New isolate genomes and global marine metagenomes resolve ecologically relevant units of SAR11

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Running Title: SAR11 genomes from the tropical Pacific

Abstract

The bacterial order *Pelagibacterales* (SAR11) is among the most abundant and widely distributed microbial lineages across the global surface ocean, where it forms an integral component of the marine carbon cycle. However, the limited availability of high-quality genomes has hampered comprehensive insights into the ecology and evolutionary history of this critical group. Here, we increase the number of complete SAR11 isolate genomes fourfold by describing 81 new SAR11 strains from seven distinct lineages isolated from coastal and offshore surface seawater of the tropical Pacific Ocean. We leveraged comprehensive phylogenomic insights afforded by these isolates to characterize 24 monophyletic, discrete ecotypes with unique spatiotemporal patterns of distribution across the global ocean, which we define as genera. Our data illustrate fine-scale differentiation in patterns of detection with ecologically-relevant gene content variation for some closely related genomes, demonstrating instances of ecological speciation within SAR11 genera. Our study provides unique insight into complex environmental SAR11 populations, and proposes an ecology-informed hierarchy to pave a path forward for the systematic nomenclature for this clade.

Main

SAR11 marine bacteria are a genetically diverse, order-level lineage of heterotrophs within the *Alphaproteobacteria* known as the *Pelagibacterales* (Grote et al. 2012) that numerically dominate planktonic communities across the global ocean (Morris et al. 2002; Carlson et al. 2009; Eiler et al. 2009; Schattenhofer et al. 2009; Becker et al. 2019). Associations between the spatiotemporal distribution of operationally defined subclades and environmental variables suggest the presence of distinct ecotypes within SAR11 (Carlson et al. 2009; Eren et al. 2013a; Delmont et al. 2019; Tucker et al. 2021). Previous studies further support the functional differentiation of subclades (Grote et al. 2012; Thrash et al. 2014), even across short biogeographical distances (Tucker et al. 2024a). While limited in number, the available high-quality SAR11 genomes have demonstrated that this group is a remarkably cohesive genetic assemblage (Grote et al. 2012), making it an attractive model to study the capacity of a minimalist genome to reach stunning levels of success.

Since the first observation of SAR11 through environmental 16S rRNA gene fragments over three decades ago (Giovannoni et al. 1990), microbiology has benefited from a dramatic increase in microbial sequence data recovered directly from the environment, offering representative genomes for many difficult to cultivate microbial lineages (Hug et al. 2016). However, even the most comprehensive genome-resolved surveys of marine metagenomes have failed to yield high-quality SAR11 genomes (Paoli et al. 2022), resulting in limited insights into what constitutes ecologically meaningful units within this broad group. The extensive intra-clade diversity of SAR11 (Tsementzi et al. 2016; Kiefl et al. 2023) confounds the ability to reconstruct

environmental genomes from metagenomes (Delmont et al. 2018; Tully et al. 2018), which is why one of the most abundant microbial clades in marine systems suffers from poor representation in genome-resolved metagenomics surveys (Chang et al. 2024). Circumventing the need to assemble complex metagenomes first for genome recovery, single-cell sorting techniques have been much more effective in sampling environmental SAR11 populations through single-amplified genomes (SAGs). However, in an extensive effort to characterize surface ocean microbes, the estimated genome completion of SAGs that could be affiliated with SAR11 remained below 60% (Pachiadaki et al. 2019), a level that prevents robust phylogenomic insights. Such barriers have led to a reliance on isolate genomes to investigate the evolution of SAR11 populations (Vergin et al. 2007; Wilhelm et al. 2007; Thrash et al. 2011; Grote et al. 2012; Muñoz-Gómez et al. 2019), yet following this path has been impeded by another formidable challenge: the difficulty of cultivating SAR11 in the laboratory, even with genomic insights regarding its unique growth requirements (Tripp et al. 2008; Carini et al. 2013; Sun et al. 2016).

The first successful cultivation of SAR11 in 2002 resulted in the isolation of *Pelagibacter ubique* strain HTCC1062 (Rappé et al. 2002), followed by the publication of its complete genome (Giovannoni et al. 2005). Over the past two decades, additional isolate genomes have been few, with only 25 currently available. Despite their rarity, high-quality genomes from isolated strains not only shed light on SAR11 biology (Schwalbach et al. 2010; Sun et al. 2011; Carini et al. 2013) and the origins of this lineage within the *Alphaproteobacteria* (Thrash et al. 2011; Grote et al. 2012; Muñoz-Gómez et al. 2019), but also have made it possible to establish key concepts in biology such as genome streamlining (Schwalbach et al. 2010; Sun et al. 2011;

Grote et al. 2012; Viklund et al. 2012; Giovannoni et al. 2014; Giovannoni 2017) and investigate the evolutionary processes that shape protein evolution (Delmont et al. 2019; Kiefl et al. 2023).

Here we report 81 high-quality genomes from SAR11 strains, increasing the number available for SAR11 isolates by fourfold, and leverage this new collection to build a robust genome phylogeny for the order *Pelagibacterales*. By incorporating publicly available, high-quality single-cell genomes and surface ocean metagenomes from both a steep, nearshore to open-ocean local environmental gradient and elsewhere from around the globe, we reveal cohesive patterns of genomic and ecotypic diversification. We propose a framework through which to characterize and interpret genome heterogeneity at multiple stages along the evolutionary history of SAR11 marine bacteria, and establish a roadmap for future efforts to organize this globally abundant bacterial clade.

