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Geographic distribution at subspecies resolution level: closely related Rhodopirellula species in European coastal sediments

Zure, M

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1 **Title:** Geographic distribution at a sub-species resolution level: closely related *Rhodopirellula*
2 species in European coastal sediments

3 **Running title:** Biogeography of *acsA* sequences of *Rhodopirellula*

4 **Authors:** Marina Žure¹, Antonio Fernandez-Guerra^{2,3}, Colin B. Munn⁴ and Jens Harder¹

5 ¹Department of Microbiology, Max Planck-Institute for Marine Microbiology, Bremen,
6 Germany

7 ²Oxford e-Research Centre, University of Oxford, 7 Keble Road, OX1 3QG, Oxford, United
8 Kingdom

9 ³Microbial Genomics and Bioinformatics Research Group, Max Planck-Institute for Marine
10 Microbiology, Bremen, Germany

11 ⁴School of Marine Sciences and Engineering, University of Plymouth, Plymouth, United
12 Kingdom

13 Correspondence: Jens Harder, Department of Microbiology, Max Planck-Institute for Marine
14 Microbiology, Celsiusstrasse 1, 28359 Bremen, Germany

15 Email: jharder@mpi-bremen.de, phone: ++49 421 2028 750, fax: ++49 421 2028 590

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18

19 **Subject Categories:** Microbial population and community ecology

20

21

22 **Abstract**

23 Members of the marine genus *Rhodopirellula* are attached living bacteria and studies based on
24 cultured *Rhodopirellula* strains suggested that three closely related species *R. baltica*, '*R.*
25 *europaea*' and '*R. islandica*' have a limited geographic distribution in Europe. To address this
26 hypothesis, we developed a nested PCR for a partial acetyl CoA synthetase (*acsA*) for a single
27 gene copy detection in DNA preparations from intertidal sediments collected from all around
28 Europe. Furthermore, we performed growth experiments in a range of temperature, salinity and
29 light conditions. A combination of BLAST and minimum entropy decomposition (MED) was
30 used to analyze the sequences with the aim to explore the geographical distribution of the species
31 and subspecies. MED has been mainly used for the analysis of the 16S rRNA gene and here we
32 propose a protocol for the analysis of protein coding genes taking into account the degeneracy of
33 the codons and a possible overestimation of functional diversity. The high resolution analysis
34 revealed differences in the intraspecies community structure in different geographic regions,
35 however, we found all three species present in all regions sampled and in agreement with growth
36 experiments, we demonstrated that *Rhodopirellula* species do not have a limited geographic
37 distribution in Europe.

38

39 **Introduction**

40 The genus *Rhodopirellula* is a marine representative of a globally distributed and
41 environmentally important bacterial phylum, *Planctomycetes*, in the *Planctomycetes*-
42 *Verrucomicrobia-Chlamydiae* (PVC) super-phylum (Wagner and Horn, 2006). Members of this
43 group show some exceptional properties, such as budding reproduction and species-specific

44 intracellular membrane-separated compartmentalization (Fuerst and Sagulenko, 2011; Speth *et*
45 *al.*, 2012) and they are proposed to play an important role in the global carbon and nitrogen
46 cycles (Strous *et al.*, 2002; Glöckner *et al.*, 2003). The genus *Rhodopirellula* and the type strain
47 *Rhodopirellula baltica* SH1^T were first described by Schlesner *et al.*, (2004). This aerobic
48 chemoorganotrophic bacterium attaches and then reproduces by budding which results in motile
49 and sessile periods of life. The adult cells can be found attached on sediment particles
50 (Winkelmann and Harder, 2009) and in association with organisms, e.g. phytoplankton, sponges,
51 and macroalgae (DeLong *et al.*, 1993; Pimentel-Elardo *et al.*, 2003; Fuerst *et al.*, 1997;
52 Bengtsson and Øvreås, 2010). Besides *R. baltica*, the genus comprises many strains awaiting a
53 full taxonomic description (e.g. Winkelmann and Harder, 2009) and four validly described
54 species isolated from different habitats around the world (Roh *et al.*, 2013; Bondoso *et al.*, 2014;
55 Yoon *et al.*, 2015). A systematic isolation resulted in a strain collection of the genus
56 *Rhodopirellula* from European coastal seas and sediments, with many strains affiliating according
57 to 16S rRNA gene sequence identity of 99.5-100% to *R. baltica* SH1^T (Winkelmann and Harder
58 (2009). However, a multilocus sequence analysis (MLSA) and DNA–DNA hybridization
59 experiments revealed the presence of several species (Winkelmann *et al.*, 2010). This
60 observation was confirmed by draft genome sequences: the average nucleotide identity (ANI) of
61 shared genes between the strains revealed three species; *R. baltica* (strains SH1^T, SH28, WH47,
62 SWK14 (Richter *et al.*, 2014b)), ‘*R. europaea*’ (strains SH398 and 6C (Richter-Heitmann *et al.*,
63 2014)) and ‘*R. islandica*’ (strain K833 (Kizina *et al.*, 2015)).
64 Cultivation independent molecular approaches employing next generation sequencing (NGS)
65 technologies enable an in-depth coverage of occurrence and diversity of microorganisms in
66 environmental samples, well beyond the study of strains and type strains. However, an adequate

67 gene marker is necessary to circumvent the low resolution of the 16S rRNA gene and to resolve
68 fine-scale biogeographic structures in environmental studies (Ramette and Tiedje, 2007). So far,
69 *rpoB* coding for the beta subunit of the RNA polymerase (Bondoso *et al.*, 2013) and *carB* coding
70 for the large subunit of carbamoylphosphate synthetase (Žure *et al.*, 2015) have been used for the
71 genus *Rhodopirellula*. These amplicons were designed to cover the whole genus. In this study
72 with a phylogenetically more closely related target group of organisms, we analyzed the
73 genomes, designed primer for a nested PCR and amplified a part of the acetyl CoA synthetase
74 gene (*acsA*) to investigate the hypothesis of Winkelmann *et al.* (2010) that *Rhodopirellula*
75 species have a limited geographic distribution in European coastal areas, with *R. baltica* limited
76 to parts of the Baltic Sea and the east coast of the North Sea, '*R. europaea*' to southern North
77 Sea to the Mediterranean Sea and '*R. islandica*' to North European Atlantic regions.
78 Furthermore, we analyzed the microdiversity in the *acsA* gene sequences using the minimum
79 entropy decomposition (MED), an automated oligotyping method (Eren *et al.*, 2013, 2014b). The
80 'oligotyping' utilizes Shannon entropy (Shannon, 1948) to identify the variability in each
81 nucleotide position and uses the high entropy positions to partition sequence datasets into high
82 resolution OTUs. Position-specific variations up to a single nucleotide resolution in the
83 sequenced region can resolve ecologically meaningful differences between closely related
84 organisms (McLellan *et al.*, 2013; Buttigieg and Ramette, 2015; Eren *et al.*, 2014a, 2015).
85 Additionally, we performed growth experiments in wide range of temperature, salinity and light
86 conditions to demonstrate that strains of these three species are capable to grow at environmental
87 relevant conditions..

