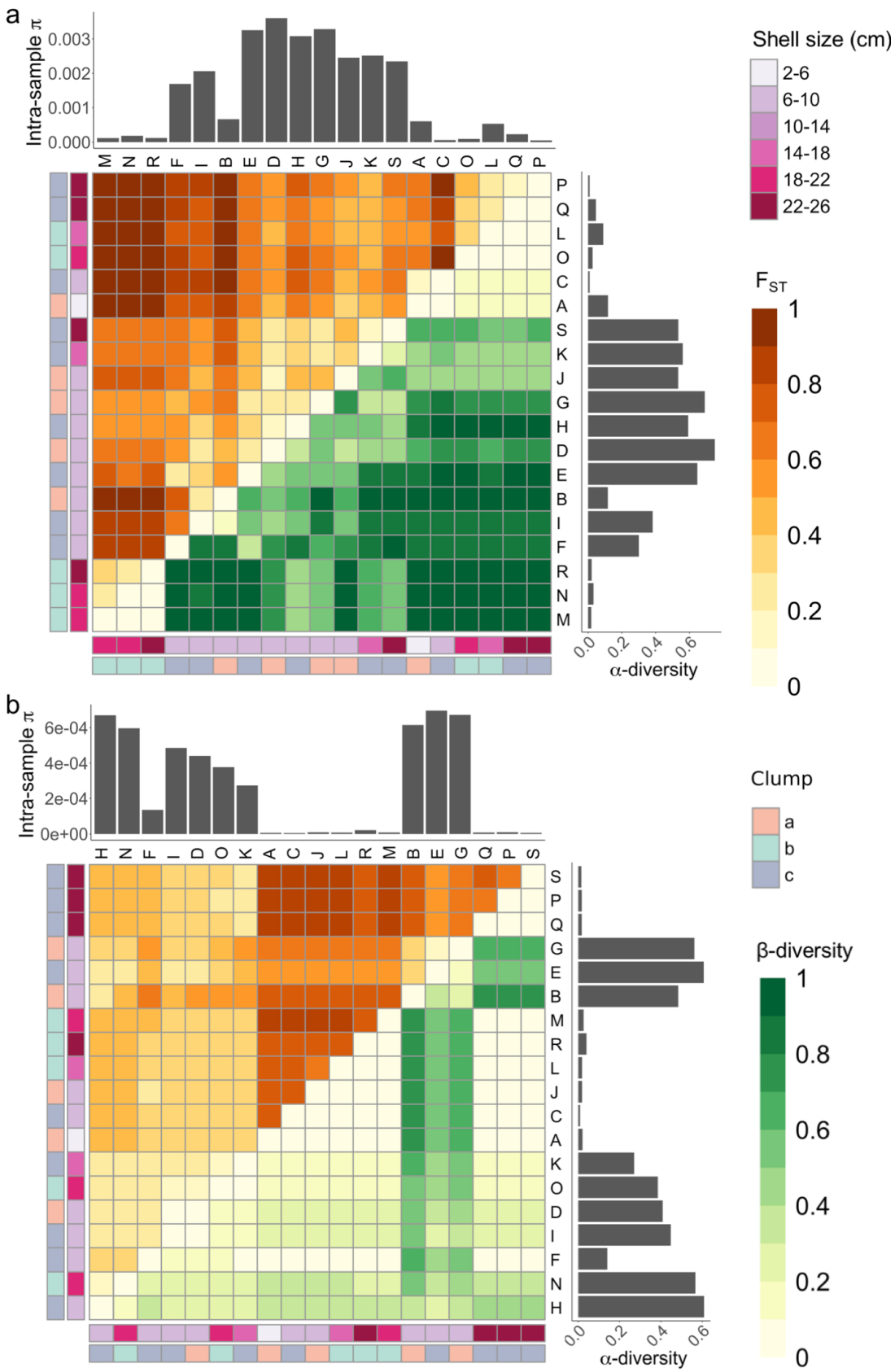
the data, DRP, AK, and TD interpreted the results with contribution from RA, DRP, AK, and TD wrote the manuscript with contributions from all authors.