Results

Eighty-one high-quality genomes sequenced from 206 newly isolated SAR11 strains and co-cultures

Three dilution-to-extinction culturing experiments using surface seawater collected from nearshore and adjacent offshore environments of O‘ahu, Hawai‘i, in the tropical Pacific Ocean resulted in 916 isolates from 2,102 inoculated cultures (Table 1; Supplemental Fig. 1). Using a streamlined isolate-to-genome approach, we identified 206 cultures as either pure SAR11 strains or mixed cultures with at least 50% of the total reads matching a SAR11 strain via 16S rRNA gene amplicon sequencing (Supplemental Table 1), and sequenced draft genomes from 90. Manual curation resulted in 79 high-quality SAR11 isolate genomes. The genomes from two

100 strains (HIMB123 and HIMB109) isolated from a previous culture experiment were also added
101 (Brandon 2006), resulting in 81 new SAR11 genomes from isolates. The majority of these
102 (n=60) assembled into ten contigs or less, including 24 closed genomes and an additional 30
103 containing one to three contigs. They ranged from 1.00 to 1.54 Mbp in size and GC content of
104 28.5 to 30.7% (Supplemental Table 2). The median pairwise genome-wide average nucleotide
105 identity (gANI) value across all genomes was 81.8% and none of the 81 new isolate genomes
106 were identical. Having captured a genetically diverse array of SAR11 isolates, we used a
107 phylogenomic approach to characterize evolutionary relationships between these genomes and to
108 high-quality single-cell and isolate genomes previously retrieved from seawater.

109

110 **Table 1. Summary of high-throughput culturing (HTC) experiments.**

111

Site	Inoculum source	Inoculum size (# of cells)	Cultures screened	Positive cultures	SAR11 genomes
SB	raw seawater	5	576	339	53
STO1	raw seawater	5	576	126	16
STO1	cryopreserved seawater	5	480	142	9
STO1	cryopreserved seawater	100	470	343	1

112

113 **A comprehensive genome phylogeny reveals a robust evolutionary backbone populated by**
114 **clusters of closely related genomes**

115 We first sought to resolve relationships between the strains isolated in this study and
116 other publicly available high-quality *Pelagibacterales* genomes to precisely establish where the
117 new genomes originate from within the broad spectrum of known SAR11 diversity. For this, we
118 created a database that, in addition to the 81 genomes presented here, included 25 public SAR11
119 isolate genomes, 8 of which were also isolated from off the windward coast of O‘ahu, Hawai‘i,
120 and 375 SAR11 single-amplified genomes (SAGs) estimated to be ≥85% complete with a

121 redundancy <5% (Supplemental Table 3). We also included five additional SAR11 SAGs of
122 potentially unique evolutionary origin in this collection (Vergin et al. 2013; Thrash et al. 2014),
123 though we excluded genomes from putative SAR11 subgroups IV (Vergin et al. 2013) and V
124 (Thrash et al. 2011) due to their unlikely or, at a minimum, uncertain shared common ancestry
125 with SAR11 (Thrash et al. 2011; Viklund et al. 2013; Haro-Moreno et al. 2020; Muñoz-Gómez et
126 al. 2022). This resulted in a curated collection of 481 SAR11 genomes to assess the evolutionary
127 backbone for SAR11.

128 Previous studies investigating phylogenomic relationships within the
129 *Alphaproteobacteria* utilized a curated set of 200 single-copy core genes (SCGs) for this
130 bacterial class (Wang and Wu 2013; Muñoz-Gómez et al. 2019). We evaluated the presence of
131 these 200 SCGs across our genome dataset, and excluded genes missing in more than 90% of the
132 481 SAR11 genomes. This resulted in a SAR11-specific SCG set of 165 genes for downstream
133 phylogenomic analyses, referred to hereafter as the SAR11_165 core gene set (Supplemental
134 Table 4).

135 Our analysis of the 481 genomes using the SAR11_165 gene set revealed that the SAR11
136 clade consists of four robust, deeply-branching sublineages (Fig. 1; Supplementary Fig. 2). Three
137 of these branches were the previously characterized subclades Ic (Vergin et al. 2013), II (Suzuki
138 et al. 2001), and III (Morris et al. 2005), while the fourth was a combination of established
139 SAR11 subclades Ia and Ib (Suzuki et al. 2001), which did not form separate monophyletic
140 subclades in this comprehensive genomic dataset and robust phylogenetic analysis. If the SAR11
141 clade is assigned to the taxonomic level of a bacterial order, then these four lineages logically
142 resolve to the taxonomic level of families.

143 We further removed genomes from this initial tree in two steps. First we excluded SAGs
144 that did not fall into a 90% gANI cluster of at least three genomes to focus our analyses on
145 well-resolved regions of the tree. Second, we de-replicated the remaining genomes using a
146 conservative cutoff of 95% gANI to minimize subsequent competitive metagenomic recruitment
147 steps splitting reads among closely related genomes (Evans and Denef 2020). While the 95%
148 ANI cutoff is broadly recognized in contemporary microbiology as a threshold to identify
149 microbial species, it over-splits ecologically and evolutionarily cohesive units in SAR11 and
150 does not delineate species-like groups. We note that the reason behind our use of the 95% ANI in
151 this step of our analysis was solely to establish a technically robust workflow prior to
152 competitive read recruitment rather than a biologically meaningful partitioning of our genomes, a
153 challenge our study focuses on later.

154 We then turned our attention to the distal end of the phylogeny, which contained a large
155 number of well supported clusters of closely related genomes, particularly within the Ia/Ib
156 subgroup that contained 78 of the 81 new isolate genomes. A phylogeny of the resulting 268
157 genomes revealed 24 monophyletic clusters within the historical Ia/Ib subgroup that were
158 characterized by a range of gANI values from 84% to 96% (92.1 ± 2.94%; mean ± SD) (Fig. 1,
159 Supplemental Fig. 3). While a handful of these clusters were recognized previously, we defined
160 an additional 11 here (Fig 1; Supplemental Table 5). Twelve of the 24 clusters contained an
161 isolated representative, and eight contained at least one isolate from our study area in the tropical
162 Pacific.

163 In summary, our extensive phylogenomic analysis of SAR11 revealed 24 monophyletic
164 clusters within the historical Ia/Ib subgroup which included the majority of SAR11 SAGs and the

new and previously published isolate genomes. The non-uniform minimum gANI estimates suggest that the application of sequence-based ANI thresholds to demarcate SAR11 diversity may obscure important evolutionary signals. Hypothesized drivers of the maintenance and partitioning of genomic diversity in SAR11 include niche-based processes, where genetically cohesive clusters also display ecological homogeneity and the underlying genetic diversity is maintained by similar forces of selection, recombination, and drift. To understand the potential eco-evolutionary forces that shape SAR11 diversification, we turned to metagenomic read recruitment analysis to recover biogeographical distribution patterns for our genomes across the globe.