88

89 **Material and Methods**

90 **Sampling and DNA extraction.** The upper two centimeters of intertidal sandy sediments were
91 sampled in a period from 2005 until 2013 from 147 locations around the European coast and
92 frozen at -80°C until further processing. Genomic DNA was extracted from 500 mg of sediment
93 using the FastDNA[®] Spin Kit for Soil (MP Biomedicals, Santa Ana, California, USA) and
94 characterized by spectroscopy (NanoDrop 100, Thermo Fisher Scientific, Waltham,
95 Massachusetts, USA) and agarose gel electrophoresis with ethidium bromide (EtBr) staining.
96

97 **Primer design and amplification of *acsA* gene.** Amplicon sequences (Winkelmann *et al.*, 2010)
98 and genomes of nine *Rhodopirellula* and six other planctomycetes genomes (*Blastopirellula*
99 *marina*, *Gemmata obscuriglobus*, *Planctomyces maris*, *Planctomyces limnophilus*, *Planctomyces*
100 *staley* and *Candidatus Kuenenia stuttgartiensis*) were used to design degenerated nested PCR
101 primers. Partial gene amplicons of *acsA*, *carB*, *fumC*, *glyA*, *guaA*, and *icd* were *in silico* specific
102 for *R. baltica*, '*R. europaea*' and '*R. islandica*' and had several mismatches to other
103 *Rhodopirellula* strains. Optimisation of PCR conditions gave the best results for nested primers
104 of the *acsA* gene (NCBI GenID:1792396 in SeqID NC_005027.1). The external *acsA* primers
105 targeted a 586 bp long amplicon from the position 1079 to 1663: forward primer 5'-
106 GACDTGYTTGATGTACGAAG-3' and reverse primer 5'- TCSACYTCGATCGTGCTCA-3'.
107 The internal *acsA* primers targeted a 487 bp long amplicon from the position 1139 to 1625:
108 forward primer 5'-GTYGAGCGKCACAARGTCA-3' and reverse primer 5'-
109 GATCACATCRTCGATGCGT-3'. Fifteen barcoded internal primers were used for Illumina
110 MiSeq sequencing (Supplementary Table S1). In the first amplification, the PCR mixture
111 contained 10-15 ng of genomic DNA, 3.3 µM of each *acsA* external primer, and a 2x PCR
112 master mix (Promega, Madison, Wisconsin, USA) in 30 µl volume. The template DNA was

113 denatured for 4 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, 3 min at
114 72°C, and a final extension of 10 min at 72°C. Amplicons were purified with a PCR purification
115 kit (Qiagen, Hilden, Germany), quantified by spectroscopy and visualized on 1% agarose gel
116 stained with EtBr. Depending on the amplicon quantity, 1 µl of non-diluted or diluted (1:10,
117 1:100) purified PCR amplicon served as template for amplification with internal *acsA* primers
118 (3.3 µM) and a 2x PCR master mix (Promega Corporation, USA) in 30 µl volume. The PCR
119 protocol was 4 min at 94°C, followed by 20 or 30 cycles of 1 min at 94°C, 1 min at 58°C, 3 min
120 at 72°C, and a final extension of 10 min at 72°C (Supplementary Table S2). After amplicon
121 purification and quantification, 43 out of 95 amplicons obtained after the second PCR were
122 selected for sequencing on a MiSeq platform (Illumina, San Diego, California, USA) at the Max
123 Planck-Genome-Centre, Cologne, Germany (Table 1).

124 **Preprocessing of Illumina raw reads.** MiSeq sequencing yielded 4 713 544 paired-end reads.
125 Linker and primer sequences were removed using Cutadapt 1.8
126 (<https://github.com/marcelm/cutadapt>; (Martin, 2011)) and the reads were demultiplexed using
127 Sabre 1.000 (<https://github.com/najoshi/sabre>). Pair-end reads were merged with PEAR 0.9.5
128 (Zhang *et al.*, 2014) and quality trimmed to a phred quality of 20 using the BBDuk program
129 from the BBMAP 34.00 suite (<http://sourceforge.net/projects/bbmap/>). The open reading frames
130 were identified using the getorf program from the EMBOSS suite 6.6.0 (Rice *et al.*, 2000) and
131 sequences with stop codons were removed. Furthermore, the *acsA* sequences were translated and
132 aligned using MAFFT version 7 (Kato and Standley, 2013). The amino acid alignment was
133 back-translated to nucleotides and visually inspected and refined using Seawiew version 4 (Gouy
134 *et al.*, 2009). The preprocessing steps resulted in 1 493 643 high quality sequences (483bp) from
135 43 sample sites (Supplementary Table S2). Raw sequence reads were deposited to the NCBI

136 (<http://www.ncbi.nlm.nih.gov>), Bioproject ID; PRJNA293505.

137

138 **Taxonomic classification of partial *acsA* gene sequences.** The high quality reads were
139 taxonomically assigned using the Basic Local Alignment Search Tool version (BLAST) 2.2.30+
140 (Camacho *et al.*, 2009). Sequences were compared to a database containing *acsA* sequences of
141 *Rhodopirellula* strains (Supplementary Table S3) using nucleotide-nucleotide BLAST (blastn).
142 Sequences that had more than 94% identity to strains of *R. baltica*, '*R. europaea*' or '*R.*
143 *islandica*' were assigned to these species. The threshold was inferred from the intraspecies
144 pairwise identities of the *acsA* amplicon sequence (483bp) of four *R. baltica* strains (94.8-97.3%)
145 and the interspecies identities (less than 92%). Sequences with identities lower than 94% to
146 strains of the three *Rhodopirellula* species were further analyzed using the non-redundant
147 sequence database of NCBI (June 2015 version).

148 **Minimum entropy decomposition (MED) analysis.** The subspecies diversity (microdiversity)
149 in the *acsA* gene sequences affiliated to *R. baltica*, '*R. europaea*' or '*R. islandica*' was studied
150 using the MED pipeline version 2.2. MED is an automated oligotyping method that partitions the
151 whole datasets into homogenous OTUs (MED nodes). To account for singularities of the protein
152 coding gene, i.e. the degeneracy of the genetic code, we developed a protocol and set of
153 diagnostics prior to the application of oligotyping. First, we calculated the entropy of each
154 position as described in the oligotyping pipeline. Then we analyzed the distribution of the
155 entropy values for each codon position to observe a possible entropy bias towards the third
156 codon position due to the much faster evolution rate of the third position in comparison with the
157 first and the second codon position. Afterwards, we calculated the frequencies of transitions and
158 transversions to unravel the nature of the nucleotide variations observed in the third codon

159 position and plotted the frequencies against the K80 genetic distance (Kimura's 2-parameters
160 distance (Kimura, 1980)) in the so-called saturation plot. Due to the large size of the NGS
161 datasets, we estimated the saturation plot by performing 100 random subsamples of 10 000
162 sequences each. Those diagnostics have been implemented in the R package oligo4fun
163 (<http://genomewalker.github.io/oligo4fun/>). Oligo4fun relies on the R packages SPIDER (Brown
164 *et al.*, 2012) and APE (Paradis *et al.*, 2004). The dataset of sequences affiliating to *R. baltica*, '*R.*
165 *europaea*' and '*R. islandica*' were analyzed with this protocol. We studied the effect of the
166 higher entropy in the third codon position on the final number of nodes by MED analyses using
167 two datasets: one using all base positions of the *R. baltica*-affiliated sequences and the same
168 dataset where we removed the third codon position. The representative nucleotide sequences of
169 the final nodes obtained in both MED analyses were translated to amino acids (the third codon
170 position was restored). The resulting proteins were clustered at the 100% identity level using
171 CD-HIT v4.5.6 (Li and Godzik, 2006) to reveal the effect of silent substitutions on the MED
172 partitioning at the functional level. A comparison of the cluster representatives with CD-HIT-2d
173 allowed us to identify shared fractions between both MED analyses.