Figure Legends


a
SOX
Relative frequency
Mussel individual
S4
S3.3
S3.2
S3.1
S2.3
S2.2
S2.1
S1.4
S1.3
S1.2
S1.1
b
c
E_c7.2
count
frequency
Type of SNV
clade1
clade2
clade3
clade4
multiple_clades
polymorphic_variant
polymorphic_invariant
d
MOX
M2.3
M2.2
M2.1
M1.3
M1.2
M1.1
e
f

592

Figure 1: Symbiont strain abundances (a, d), symbiont strain relationships (b, e) and example allele frequency spectra (c, f). a, b, c, 11 strains reconstructed for SOX. These cluster into four clades, with two times four, three and one strain per clade, labelled by shades of green, red, blue, and yellow. The strains differ by between 669 SNVs (strains S2.2 and S2.3, sequence identity 99.95%) and 8,171 SNVs (strains S3.2 and S4 sequence identity 99.36%). Minimum number of SNVs between strains of different clades is 6,451 (strains S1.1 and S2.1, sequence identify 99.49%). **d, e, f,** 6 strains reconstructed for MOX. These cluster into two clades, labelled by shades of red and blue. Strains differ by between 105 (strain M2.2 and M2.3, sequence identity 99.99%) and 2,677 SNVs (strain M1.1 and M2.1, sequence identity 99.81). The minimum number of SNVs differentiating strains from different clades is 2,224 (strains M2.2 and M1.3, sequence identity 99.85%). **a, d,** Stacked barplot of strain relative abundances for each individual mussel. Mussel individuals are labeled with an assigned letter (A-S), followed by the sampling clump (a, b or c) and the shell size (cm). **b, e,** Splits network of the strain genome sequences. Scale bar shows the number of differences per site. The red dots indicate the position of the root. **c, f,** Example of derived allele frequency spectra (sample E). Different colors represent different strain clades (see also Supplementary Fig. 6).


a
Intra-sample π
0.003
0.002
0.001
0.000
M N R F I B E D H G J K S A C O L Q P
P Q L O C A S K J G H D E B I F R N M
0.0 0.2 0.4 0.6
α-diversity
Shell size (cm)
2-6
6-10
10-14
14-18
18-22
22-26
F_ST
1
0.8
0.6
0.4
0.2
0
b
Intra-sample π
6e-04
4e-04
2e-04
0e+00
H N F I D O K A C J L R M B E G Q P S
S P Q G E B M R L J C A K O D I F N H
0.0 0.2 0.4 0.6
α-diversity
Clump
a
b
c
β-diversity
1
0.8
0.6
0.4
0.2
0

609

610 **Figure 2: Symbiont population structure for a, SOX and b, MOX.** Top left triangle: Intra-
611 sample π and symbiont fixation index (F_{ST}) based on SNVs. Lower right triangle: α- and β-
612 diversity based on reconstructed strains. Rows and columns are labelled by sample name,
613 sample location, and shell size. Heatmap hierarchical clustering is based on Euclidean distance
614 of F_{ST}. **a,** SOX: mean pairwise F_{ST} is 0.618. Two subpopulations show an extreme degree of
615 isolation: mean pairwise F_{ST} of subpopulation composed of M, N, R, is 0.313; mean pairwise F_{ST}
616 of subpopulation composed of L, O, P, Q is 0.308; mean F_{ST} of sample pairs where one sample
617 is M, N, or R and the other sample is L, O, P, or Q is 0.969. **b,** MOX: mean pairwise F_{ST} is
618 0.495. The clustering displays two groups: mean pairwise β-diversity of subpopulation
619 composed of A, B, C, D, E, F, G, H, I, J, L is 0.099; mean pairwise β-diversity of subpopulation
620 composed of K, M, N, O, P, Q, R, S is 0.383.