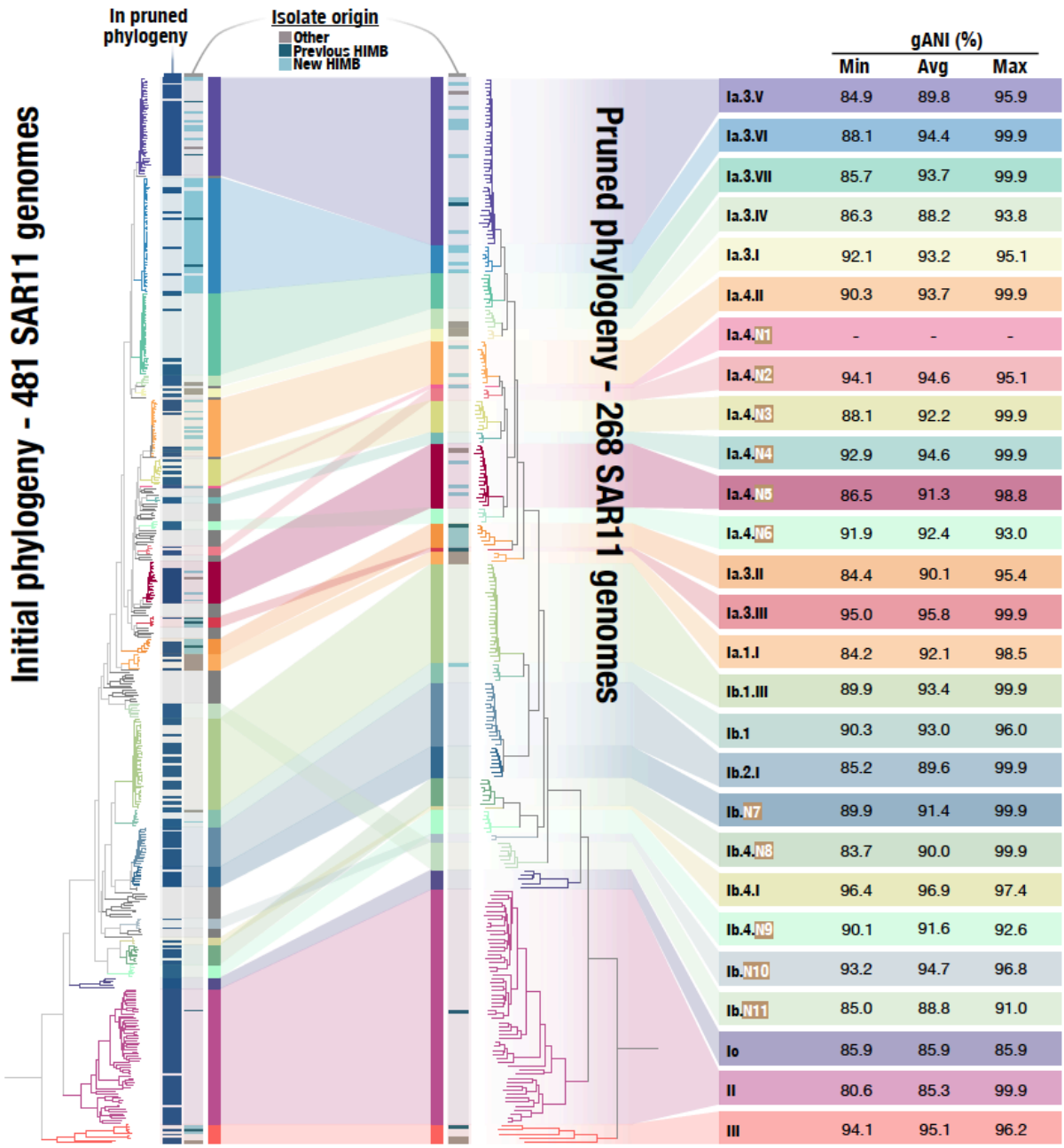


Fig 1. Comprehensive phylogenies of the *Pelagibacterales*. A comparison between an exhaustive phylogeny (left panel) with 481 SAR11 genomes (106 isolates and 375 SAGs) and a pruned phylogeny (right panel) with 268 genomes (50 isolates and 218 SAGs), based on a curated SAR11-specific set of 165 genes. Genomes included in the pruned phylogeny are indicated with a dark blue bar in the left panel, and the origin of isolate genomes is indicated for both phylogenies.

Global read recruitment from the surface ocean reveals broadly congruent phylogenetic and ecotypic diversification across SAR11

Our competitive metagenomic read recruitment assessed the distribution of the 268 SAR11 genomes around the globe and relied upon 950 publicly-available marine metagenomes, as well as metagenomes from the Kāneʻohe Bay Time-series (KByT), the location of isolation for the 81 new and 9 of the 25 existing isolate genomes (Supplemental Table 6; Supplemental Table 7). These data enabled us to investigate whether cohesive genomic and ecological groups, or ecotypes, could be discerned by combining SAR11 phylogeny and biogeography.

Our first priority was to establish whether genome clusters within a given SAR11 clade showed cohesive read recruitment profiles across metagenomes, or, in other words, whether the ecological patterns revealed by a single genome were similar to all genomes within the group to which it belonged. Detection values for multiple genomes within a genome cluster showed a high degree of cohesion (Fig. 2; Supplemental Table 8; Supplemental Table 9). For example, representatives from Ia.3.IV, Ia.3.I, Ia.4_II, Ia.4.N2, Ia.4.N5, Ib.1.III, and Ib.4.N9 are particularly consistent within the genome clusters (Fig. 2). Consistent overlap between SAGs and isolate genomes within the same clade demonstrated that both genome types accurately reflect distribution patterns for closely related populations as inferred by phylogeny (Fig. 2). A non-metric multidimensional scaling (NMDS) analysis of the overall detection patterns of genomes across metagenomes consistently grouped genomes within a given clade more closely compared to those that belonged to other genome clusters (Supplemental Fig. 4), further supporting a high degree of intra-clade ecological cohesion.