174 Based on results of these preliminary MED analyses, the third position in the codon was
175 removed from all sequences and MED analyses were performed for each species-affiliated
176 sequence dataset separately. Different absolute sequence numbers required different M values
177 which serve as noise filter: a node will be eliminated if the abundance of the sequence is below
178 the M value. The M values were calculated dividing the number of sequences by 10 000 as
179 recommended by Eren *et al.*, (2014b). For *R. baltica*, '*R. europaea*', and '*R. islandica*', M values
180 of 75, 17, and 22 were used, respectively. The number of discriminant locations used for entropy
181 decomposition discriminant (d) was set to 1 for all species. Maximum variation allowed in each

182 node (V) was computed in the MED pipeline and was set to 3 nucleotides. A summary of MED
183 analyses is given in Supplementary Table S4.

184 **Diversity within *Rhodopirellula* species.** The sequence counts within the datasets of *R. baltica*,
185 '*R. europaea*' and '*R. islandica*' were normalized using a cumulative-sum scaling (CSS), which
186 divides the counts by the cumulative sum of counts up to a percentile determined by a data-
187 driven approach (Barberán et al. 2015; Paulson *et al.*, 2013). We estimated the inverse Simpson
188 diversity index for each *Rhodopirellula* species using the interpolation and extrapolation with
189 Hill numbers methodology as described by Chao *et al.* (2014) and implemented on the iNEXT
190 package for the R environment (<https://github.com/JohnsonHsieh/iNEXT>). For the sample-size-
191 based rarefaction approach and extrapolation we discarded all samples with abundance smaller
192 than the median and we defined a base sample size, two times larger than the mean abundance of
193 the remaining samples. The 95% confidence intervals were obtained using the bootstrap method
194 developed by Chao *et al.* (2014) with 200 replicates. In addition, species richness and Shannon
195 diversity indices were estimated for each sample. Sample-size-based rarefaction curves for *R.*
196 *baltica*, '*R. europaea*' and '*R. islandica*' are shown in Supplementary Figure S1. Estimated
197 species richness and Shannon diversity indices are provided in Supplementary Table S5.

198 **Species community analysis.** For the analysis we discarded samples where the number of
199 sequences were less than the median value of all samples and all the MED nodes with prevalence
200 lower than 10%. After the filtering, 22 samples remained for *R. baltica*, 20 for '*R. europaea*' and
201 22 for '*R. islandica*'. Using the CSS normalized counts (see diversity section), we \log_2
202 transformed the normalized counts (Paulson *et al.*, 2013). We calculated the species community
203 similarity using the Bray-Curtis dissimilarity distance implemented in the vegan R package
204 (<http://vegan.r-forge.r-project.org/>). We used the Sørensen similarity coefficients (1 – Bray-

205 Curtis dissimilarity) to construct a graph using the samples as nodes and the similarities as edges,
206 then we superimposed the graph on a map. To simplify the graph, we implemented a trimming
207 algorithm that tries to maintain the connectivity of the graph with the least number of edges, to
208 highlight the most similar associations without the need of applying any arbitrary threshold.

209 **Growth experiments.** *R. baltica* SH1^T, '*R. europaea*' 6C and '*R. islandica*' K833 were grown
210 in three replicates at different temperature, salinity and light conditions in 250 ml flasks
211 containing 50 ml of liquid M13a medium (DSMZ medium 600a) (Schlesner, 1994), with
212 modifications described by Winkelmann and Harder (2009) and shaken at 50 rpm. Standard
213 conditions for growth were dark, 28°C and salinity 35 PSU (100% artificial sea water, ASW).
214 Experimental conditions included four temperatures (14, 21, 28, 37°C), four salinities (25, 50,
215 100, 125, 150% ASW) and three light conditions (24h dark, 24h light and 12h light/12h dark).
216 Cultures were sampled daily for optical density measurements at 600 nm (OD₆₀₀) to generate
217 growth curves and determine growth rates.

218 **Results**

219 **Taxonomic classification of *acsA* gene sequences.** BLAST analysis revealed that 75% of 1 493
220 643 amplicon sequences in the whole dataset affiliated with the species *R. baltica*, '*R. europaea*'
221 and '*R. islandica*', thus they had more than 94% identity to a reference sequence (Supplementary
222 Table S2). The rest of the sequences were assigned to *Planctomycetaceae* affiliating to other
223 *Rhodopirellula* (18% of all sequences, no affiliation to known strains on the species level) or to
224 the genera *Blastopirellula* (5%) and *Pirellula* (2%) (data not shown).

225 In the 43 sample sites, sequences of *R. baltica* and '*R. islandica*' were found in all samples and

226 sequences of '*R. europaea*' were found in 40 samples, with relative sequence abundances
227 ranging from 0.01% to 99% in each sample (Figure 1). *R. baltica* was detected with high relative
228 abundance (43-99%) in 23 samples covering all regions sampled. In samples from Scotland (101
229 (sample ID, see Table 1)) and Norway (76) this species accounted for 7 and 10% respectively,
230 and in the remaining 19 samples the relative abundances were less than 1%. High abundances of
231 '*R. islandica*' were found in six samples, from Iceland (23), Ireland (129), Scotland (101, 104
232 and 109) and Norway (76), with relative abundances of 74-90%. In other samples, '*R. islandica*'
233 accounted for 0.02-2% of total sequences. '*R. europaea*' dominated in four samples, two from
234 the Mediterranean Sea (19 and 123) and two from France (117 and 38) with relative abundances
235 of over 97%. '*R. europaea*' was also found in samples from the Mediterranean Sea (49), France
236 (112) and North Sea (11), with relative abundances of 24, 14 and 5% respectively. In the rest of
237 the dataset, '*R. europaea*' related sequences accounted for 0.02-2%.

238 **Minimum entropy decomposition (MED) analysis.** The distribution of the entropy values for
239 each codon position showed a clear bias towards the third position in the codon for the whole
240 dataset (Supplementary Figure S2) and the *R. baltica* dataset (Figure 2a). The saturation
241 substitution analyses revealed that transitions are more frequent than transversions in the third
242 position of the codon for the whole dataset (Supplementary Figure S3) and the *R. baltica* dataset
243 (Figure 2b). MED analysis of *R. baltica* sequences with the third position ("all") in the codon
244 gave approximately 1.8 times more nodes than MED analysis of the dataset where the third
245 position in the codon was removed ("no3rd"), although both approaches harbored a similar
246 number of sequences (Figure 2c,d). Translation of the node representative sequences yielded
247 clusters at 100% protein sequence identity. This revealed the effect of higher frequencies of
248 transitions in the third codon position resulting in silent substitutions. As shown in Figure 2e,

249 MED analysis of the “all” dataset showed that 40% of the node representatives had the same
250 amino acid sequence (84 non-identical MED nodes). In contrast, only 1% of the translated node
251 representatives were identical (81 non-identical MED nodes) when the MED analysis was
252 performed with only the first two bases of each codon (“no3rd”). The composition of the 100%
253 identity clusters showed that both approaches shared 58 of the final node representative
254 sequences (Figure 2f). All of the 26 non-shared representative nodes from the “all” dataset were
255 included in one of the “no3rd” representative nodes. The fraction not shared in the “no3rd”
256 dataset corresponds to non-representative sequences (13 MED nodes, 16%) and outliers due the
257 M parameter (10 MED nodes, 12%) in the “all” dataset. Those results showed how the removal
258 of the third codon position increased the resolution of the MED analyses obtaining a higher
259 number of functionally non-redundant MED nodes, hence, the removal of the third codon
260 position to account for the bias of the third codon position and a possible overestimation of the
261 diversity was well justified.