621

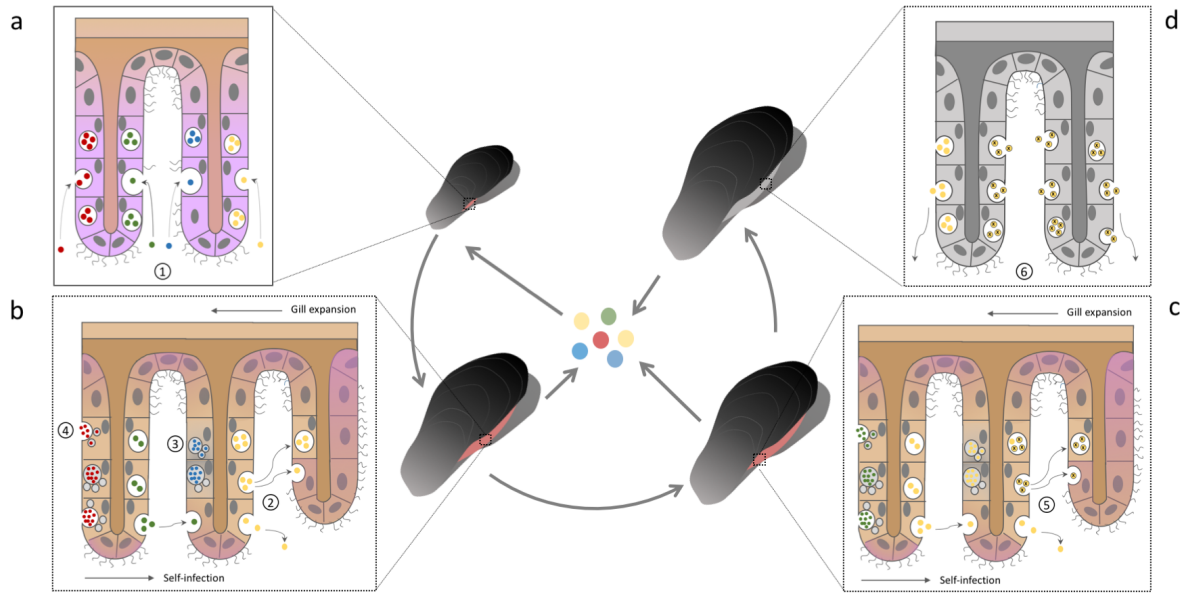


Figure 3. Symbiont colonization dynamics. a, The post larvae mussel gill does not take up endosymbionts until the gill presents several filaments and the gill epithelial cells reach a determined developmental stage[26]. At this time point, the filaments are simultaneously infected by different strains via endocytosis (1). This imposes the first bottleneck in the symbiont population, since most likely, not all the strains from the environmental pool can infect the tissue. **b,** Bacteria are released from the host tissue to the environmental pool. As the mussel grows, new filaments are continuously formed in the gill throughout the mussel life span (growing cells shaded in purple). The new tissue is colonized by a self-infection process[26], which involves infection of the newly formed filaments via endocytosis with bacteria that are released from old tissue via exocytosis (2). The spatial distribution of strains within the gill tissue also supports self-infection[45]. The continuous self-infection process imposes serial founder effects that lead to a reduction in strain diversity, which is mostly driven by drift. Additional sources of diversity loss are: tissue replacement (3) and regulated lysosomal digestion of symbionts[58] (4). **c,** In older mussels, a unique strain dominates the gill. In addition, *de novo* mutations occur in symbiont genomes (marked by x). Due to serial founder effects within the same mussel, those variants can be quickly fixed inside the mussel (5). **d,** As the mussel dies, bacteria are released from the gill, going back to the environmental pool (6).

642 # Tables

643 **Table 1. Neutrality index (NI) for the symbiont core genomes.**

644 **a**

	SOX		MOX	
	divergent	**polymorphic**	**divergent**	**polymorphic**
nonsynonymous SNVs	5004	990	2115	704
synonymous SNVs	10577	1450	1313	515
nonsynonymous SNVs/synonymous SNVs	0.47	0.68	1.61	1.37
NI	1.44		0.85	

645 **b**

	SOX		MOX	
	divergent	**polymorphic**	**divergent**	**polymorphic**
nonsynonymous SNVs	2549	3455	1041	1778
synonymous SNVs	6370	5657	649	1179
nonsynonymous SNVs/synonymous SNVs	0.40	0.61	1.60	1.51
NI	1.52		0.94	

646 **a,** divergent SNVs are all those SNVs that differ between at least two strains, i.e., all identified
647 strains are considered as diverged taxonomic units, and polymorphic SNVs are all the invariant
648 SNVs. **b,** Divergent SNVs have the same state inside a strain clade and are not invariant and
649 polymorphic SNVs are all the remaining, i.e., only the clades are considered as diverged
650 taxonomic units.

651

652 **Table 2. Nucleotide diversity (π), Fixation Index (F_{ST}), and pN/pS calculations for both**
653 **symbiont populations.**

	SOX	MOX
Intra-sample π range	$5.2x10^{-5}$ - $3.6x10^{-3}$	$5.6x10^{-6}$ - $7.0x10^{-4}$
intra-sample π mean	$1.4x10^{-3}$ ± $1.3x10^{-3}$ (s.d)	$2.7x10^{-4}$ ± $2.8x10^{-4}$ (s.d)
intra-sample π median	$6.7x10^{-4}$	$1.4x10^{-4}$
Pairwise F_{ST} range	0.151- 0.986	0.096- 0.898
Mean pairwise F_{ST}	0.618	0.495
pN/pS	0.137	0.425