Our second priority was to establish insights into whether SAR11 genome clusters differed in their biogeographical patterns, and whether genome clusters identified SAR11 populations of distinct ecology. Hierarchical clustering of metagenomes based on SAR11 detection patterns revealed four groups: metagenomes that originated from (1) low-latitude samples, (2) high-latitude samples with low SAR11 diversity, (3) low-latitude samples with high SAR11 diversity, as well as (4) samples from coastal Kāneʻohe Bay (Fig. 2). Many SAR11 genome clusters were indeed differentially distributed across these metagenome groups. For example, Ia.4.II and Ia.4.N5 were only consistently found in groups 3 and 4, while Ia.3.IV was found across group 1 and only in select sites in groups 3 and 4 (Fig 2). However, in multiple cases, the environmental detection patterns of different phylogenomic genome clusters overlapped; while there was some degree of inter-clade ecological differentiation, distinct SAR11 genome clusters frequently co-occurred (Fig. 2, Supplemental Fig. 5). This observation suggests that patterns of distribution alone cannot discern the boundaries of cohesive ecological units within SAR11, a task that evidently requires the integration of biogeographical patterns through metagenomic read recruitment with ancestral relationships among genomes though phylogenomics.

Finally, we used the read recruitment analysis to assign ecological patterns to specific SAR11 genome clusters. While multiple broad patterns were clear from the pairing of the phylogenomic relationships and read recruitment data, we focused our investigation on whether the genome clusters within the SAR11 Ia/Ib lineage that appeared to be confined to the coastal end of the KByT environmental gradient (Ia.3.VI, Ia.3.II, and Ia.3.III; Supplemental Fig. 1; also see (Tucker et al. 2024a)) were similarly constrained to coastal areas globally. Indeed, two of the

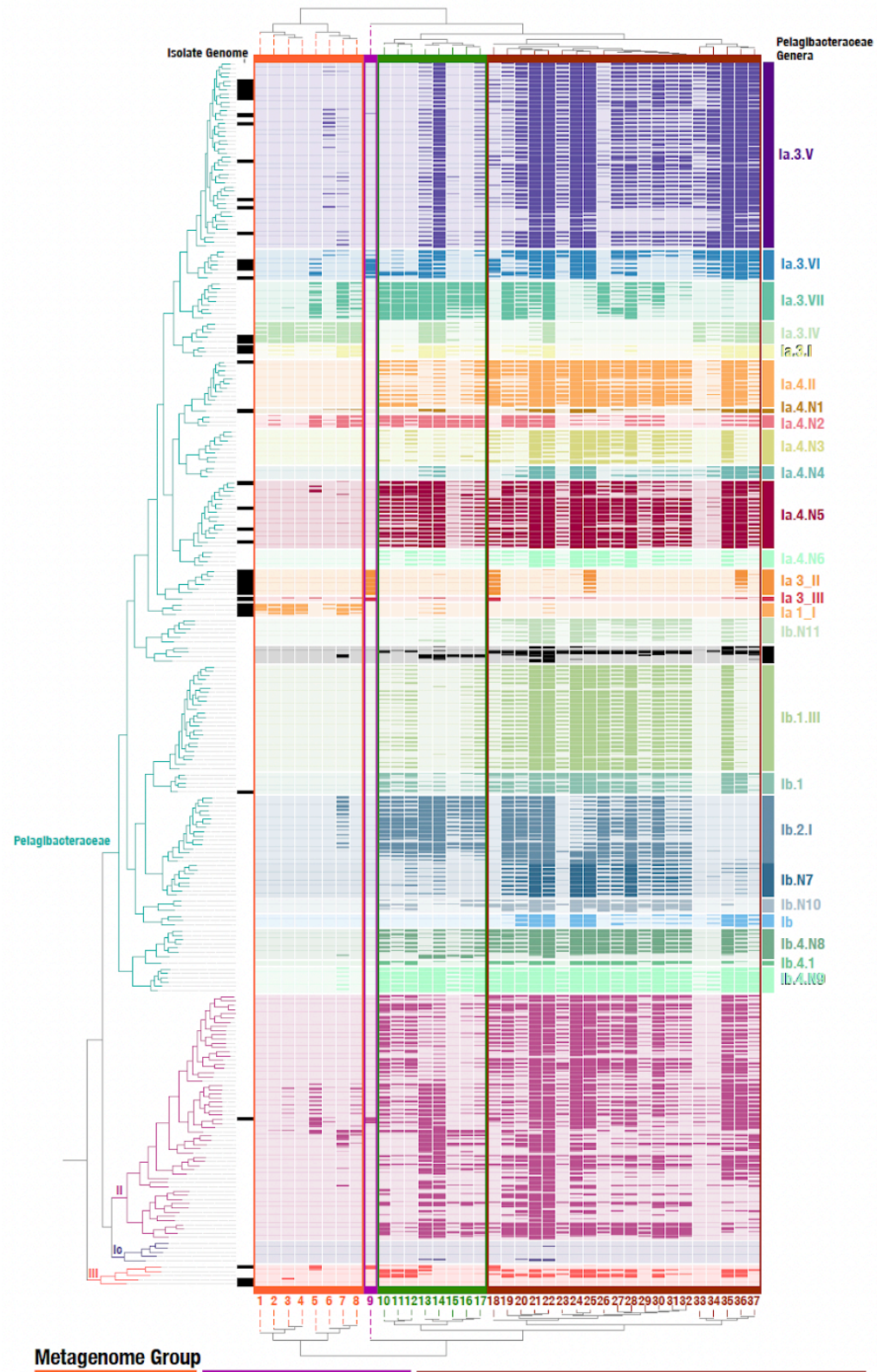
225 three genome clusters, Ia.3.II and Ia.3.III, were detected almost exclusively in metagenomes
226 sourced from coastal environments (e.g., KByT, the north coast of Panama, the Chesapeake Bay,
227 and the Atlantic coast of Portugal). Interestingly, while the clade Ia.3.VI was restricted to
228 nearshore metagenomes across KByT, it was well-detected in both coastal and offshore
229 environments in other oceanic regions (Fig. 2). Genome clusters Ia.3.II and Ia.3.III did not
230 include any SAGs and were only composed of isolates from coastal Kāneʻohe Bay. Yet, we could
231 detect them in other oceans, which confirms their global relevance as representatives of SAR11
232 populations adapted to coastal ecosystems.