262 MED analysis of the individual species *R. baltica*, '*R. europaea*' and '*R. islandica*' resulted in 82,
263 221 and 82 final nodes, respectively (Figure 3). The total node abundance in each sample site for
264 each species is given in Supplementary Table S6. For the *R. baltica* sequence dataset, one node
265 (Rb-168) was dominant in all samples with relative abundance of 60-100%, except in sample
266 143 from the North Sea, where node Rb-43 was dominant with a relative abundance of 89%.
267 Node Rb-43 was also found in thirteen other samples of the regions sampled. Other nodes were
268 present with lower relative abundances; 14 nodes were found in more than 30 samples and 46
269 nodes in more than 20 samples. The rest were nodes present in 2-19 samples. *R. baltica* had the
270 lowest *acsA* gene diversity in comparison with the other two species and had a relatively low
271 diversity regardless of the sequence abundance of the species in the sample. '*R. islandica*' and

272 '*R. europaea*' had a lower genetic diversity in the samples where those species were abundant
273 (Figure 4). The low values of diversity on *R. baltica* are in concordance with the community
274 structure along the European coasts; Sørensen similarity coefficient suggests that all European
275 regions exhibit a similar community structure for *R. baltica* (Figure 5).

276 According to BLAST analysis, '*R. islandica*' was abundant in samples from Iceland (23), Ireland
277 (129), Scotland (101, 104, 109) and Norway (76). One node, Ri-168, was dominant in those
278 samples with relative abundances of 66-82%. In total, this node was found in 41 samples with
279 relative abundance of 10-82%. The second most abundant node (Ri-105) was found in 38
280 samples with relative abundance of 1-70%. From 82 final nodes, 37 were present in at least ten
281 different samples (10- 41). Although '*R. islandica*' showed no habitat limitation, the species
282 community analysis revealed a clear geographical clustering, separating the communities from
283 the Northern Atlantic from the rest of Europe (Figure 5).

284 '*R. europaea*' is the species with the largest diversity values (Figure 4), and different nodes were
285 dominant in different samples with high abundances of the species (19, 123, 38, 117, 49, 112), in
286 contrast to '*R. islandica*'. Eight nodes found abundant in those samples were also abundant in 12
287 to 36 samples, being well distributed on the European coasts. Furthermore, the low values of
288 similarities of '*R. europaea*' communities suggest that this species with its subspecies is highly
289 adapted to its geographical location, presenting a limited dispersion (Figure 5).

290 **Growth experiments.** Under the standard growth conditions the growth rates of species *R.*
291 *baltica*, '*R. europaea*' and '*R. islandica*' were 0.045, 0.035 and 0.059 h⁻¹, with doubling times
292 of 15, 19 and 12 hours, respectively. All strains grew in all the conditions tested with different
293 rates, except for '*R. islandica*', which did not grow at 37°C (Figure 6).

294 Discussion

295 The occurrence of three attached-living, closely related species of the genus *Rhodopirellula*, *R.*
296 *baltica*, '*R. europaea*' and '*R. islandica*', in European coastal regions was investigated by
297 BLAST and MED analysis of a partial *acsA* amplicon, enabling an in-depth coverage of the
298 diversity of these three species in intertidal sediments. Cultivation-independent studies based on
299 protein coding genes need a number of genomes as reference basis which enables the
300 development of primers for a small group of taxa with a discriminative power against the rest of
301 the organisms (Bondoso *et al.*, 2013; Žure *et al.*, 2015). In this case, *acsA* gene resulted as a good
302 candidate to delimitate three species with a 16S rRNA gene identity of more than 99.5%.
303 Furthermore, 75% of the sequences affiliated to the targeted species and 18% were distantly
304 related to other *Rhodopirellula* strains, suggesting a highly selective amplification, as cells of
305 *Rhodopirellula* represented only 2% of planctomycetal cells in a coastal sediment (Žure *et al.*,
306 2015)

307 Oligotyping was originally developed to detect subtle nucleotide differences in the 16S rRNA
308 gene, which is a non-protein coding gene and functional selection is characterized by strongly
309 conserved regions flanking hypervariable regions that have diverged over evolutionary time.
310 Different patterns of functional selection are observed in protein coding genes, where changes at
311 the third position in the codon are often non-selective due to the degenerated nature of the
312 genetic code, while changes in the first or second nucleotide are likely to produce a change in
313 amino acidic sequence. Using a high-resolution, entropy-based method like MED on coding
314 sequences can result in an overestimation of the effective genetic diversity in the environment as
315 a result of the neutral character of changes in the third position in the codon. Indeed, the largest
316 entropy values were observed in the third codon position. We also observed that transitions

317 outnumbered transversions on the third codon positions, resulting in silent substitution on the
318 *acsA* gene. MED analysis of sequences including the third position in the codon gave almost
319 twice as many nodes, but on the amino acid sequence level, 40% of node representative
320 sequences were identical. Furthermore, with the removal of the third codon position we obtained
321 a higher number of non-redundant MED nodes. For functional studies involving oligotyping
322 where the ecologically meaningful unit is the protein sequence, we recommend to perform a pre-
323 study including either the first two or all three codon positions to reveal the ecological
324 importance of the third codon position in the gene under study. In our case study of a partial
325 *acsA* amplicon, we removed the third position in the codon to avoid the overestimation of the
326 functional diversity.

327 Oligotyping can also be used for analysing amino acid sequences (Sintes *et al.*, 2016), however
328 protein alignments do not have the resolution to resolve relationships on the species level or
329 below, and using oligotyping on amino acid sequences of *acsA* gene of *Rhodopirellula* species
330 demonstrated an underestimation of the diversity in the environment. For example, oligotyping
331 analysis of the '*R. islandica*' dataset on amino acid sequences resulted in ten times fewer
332 oligotypes with one node dominating in all regions (data not shown).