654

References

1. McFall-Ngai, M. *et al.* Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci.* **110**, 3229–3236 (2013).
2. McFall-Ngai, M. J. The Importance of Microbes in Animal Development: Lessons from the Squid-Vibrio Symbiosis. *Annu. Rev. Microbiol.* **68**, 177–194 (2014).
3. Shabat, S. K. B. *et al.* Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *Isme J.* **10**, 2958 (2016).
4. Schretter, C. E. *et al.* A gut microbial factor modulates locomotor behaviour in Drosophila. *Nature* **563**, 402–406 (2018).
5. Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M. & Relman, D. A. The Application of Ecological Theory Toward an Understanding of the Human Microbiome. *Science* **336**, 1255–1262 (2012).
6. David, L. A. *et al.* Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559–563 (2014).
7. Sommer, F. *et al.* The Gut Microbiota Modulates Energy Metabolism in the Hibernating Brown Bear *Ursus arctos*. *Cell Rep.* **14**, 1655–1661 (2016).
8. Koren, O. *et al.* Host Remodeling of the Gut Microbiome and Metabolic Changes during Pregnancy. *Cell* **150**, 470–480 (2012).
9. Jones, R. J., Hoegh-Guldberg, O., Larkum, A. W. D. & Schreiber, U. Temperature-induced bleaching of corals begins with impairment of the CO2 fixation mechanism in zooxanthellae. *Plant Cell Environ.* **21**, 1219–1230 (1998).
10. Riou, V. *et al.* Influence of CH_4 and H_2S availability on symbiont distribution, carbon assimilation and transfer in the dual symbiotic vent mussel *Bathymodiolus azoricus*. *Biogeosciences* **5**, 1681–1691 (2008).
11. Bright, M. & Bulgheresi, S. A complex journey: transmission of microbial symbionts. *Nat. Rev. Microbiol.* **8**, 218–30 (2010).
12. Wernegreen, J. J. Endosymbiont evolution: predictions from theory and surprises from genomes: Endosymbiont genome evolution. *Ann. N. Y. Acad. Sci.* **1360**, 16–35 (2015).
13. Woyke, T. *et al.* One Bacterial Cell, One Complete Genome. *PLoS ONE* **5**, e10314 (2010).
14. Guyomar, C. *et al.* Multi-scale characterization of symbiont diversity in the pea aphid complex through metagenomic approaches. *Microbiome* **6**, 181 (2018).
15. Boscaro, V. *et al.* Parallel genome reduction in symbionts descended from closely related free-living bacteria. *Nat. Ecol. Evol.* **1**, 1160 (2017).
16. Klose, J. *et al.* Endosymbionts escape dead hydrothermal vent tubeworms to enrich the free-living population. **112**, 11300–11305 (2015).
17. Hagen, M. J. & Hamrick, J. L. Population level processes in *Rhizobium leguminosarum* bv. *trifolii*: the role of founder effects. *Mol. Ecol.* **5**, 707–714 (1996).
18. Vega, N. M. & Gore, J. Stochastic assembly produces heterogeneous communities in the *Caenorhabditis elegans* intestine. *PLOS Biol.* **15**, e2000633 (2017).
19. Schloissnig, S. *et al.* Genomic variation landscape of the human gut microbiome. *Nature* **493**, 45–50 (2013).
20. Ellegaard, K. M. & Engel, P. Genomic diversity landscape of the honey bee gut microbiota. *Nat. Commun.* **10**, 446 (2019).
21. Wollenberg, M. S. & Ruby, E. G. Population Structure of *Vibrio fischeri* within the Light Organs of *Euprymna scolopes* Squid from Two Oahu (Hawaii) Populations. *Appl. Environ. Microbiol.* **75**, 193–202 (2009).
22. Won, Y.-J. *et al.* Environmental Acquisition of Thiotrophic Endosymbionts by Deep-Sea Mussels of the Genus *Bathymodiolus*. *Appl. Environ. Microbiol.* **69**, 6785–6792 (2003).
23. Dubilier, N., Windoffer, R. & Giere, O. Ultrastructure and stable carbon isotope composition of the hydrothermal vent mussels *Bathymodiolus brevior* and *B.* sp. affinis *brevior* from the North Fiji Basin, western Pacific. *Mar. Ecol. Prog. Ser.* **165**, 187–193 (1998).
24. Duperron, S. *et al.* Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel species from cold seeps in the Gulf of Mexico. *Environ. Microbiol.* **9**, 1423–1438 (2007).