233 Through the combination of global metagenomic read recruitment and phylogenomics,
234 we show that SAR11 genome clusters contain genomes with a high degree of intra-clade
235 ecological cohesion. These genome clusters were often distinguished by their ecological
236 distributions and demonstrated notable inter-clade ecological differentiation. Finally, we applied
237 this framework to understand how SAR11 genetic and ecological diversity partitions among
238 ocean biomes, in particular coastal ocean and open ocean environments.

239 The integrated ecological and evolutionary framework here is supported by high-quality
240 genomes that span the known diversity of the *Pelagibacterales*, providing a critical opportunity
241 to discern distinct ecologically meaningful genome clusters within SAR11. We show that the 24
242 distinct genome clusters represent groups sharing cohesive ecological patterns and evolutionary
243 relationships, not at the finest tips of the phylogenomic tree, but at relatively deeper branches
244 that encompass gANI values ranging between 84% and 96%. This suggests it is unlikely that
245 these genome clusters represent SAR11 diversity at the level of ‘species’. This conclusion is
246 further supported by our companion work (Tucker et al. 2024a), which reveals systematic

differences in the metabolic potential of SAR11 genome clusters that likely support distinct ecological distributions in immediately adjacent coastal and open ocean surface seawater with habitat-specific metabolic genes that are under higher selective forces. With the combined evidence presented here and in the work by Tucker et al. (2024a) that unite SAR11 diversity into distinct genome clusters with ecotype properties supported by SAR11 phylogenomics, ecology, metabolic potential, as well as population genetics, we argue that the most conceivable taxonomic rank at which SAR11 genome clusters can be described in a conventional framework emerges as the 'genus' level.

This genus-level designation is ideal as it encompasses a degree of diversity previously designated by SAR11 subgroups and has the flexibility to account for subtle variation in ecology recognized between closely related genomes. We identified the highest quality genome representatives (electing for isolates when possible) to assign as type genomes for each genus (Fig. 4), which establishes a roadmap to rationally designate new genera as they are identified in the future.



Metagenome Group

High-latitude, low-diversity

1 Temperate Pacific Ocean
2 Temperate Atlantic Ocean
3 English Channel
4 Temperate East Pacific
5 Mediterranean
6 Temperate East Pacific
7 Atlantic Ocean South Africa
8 Temperate Atlantic & Pacific Ocean

Coastal Kāne'ohe Bay

9 Coastal O'ahu Kāne'ohe Bay

Low-latitude

10 Atlantic Ocean North West Central
11 Summer BATS
12 Early Fall BATS
13 Mediterranean
14 North Atlantic & Mediterranean
15 Transect South West Pacific Ocean
16 South West & Central Pacific Ocean
17 South West Pacific Ocean

Low-latitude, high-diversity

18 Offshore & Transition Kāne'ohe Bay
19 Late Fall BATS
20 Winter & Spring BATS
21 South Indian Ocean
22 Atlantic Ocean
23 South Central Atlantic Ocean
24 Indian Ocean
25 Red Sea
26 Atlantic Ocean West
27 North West Atlantic Ocean
28 Summer & Fall BATS
29 Coastal Australia & Central Pacific Ocean
30 Station ALOHA
31 Coastal Australia
32 Offshore Kāne'ohe Bay
33 Atlantic Ocean, South Africa
34 North & South East Pacific Ocean
35 Pacific Ocean Central & North
36 Indian Ocean
37 North & South East Pacific Ocean

Fig. 2: Global metagenome read recruitment to 268 *Pelagibacterales* genomes. A clustering analysis reveals that the distribution of metagenomes from the same geographic location have characteristic patterns of detection. Detection values from 0.25 to 0.75 are shown.

Evidence for ecological speciation within closely related genome clusters

Despite broad ecological cohesion within what we have designated as *Pelagibacterales* genera, some notable differences highlight underlying complexities in defining the finest scales of divergence. The Ia.3.VI genus includes genomes from strains of Kāneʻohe Bay origin as well as SAGs from other regions of the global ocean and encompasses significant genomic diversity (minimum gANI 88%) and phylogenomic structure (Fig. 3a). Through read recruitment, we observed notable differences in detection patterns of genomes across metagenomic samples. Isolate genomes from the bay harbored the highest detection values of the Ia.3.VI genus from metagenomes in the bay, while a SAG from the BATS site in the Atlantic Ocean (GCA_902533445.1) had the highest detection values at the BATS site (Fig. 3a), particularly in the summer and fall (Fig. 2).