333 Based on the BLAST results, the three species showed a wide geographical distribution with
334 high abundances of *R. baltica* found in samples covering all European regions, whereas high
335 abundances of '*R. europaea*' and '*R. islandica*' were found only in the samples from the regions
336 where Winkelmann *et al.* (2010) had isolated the strains and which had been suggested as a
337 preferable habitat. However, as we found these species in almost all samples it is likely that upon
338 favorable change in the environment they could become abundant in other regions. Intertidal
339 marine systems are highly dynamic systems and microorganisms living there are exposed to high

340 fluctuations in environmental conditions so it is likely that observed abundances of different
341 *Rhodopirellula* species in different samples reflect the conditions present in the environment at
342 the time of the sampling. Change in the environment may result in the shift of the rare and
343 abundant species in a particular environmental condition (Caporaso *et al.*, 2012). As the present
344 results deal only with the diversity and distribution of *Rhodopirellula* species from samples taken
345 at a single time point, future studies should address temporal variability and in situ measurement
346 of environmental parameters. The experimental determination of growth rates of three
347 *Rhodopirellula* species demonstrated the broad range of temperatures, salinities and light
348 conditions in which these species are able to grow, which supports our finding that three
349 *Rhodopirellula* species were present in all regions sampled, from the Mediterranean Sea, which
350 is characterize by high salinity (~ 38 PSU) and surface water temperature (~ 16-26°C), to the
351 North Europe Atlantic regions which are characterized by lower salinity (~ 34 PSU) and
352 temperature (~ 6-16°C) (<http://www.nodc.noaa.gov/>).

353 Although all species were found in all European regions sampled, MED analysis revealed a
354 difference in community composition of the individual species. The MED nodes may be
355 interpreted as subspecies. '*R. baltica*' had the lowest diversity in all regions, and our analysis
356 showed that all sampling sites present a similar community structure of nodes suggesting this
357 species does not have any habitat limitation and is highly adapted to changing environmental
358 conditions. In a transcriptomic study of *R. baltica*, Wecker *et al.* (2009) demonstrated that the
359 species is highly responsive to its environment, adjusting the expression of more than 3,000
360 genes in response to changes of temperature or salinity. On the contrary, '*R. europaea*' had the
361 highest diversity in all samples and the low values of the Sørensen similarity coefficient suggest
362 that the different ecotypes (MED nodes) are specific to each geographical location. In the case of

363 ‘*R. islandica*’ we observed a clear clustering of the samples located in North Europe where water
364 temperatures are lower, suggesting that this species has ecotypes adapted to cold environments.
365 These differences could be explained by differences in genomes of these three species (Glöckner
366 *et al.*, 2003; Richter *et al.*, 2014b; Richter-Heitmann *et al.*, 2014; Kizina *et al.*, 2015). The size of
367 the genomes is more than 7 Mb with over 6000 predicted open reading frames, which reflects the
368 complex lifestyle of *Rhodopirellula* species and ability to colonize highly dynamic intertidal
369 coastal sediments. The gene-content comparison revealed that species share ~55% genes,
370 whereas ~ 45% are species-specific. The high number of shared genes may explain that we found
371 co-occurrence of the species in different regions as the species shared common features allowing
372 them to colonize the same habitats, whereas the species-specific genes may explain the
373 difference in community structure and adaptation.

374 Taken together, we demonstrated that a minimum entropy decomposition analysis of protein-
375 coding genes can be a powerful tool to study the within species community structure and this
376 high resolution analysis revealed differences within *Rhodopirellula* species in different
377 geographic regions. However, we found all three species present in all regions sampled and in
378 agreement with growth experiments, we demonstrated that ‘*R. baltica*’, ‘*R. europaea*’ and ‘*R.*
379 *islandica*’ do not have a limited geographic distribution in European coastal sediments as
380 previously suggested.

381

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388

389 **Conflict of interest.** The authors declare no conflict of interest.

390

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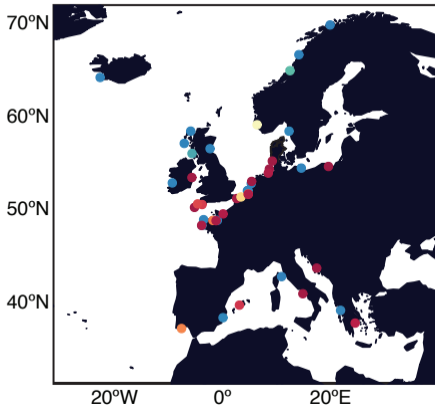
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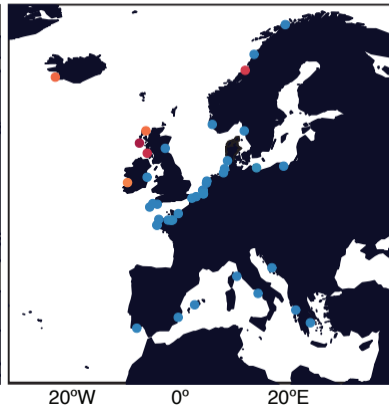
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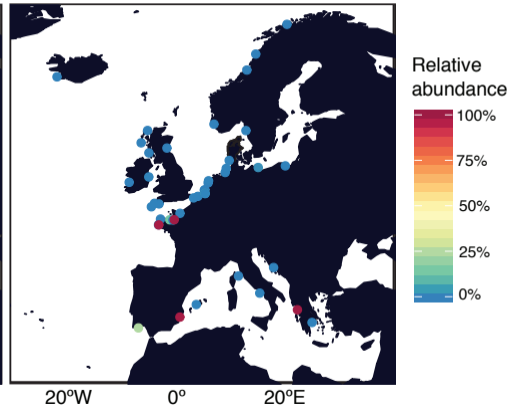
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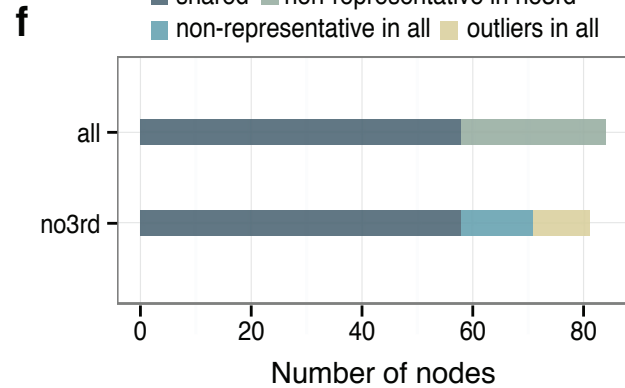
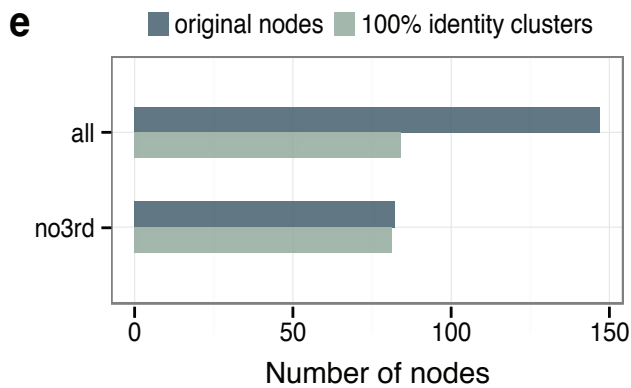
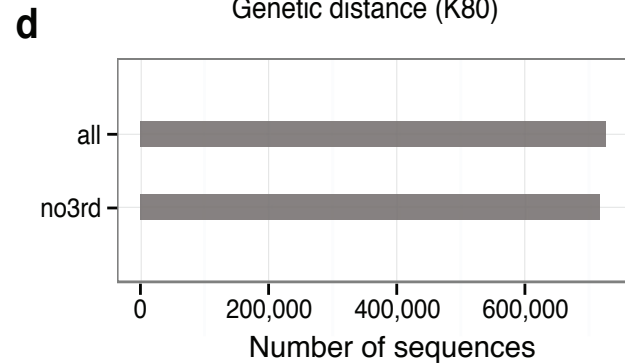
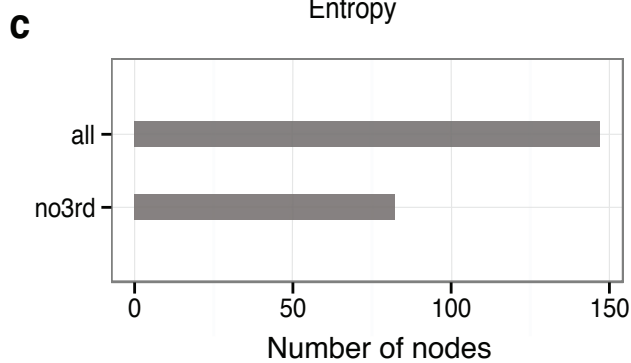
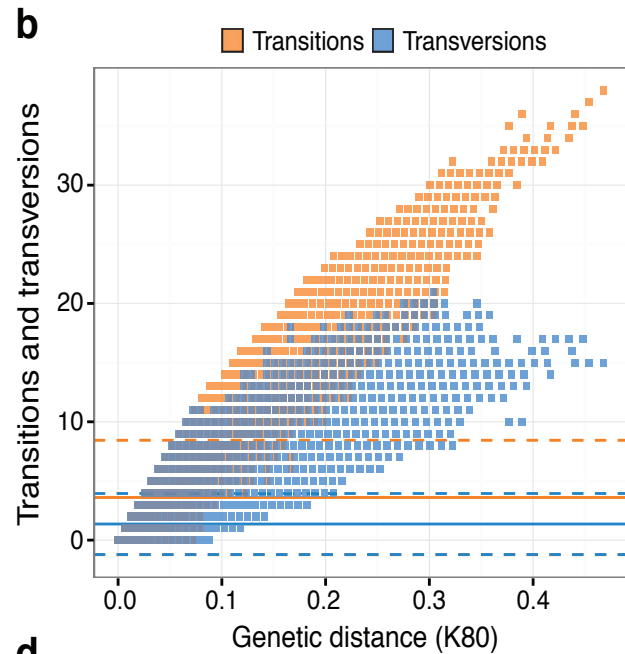
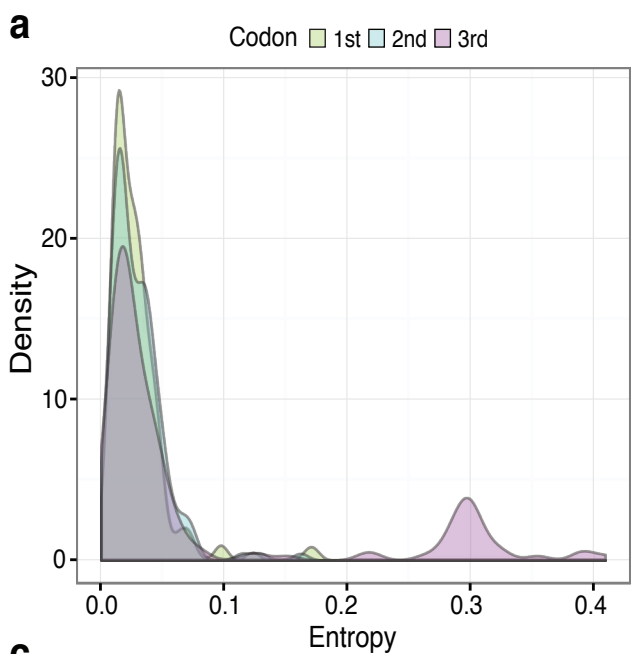