25. Ansorge, R. *et al.* Diversity matters: Deep-sea mussels harbor multiple symbiont strains. *bioRxiv* 531459 (2019).
26. Wentrup, C., Wendeberg, A., Schimak, M., Borowski, C. & Dubilier, N. Forever competent: Deep-sea bivalves are colonized by their chemosynthetic symbionts throughout their lifetime. *Environ. Microbiol.* **16**, 3699–3713 (2014).
27. Schöne, B. R. & Giere, O. Growth increments and stable isotope variation in shells of the deep-sea hydrothermal vent bivalve mollusk *Bathymodiolus brevior* from the North Fiji Basin, Pacific Ocean. *Deep Sea Res. Part Oceanogr. Res. Pap.* **52**, 1896–1910 (2005).
28. Van Dover, C. Community structure of mussel beds at deep-sea hydrothermal vents. *Mar. Ecol. Prog. Ser.* **230**, 137–158 (2002).
29. Nielsen, H. B. *et al.* Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nat. Biotechnol.* **32**, 822–828 (2014).
30. Riou, V. *et al.* Influence of CH_4 and H_2S availability on symbiont distribution, carbon assimilation and transfer in the dual symbiotic vent mussel *Bathymodiolus azoricus*. *Biogeosciences* **5**, 1681–1691 (2008).
31. Shapiro, B. J. & Polz, M. F. Ordering microbial diversity into ecologically and genetically cohesive units. *Trends Microbiol.* **22**, 235–247 (2014).
32. Kashtan, N. *et al.* Single-cell genomics reveals hundreds of coexisting subpopulations in wild *Prochlorococcus*. *Science* **344**, 416–20 (2014).
33. Quince, C. *et al.* DESMAN: a new tool for de novo extraction of strains from metagenomes. *Genome Biol.* **18**, 181 (2017).
34. Ho, P.-T. *et al.* Geographical structure of endosymbiotic bacteria hosted by *Bathymodiolus* mussels at eastern Pacific hydrothermal vents. *BMC Evol. Biol.* **17**, 121 (2017).
35. Rocha, E. P. C. *et al.* Comparisons of dN/dS are time dependent for closely related bacterial genomes. *J. Theor. Biol.* **239**, 226–235 (2006).
36. Kryazhimskiy, S. & Plotkin, J. B. The population genetics of dN/dS. *PLoS Genet.* **4**, e1000304 (2008).
37. Rand, D. M. & Kann, L. M. Excess amino acid polymorphism in mitochondrial DNA: contrasts among genes from *Drosophila*, mice, and humans. *Mol. Biol. Evol.* **13**, 735–748 (1996).
38. Lorion, J. *et al.* Adaptive radiation of chemosymbiotic deep-sea mussels. *Proc R Soc B* **280**, 20131243 (2013).
39. Achtman, M. & Wagner, M. Microbial diversity and the genetic nature of microbial species. *Nat. Rev. Microbiol.* **6**, 431–440 (2008).
40. Russell, S. L., Corbett-Detig, R. B. & Cavanaugh, C. M. Mixed transmission modes and dynamic genome evolution in an obligate animal–bacterial symbiosis. *ISME J.* **11**, 1359–1371 (2017).
41. Sprockett, D., Fukami, T. & Relman, D. A. Role of priority effects in the early-life assembly of the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* **15**, 197–205 (2018).
42. Benson, A. K. *et al.* Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc. Natl. Acad. Sci.* **107**, 18933–18938 (2010).
43. Rothschild, D. *et al.* Environment dominates over host genetics in shaping human gut microbiota. *Nature* **555**, 210–215 (2018).
44. Wentrup, C., Wendeberg, A., Huang, J. Y., Borowski, C. & Dubilier, N. Shift from widespread symbiont infection of host tissues to specific colonization of gills in juvenile deep-sea mussels. *ISME J.* **7**, 1244–7 (2013).
45. Ikuta, T. *et al.* Heterogeneous composition of key metabolic gene clusters in a vent mussel symbiont population. *ISME J.* **10**, 990–1001 (2016).
46. Fontanez, K. M. & Cavanaugh, C. M. Evidence for horizontal transmission from multilocus phylogeny of deep-sea mussel (Mytilidae) symbionts. *Environ. Microbiol.* **16**, 3608–3621 (2014).
47. Ponnudurai, R. *et al.* Metabolic and physiological interdependencies in the *Bathymodiolus azoricus* symbiosis. *ISME J.* **11**, 463–477 (2017).
48. Reuter, M., Pedersen, J. S. & Keller, L. Loss of Wolbachia infection during colonisation in the invasive Argentine ant Linepithema humile. *Heredity* **94**, 364–369 (2005).
49. Didelot, X., Walker, A. S., Peto, T. E., Crook, D. W. & Wilson, D. J. Within-host evolution of bacterial pathogens. *Nat. Rev. Microbiol.* **14**, 150–162 (2016).
50. Gonzalez, R. J., Lane, M. C., Wagner, N. J., Weening, E. H. & Miller, V. L. Dissemination of a Highly Virulent Pathogen: Tracking The Early Events That Define Infection. *PLOS Pathog.* **11**, e1004587 (2015).