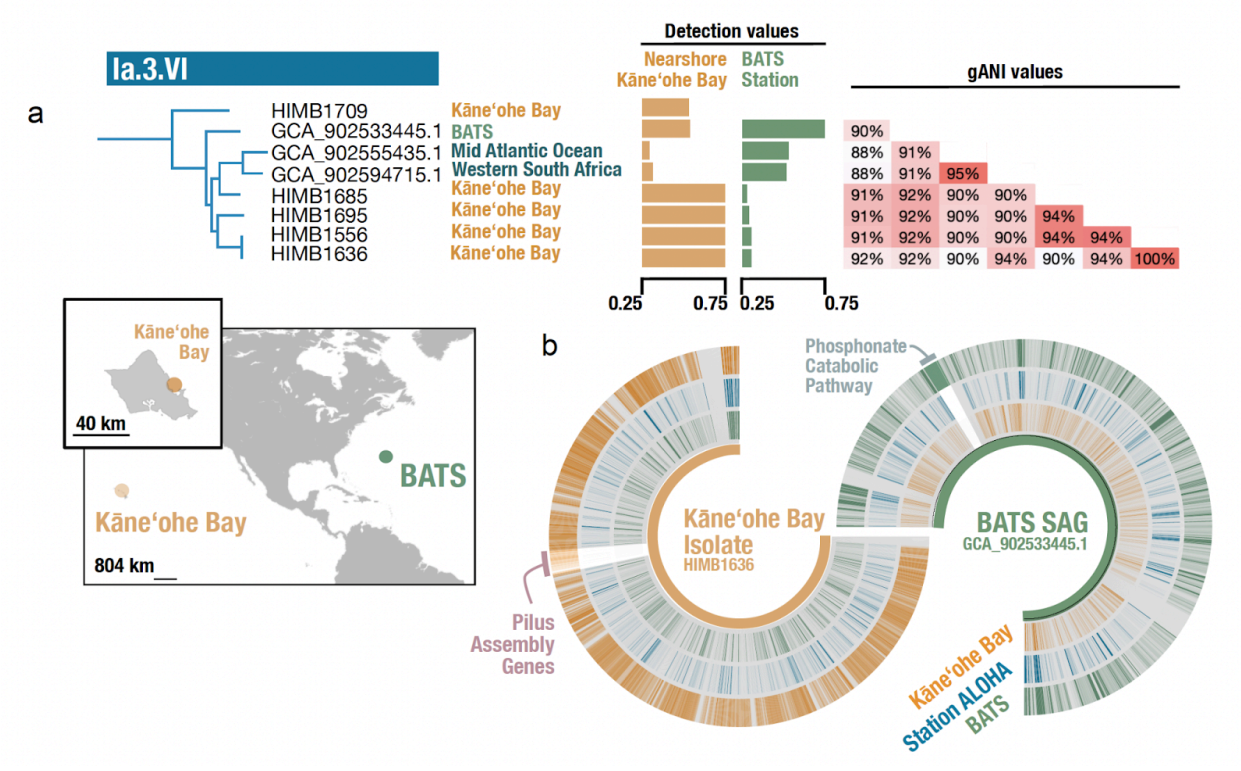


Fig. 3: Fine-scale ecological speciation between closely related SAR11 genomes. (a) Detailed view of the Ia.3.VI genus including evolutionary relationships, locations of genome origin, detection values from select locations, and within-genus gANI values. The geographic origins of two of the closely related genomes that have distinct detection patterns include Kāne‘ohe Bay in the Pacific Ocean and the Bermuda Atlantic Time-series Study (BATS) in the Atlantic. **(b)** Coverage values of isolate HIMB1636 and SAG GCA_902533445.1 of metagenomes from nearshore Kāne‘ohe Bay, Station ALOHA in the North Pacific Subtropical Gyre, and BATS highlighting the differential detection of genes for type IV pilus assembly and the phosphonate catabolic pathway.

Given the underlying genomic diversification between isolate HIMB1636 and BATS SAG GCA_902533445.1 and their distinct biogeographical distributions that peak in each of their respective source locations, we next surveyed the genomes for potentially unique metabolic capabilities. By inspecting the coverage of isolate HIMB1636 and BATS SAG GCA_902533445.1 using metagenomes from Kāne‘ohe Bay and the BATS site (Supplemental Table 10), we found one genomic region of SAG GCA_902533445.1 that had particularly high

297 coverage at BATS compared to the KByT samples and included 29 genes encoding the uptake
298 (*phnCDE*) and catabolism of phosphonates via the C-P lyase pathway (*phnGHIJKLM*) (Fig. 3d)
299 (Villarreal-Chiu et al. 2012). The *phnJ* phylogeny did not reflect the phylogenomic relationships
300 among genomes (Supplemental Fig. 6), and the entire pathway was located on a genomic island
301 similar to the marine bacterium HIMB59 (Molina-Pardines et al. 2023). The C-P lyase pathway
302 is known to be enriched in phosphate-depleted systems of the Atlantic Ocean (Sosa et al. 2019;
303 Acker et al. 2022), so the presence of the C-P lyase catabolic genes in a genome sourced from
304 BATS, but missing from a closely related genome sourced from more phosphate-replete
305 environments of Kāneʻohe Bay in the Pacific, suggests these genes provide an advantage in
306 phosphate depleted systems and that the BATS SAG GCA_902533445.1 may be locally-adapted
307 to these environments.

308 While the HIMB1636 genome lacked the C-P lyase pathway, it contained a unique
309 genomic region with particularly high coverage that was not found in SAG GCA_902533445.1,
310 and encoded genes for type IV pilus assembly. The role of type IV pilus assemblies in SAR11 is
311 unclear (Zhao et al., 2017), although in other organisms it has been associated with an array of
312 functions including DNA uptake, twitching motility, and aggregation into microcolonies (Craig
313 and Li 2008). The presence of the type IV pilus assembly genes in the Ia.3.VI genome sourced
314 from the nitrogen-limited Pacific Ocean, but not in genomes from relatively more
315 nitrogen-replete waters of BATS, along with evidence that the *Pelagibacteraceae* can utilize
316 purine nucleosides and purine-derivatives for nitrogen (Braakman et al. 2024; Tucker et al.
317 2024a), suggests that the presence of a type IV pilus may be advantageous for DNA uptake in
318 nitrogen-poor environments and that HIMB1636 may be locally-adapted. Contrary to the

319 hypothesis that genera recombine at a rate sufficient to limit the ecotypic diversification of
320 closely related genomes (Zhao et al. 2024), our read mapping instead shows that the HIMB1636
321 and SAG GCA_902533445.1 genomes within cluster Ia.3.VI have sufficiently diverged at the
322 nucleotide level to reveal clear biogeographic divergence, and that they possess sets of genes that
323 reside in hypervariable genomic regions that are clearly associated with the differences in
324 abundance.

325 We examined gANI estimates, phylogenetic branching, environmental distributions, and
326 ecologically-relevant gene content to support the characterization of ecological diversification at
327 the finest tips of the tree, a process that we theorize to represent speciation. This underscores the
328 complexity of SAR11 ecology, highlights the need to include a diversity of representative
329 genomes within even closely related genera for environmental genomics studies, and indicates
330 that continued efforts to sample SAR11 globally are key to understanding the distribution of this
331 ubiquitous clade.

332

333 **Proposed *Pelagibacterales* classification and nomenclature**

334 We leveraged the robust genome phylogeny, gANI metrics, and read recruitment to
335 establish a rational classification and nomenclature system for the *Pelagibacterales* bacterial
336 order *Pelagibacterales*. To provide a framework and vocabulary to discuss groups of SAR11 in a
337 meaningful context, we first defined four family-level monophyletic groups as the
338 *Pelagibacteraceae* (historical subgroups Ia and Ib), *Cosmipelagibacteraceae* (historical
339 subgroup II), *Allofontibacteraceae* (historical subgroup III), and the *Mesopelagibacteraceae*
340 (historical subgroup Ic) (Fig. 4). We focused our efforts primarily on classification within the

341 *Pelagibacteraceae* where the majority of cultured isolates originate. Within the
342 *Pelagibacteraceae*, we used phylogenomics and ecological data to characterize 24 genera that
343 represent cohesive genetic and ecological clades, and designate type species for each
344 (Supplemental Table 11). The primary aim of these efforts is to ensure that the taxonomic
345 hierarchy for SAR11 provides a useful and tractable reflection of the ecology and genetic
346 diversity within this globally distributed group, and establishes a rational system that future
347 efforts can build upon.