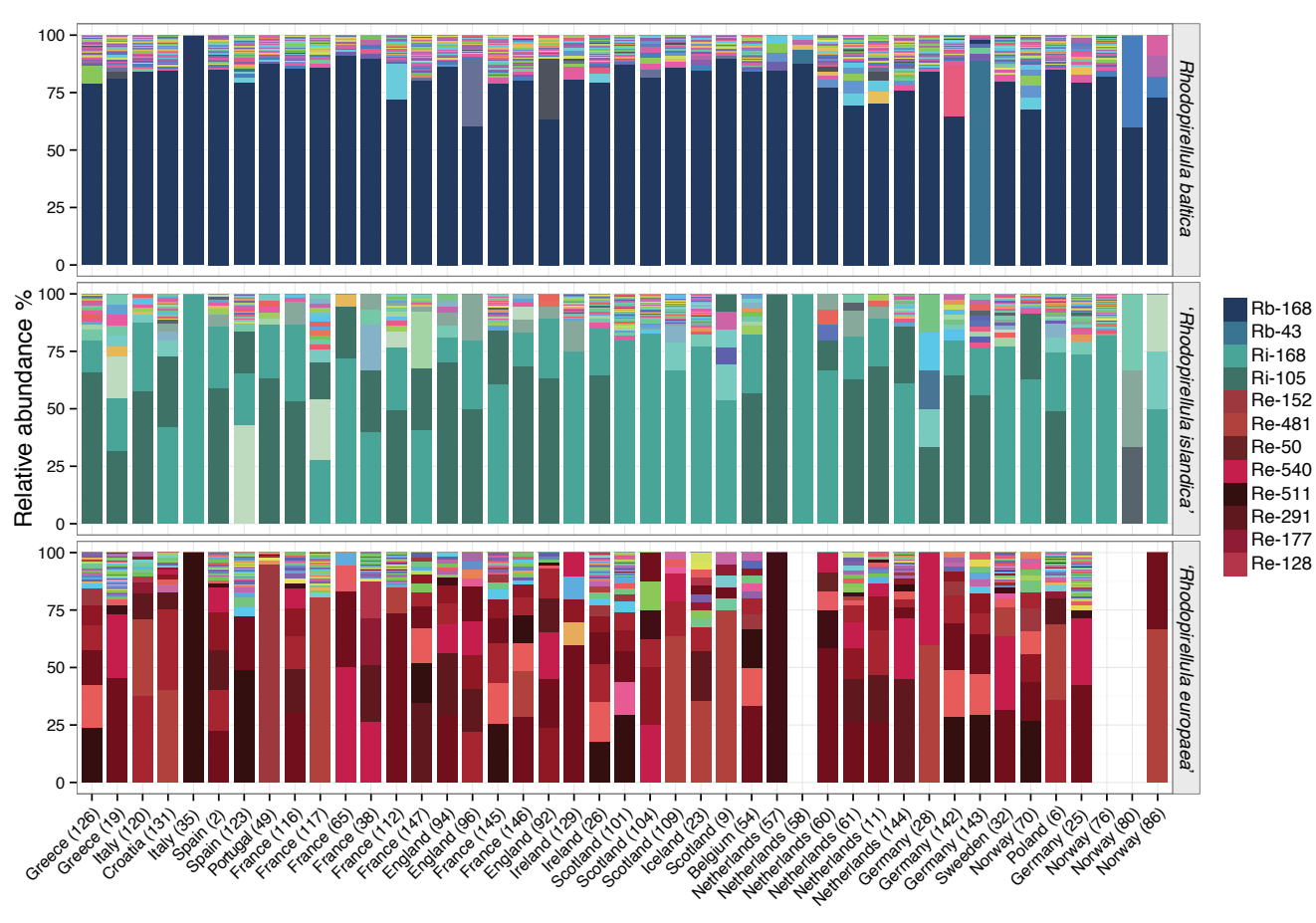
'*Rhodopirellula islandica*'