765 51. Zhang, T. *et al.* Deciphering the landscape of host barriers to *Listeria monocytogenes* infection. *Proc.*
766 *Natl. Acad. Sci. U. S. A.* **114**, 6334–6339 (2017).
767 52. Zhou, J., Bruns, M. A. & Tiedje, J. M. DNA Recovery from Soils of Diverse Composition. *Appl.*
768 *Environ. Microbiol.* **62**, 316–322 (1996).
769 53. Bushnell, Brian. BBMap. (2014). Available at: sourceforge.net/projects/bbmap/.
770 54. Nurk, S., Meleshko, D., Korobeynikov, A. & Pevzner, P. A. metaSPAdes: a new versatile
771 metagenomic assembler. *Genome Res.* **27**, 824–834 (2017).
772 55. Hyatt, D., Locascio, P. F., Hauser, L. J. & Uberbacher, E. C. Gene and translation initiation site
773 prediction in metagenomic sequences. *Bioinformatics* **28**, 2223–2230 (2012).
774 56. Kent, W. J. BLAT — The BLAST -Like Alignment Tool. *Genome Res.* **12**, 656–664 (2002).
775 57. Buchfink, B., Xie, C. & Huson, D. H. Fast and sensitive protein alignment using DIAMOND. *Nat.*
776 *Methods* **12**, 59–60 (2015).
777 58. Sun, J. *et al.* Adaptation to deep-sea chemosynthetic environments as revealed by mussel genomes.
778 *Nat. Ecol. Evol.* **1**, 0121 (2017).
779 59. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform.
780 *Bioinformatics* **25**, 1754–1760 (2009).
781 60. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079
782 (2009).
783 61. Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P. & Tyson, G. W. CheckM: assessing the
784 quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.*
785 **25**, 1043–1055 (2015).
786 62. Subramanian, S. The effects of sample size on population genomic analyses – implications for the
787 tests of neutrality. *BMC Genomics* **17**, 123 (2016).
788 63. Wilm, A. *et al.* LoFreq: A sequence-quality aware, ultra-sensitive variant caller for uncovering cell-
789 population heterogeneity from high-throughput sequencing datasets. *Nucleic Acids Res.* **40**, 11189–
790 11201 (2012).
791 64. Broad Institute. Best practices for variant calling with the GATK,
792 https://www.broadinstitute.org/partnerships/education/broade/best-practices-variant-calling-gatk-1.
793 65. Huson, D. H. SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14**, 68–73
794 (1998).
795 66. Tria, F. D. K., Landan, G. & Dagan, T. Phylogenetic rooting using minimal ancestor deviation. *Nat.*
796 *Ecol. Amp Evol.* **1**, 0193 (2017).
797 67. Nguyen, L.-T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: A Fast and Effective
798 Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol. Evol.* **32**, 268–274
799 (2015).
800 68. Helmus, M. R., Bland, T. J., Williams, C. K. & Ives, A. R. Phylogenetic Measures of Biodiversity. *Am.*
801 *Nat.* **169**, E68–E83 (2007).
802 69. Kembel, S. W. *et al.* Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* **26**,
803 1463–1464 (2010).
804 70. Chen, J. GUniFrac: Generalized UniFrac Distances. (2018). Available at: https://CRAN.R-
805 project.org/package=GUniFrac.
806 71. Stoletzki, N. & Eyre-Walker, A. The Positive Correlation between dN/dS and dS in Mammals Is Due
807 to Runs of Adjacent Substitutions. *Mol. Biol. Evol.* **28**, 1371–1380 (2011).
808 72. R Core Team. *R: A Language and Environment for Statistical Computing*. (R Foundation for
809 Statistical Computing, 2017).
810

811