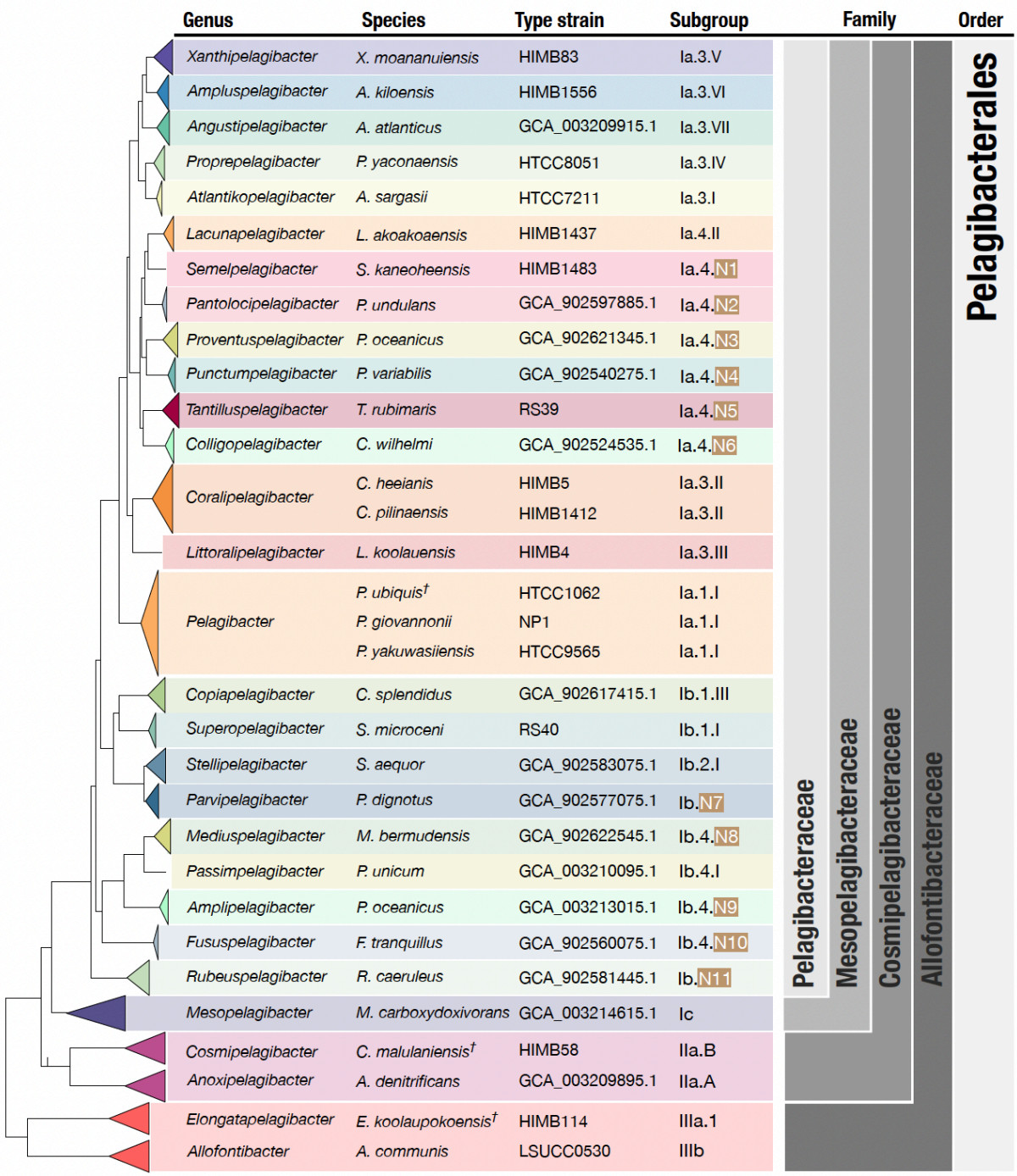


Figure 4. A proposed taxonomic framework for the SAR11 order *Pelagibacterales*. This schematic SAR11 phylogeny unites proposed genus and species names, proposed type strains, and historical reference labels.

Discussion

By integrating high-throughput cultivation experiments with publicly available genomes and metagenomes, our study provides key insights into a long-standing question: to what extent, and at what hierarchical levels, can the genomic and ecological diversity of SAR11 be partitioned into cohesive units? Through comprehensive phylogenomic analyses paired with global metagenomic read recruitment surveys, we reveal ecotypic differentiation at both relatively shallow, species-level and deeper, genus-level diversity within SAR11. This robust eco-evolutionary framework, which unifies independent yet complementary approaches to genomic diversity and biogeography, resolves the order *Pelagibacterales* into four families and the family *Pelagibacteraceae* into 24 genera, establishing a much-needed taxonomic framework that delineates SAR11 diversity into tractable units and provides a foundation for future investigations.

A tight relationship between the phylogeny and ecology of SAR11 has long been suggested (Brown et al. 2012; Vergin et al. 2013); however, the ability to associate specific SAR11 clades with distinct ecological patterns and explain forces that maintain SAR11 diversity has remained elusive. Focusing on sequence-discrete groups within deep ocean SAR11 lineages, a recent study concluded that recombination, rather than ecological speciation, was likely the major driver of species-level cohesion (Zhao et al., 2024). While this observation may explain forces that maintain species-level cohesion for some populations in this group, our study shows that the global sampling of environmental populations through metagenomes consistently supports ecological delineations that are congruent with phylogenomic clustering patterns,

373 pointing towards ecotypic differentiation as the pervasive driver of the evolution within the
374 *Pelagibacterales*. Interestingly, SAR11 genera that showed similar biogeographical distribution
375 patterns in our analysis tended to occupy distant parts of the tree. This observation suggests an
376 inverse correlation between the genetic similarity among SAR11 populations and their
377 co-occurrence, a trend known as phylogenetic overdispersion. Phylogenetic overdispersion has
378 been observed across the tree of life (Davies 2006) and is driven by forces of competitive
379 exclusion, an overarching ecological phenomenon that limits the co-occurrence of ecologically
380 similar, closely related organisms. Future analyses that aim to resolve specific genetic
381 determinants of competitive exclusion or co-existence may benefit from geographically
382 constrained time-series data, as these patterns are likely not immediately attainable from global
383 yet spatiotemporally sparse metagenomes.