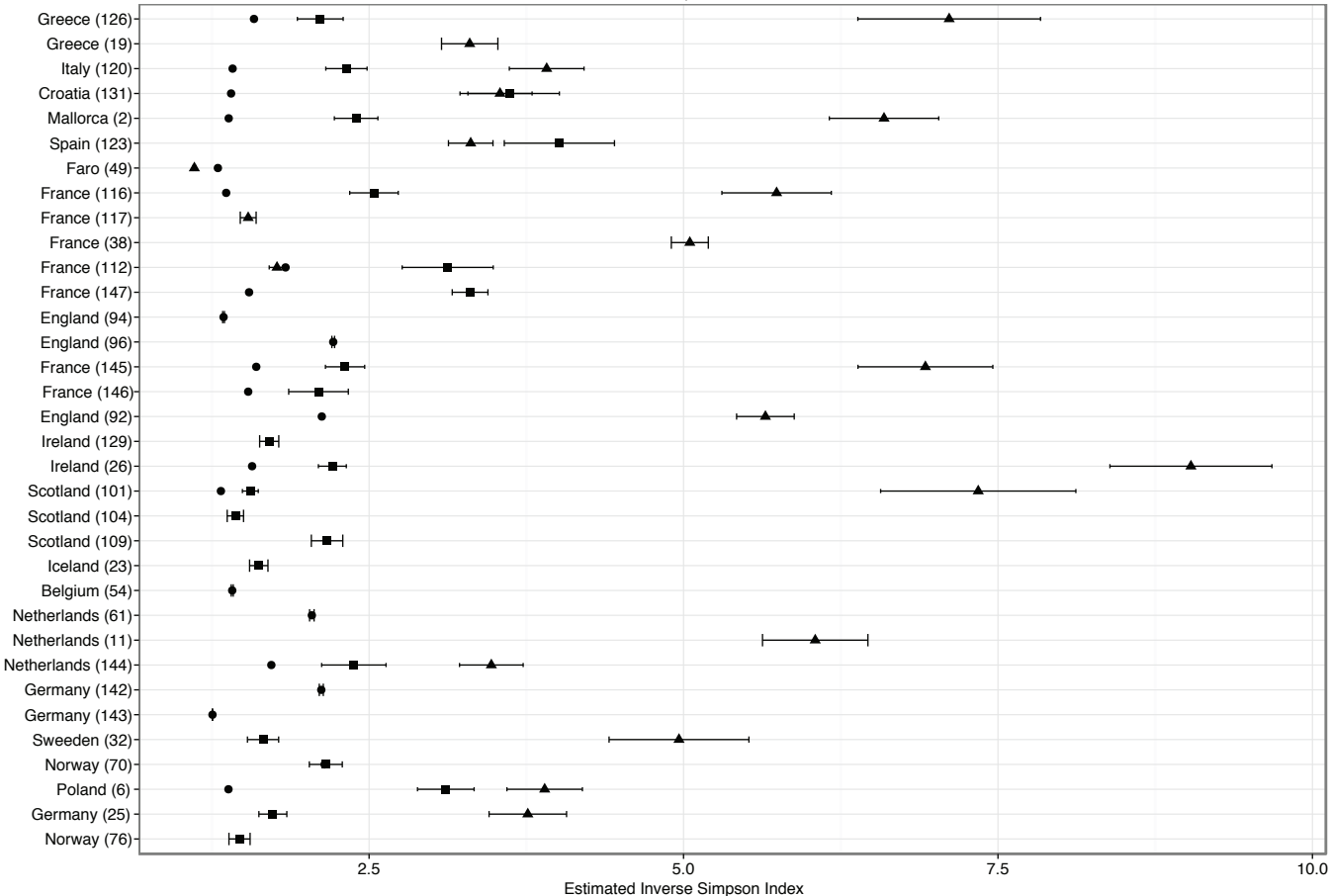
'*Rhodopirellula europaea*'



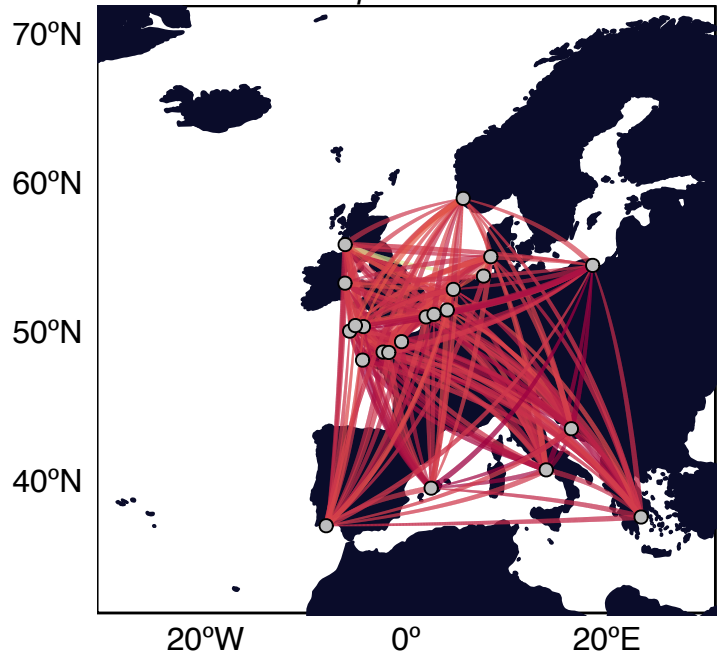




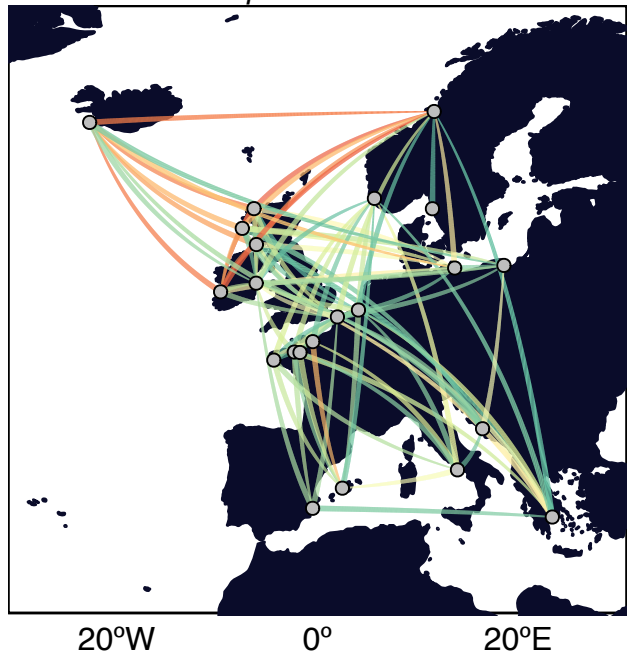
● '*R. baltica*' ▲ '*R. europaea*' ■ '*R. islandica*'



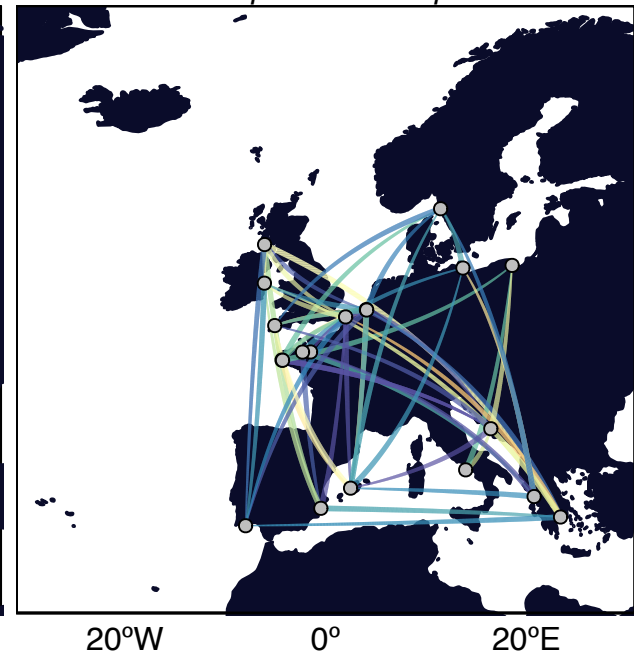
Rhodopirellula baltica



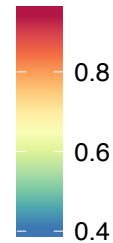
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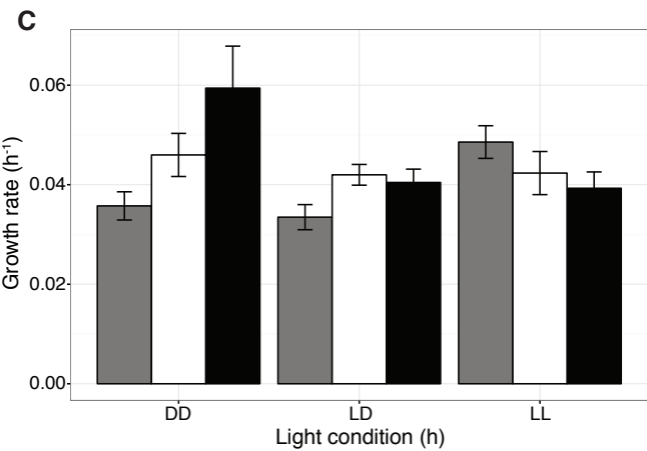
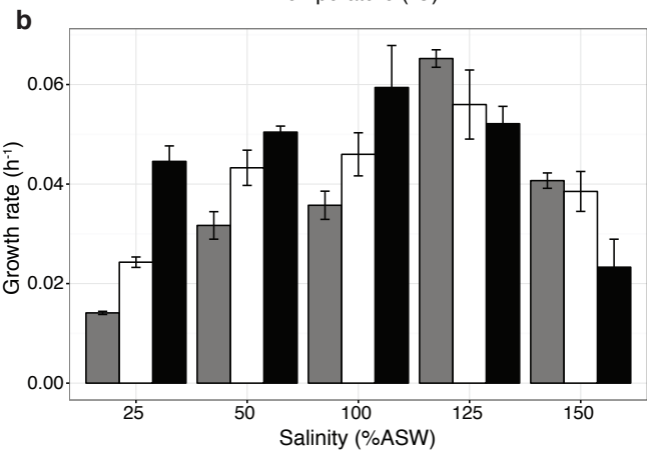
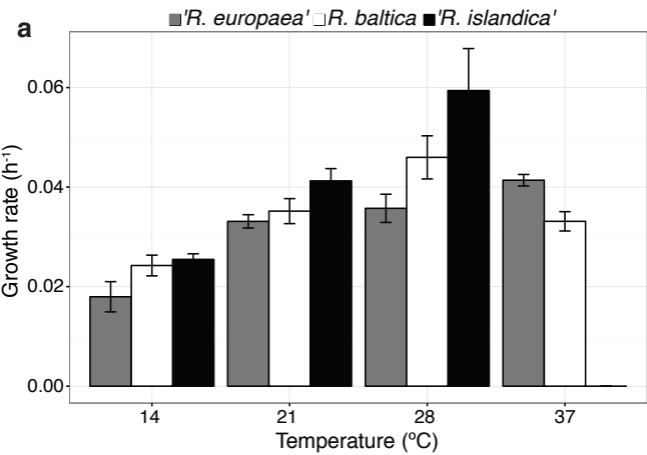


'*Rhodopirellula europaea*'



Sørensen
similarity





Sample ID	Sample location	Latitude, Longitude	Sampling date
2	Spain	39.51722, 2.47806	05.03.2005
6	Poland	54.45028, 18.57250	03.2005
9	Scotland	56.36917, -2.82833	03.2005
11	Netherlands	51.49000, 4.05500	03.2005
19	Greece	38.95583, 20.76083	04.2005
23	Iceland	64.03556, -22.69861	18.05.2005
25	Germany	54.27500, 13.66833	24.06.2005
26	Ireland	53.25972, -6.11028	29.06.2005
28	Germany	54.17300, 7.88933	06.07.2005
32	Sweedeen	58.25000, 11.45167	23.09.2005
35	Italy	42.58944, 10.09667	08.08.2005
38	France	48.64972, -1.50889	11.09.2005
49	Portugal	36.999586, -7.98027	20.06.2013
54	Belgium	51.166643, 2.761859	27.06.2013
57	Netherlands	51.833974, 3.924392	28.06.2013
58	Netherlands	51.87514, 4.040154	28.06.2013
60	Netherlands	52.621679, 4.618224	28.06.2013
61	Netherlands	52.842044, 4.691182	26.06.2013
65	France	48.719873, -3,995452	28.06.2013
70	Norway	58.94198, 5.67152	07.2012
76	Norway	64.76812, 11.62288	07.2012
80	Norway	66.48755, 13.22710	07.2012
86	Norway	69.68903, 18.86810	07.2012
92	England	50.417368, -5.075652	07.08.2013
94	England	50.043092, -5.642209	08.07.2013
96	England	50.35077, -4.26656	03.09.2013
101	Scotland	55.848596, -6.090192	02.09.2013
104	Scotland	58.256017, -6.327359	09.09.2013
109	Scotland	56.93383, -7.494062	24.07.2013
112	France	48.641524, -2.308327	29.06.2013
116	France	48.102158, -4.352176	23.08.2013
117	France	48.09098, -4.299953	24.08.2013
120	Italy	40.744985, 13.945478	13.07.2013
123	Spain	38.16703, -0.48596	16.06.2013
126	Greece	37.583647, 23.394953	09.09.2013
129	Ireland	52.678646, -9.65115	02.08.2013
131	Croatia	43.502523, 16.447045	10.04.2012
142	Germany	55.03432, 8.42428	04.2013
143	Germany	53.73668, 7.69893	04.2013
144	Netherlands	51.45950, 4.08026	10.2012
145	France	50.99695, 1.98212	10.2012
146	France	49.33955, -0.46952	10.2012
147	France	48.61387, -1.74368	10.2012