384 The practical need of microbiologists to find reasonable cutoffs to demarcate species
385 boundaries from genomic data alone and the nature of SAR11 evolution do not align seamlessly.
386 Through the analysis of genomes, a large number of anecdotal observations support 95% ANI as
387 a reasonable means to resolve archaeal and bacterial species (Jain et al. 2018; Olm et al. 2020).
388 However, SAR11 serves as a reminder that practical solutions do not necessarily apply to all
389 microbial clades (Delmont et al. 2019; López-Pérez et al. 2020). One of the implications of the
390 efforts to standardize the tree of life based on principles that work only for the majority of
391 microbial taxa is the conflation of all SAR11 genomes into two genera in the taxonomic
392 framework derived from genomes available on GTDB based on RED scores (Parks et al. 2022).
393 Indeed, while the ecologically relevant units of SAR11 described in our study are in agreement
394 with functional, evolutionary, and ecological observations, they are in disagreement with the

395 contemporary summaries of this clade based on RED- or ANI-based demarcations. The ways in
396 which evolutionary relationships between distinct clades of life intersect with taxonomic
397 classification systems will unlikely be resolved in a manner that satisfies everyone in
398 microbiology (Waite et al. 2020; Sanford et al. 2021). In this juncture, we believe that a stronger
399 motivation to understand the biological drivers that render SAR11 incompatible with our best
400 practical approaches will bring us closer to a unified solution to partition microbial diversity into
401 meaningful units, rather than casting SAR11, one of the most numerous microbial clades on our
402 planet, as a mere outlier.

403 Insights into the eco-evolutionary processes that shape SAR11 diversification in our
404 study rely heavily on the contribution of 81 new isolate genomes that represent abundant and
405 ecologically-relevant SAR11 populations across the coastal and global ocean. The
406 ecology-informed hierarchical organization of these genomes enabled us to propose SAR11
407 genera with formal names here, and investigate the likely functional determinants of ecological
408 diversification across the *Pelagibacteraceae* in our companion work (Tucker et al. 2024a). While
409 deeper understanding of the physiological, metabolic, and genetic factors that shape SAR11
410 biology will require controlled experimentation of isolated strains in the laboratory, our study
411 organizes the eco-evolutionary characteristics of known SAR11 diversity and provides a
412 roadmap for future efforts aimed to organize and understand the ubiquitous SAR11 populations
413 inhabiting the global ocean.

414

Methods

High-throughput culturing from surface seawater within and adjacent to Kāneʻohe Bay, Oʻahu

Growth medium was prepared as previously described (Monaghan et al., 2020). Briefly, 20 L of seawater was collected first on 8 July 2017 and again on 20 September 2017 from a depth of 2 meters at station SR4 (N 21º 27.699', W 157º 47.010') in acid-washed polycarbonate bottles (Supplemental Fig. 1). The seawater was then filtered, autoclaved, and sparged as previously described (Monaghan et al. 2020). After processing, the sterile seawater was stored at 4ºC until use.

Two 4 L seawater samples to be used as inoculum were collected on 26 July 2017 in acid-washed polycarbonate bottles from 2 meters from stations SB (N 21° 26.181', W 157° 46.642) and STO1 (N 21° 28.974, W 157° 45.978') (Supplemental Fig. 1) and immediately returned to the laboratory for further processing. All of the Kāneʻohe Bay Time series sampling sites were previously classified as 'nearshore', 'transition', or 'offshore', with SB and STO1 representing nearshore and offshore sites, respectively (Tucker et al. 2021). Subsamples of the raw seawater were processed as described previously (Monaghan et al. 2020). Briefly, aliquots were taken for cryopreservation in a final concentration of 10% v/v glycerol and fixed with paraformaldehyde for the enumeration of planktonic microorganisms via flow cytometry. Additionally, 0.96 L from station SB and 1.30 L from station STO1 were filtered through a 25 mm diameter, 0.1 µm pore-sized polyethersulfone membrane (Supor-100; Pall Gelman Inc., Ann

435 Arbor, MI), which was then submerged in 500 µL DNA lysis buffer and stored at -80ºC until
436 DNA extraction.

437 Subsamples of raw seawater from SB and STO1 were enumerated using microscopy,
438 diluted to 2.5 cells mL^{-1}, and plated in 2 mL volumes into a total of 1,152 wells (576 wells per
439 site) of custom-fabricated 96-well Teflon microtiter plates. This experiment is referred to here as
440 HTC2017. Plates were then sealed with breathable polypropylene microplate adhesive film and
441 incubated in the dark at 27ºC. Plates were monitored for cellular growth at 3.5 and 8 weeks using
442 flow cytometry as previously described (Tripp et al. 2008; Monaghan et al. 2020). Wells with
443 positive growth (greater than 10^4 cells mL) after 24 or 57 days of incubation were further
444 sub-cultured by transferring approximately 1 mL into 20 mL of sterile seawater media amended
445 as previously described (Monaghan et al. 2020) with 400 µM $(NH_4)_2SO_4$, 400 µM NH_4Cl, 50 µM
446 NaH_2PO_4, 1 µM glycine, 1 µM methionine, 50 µM pyruvate, 800 nM niacin (B3), 425 nM
447 pantothenic acid (B5), 500 nM pyridoxine (B6), 4 nM biotin (B7), 4 nM folic acid (B9), 6 µM
448 myo-inositol, 60 nM 4-aminobenzoic acid, and 6 µM thiamine hydrochloride (B1). These
449 subcultures were then incubated at 27ºC in the dark for an additional 33 days and then all
450 samples were processed and cataloged.

451 Cultures checked at 33 days that yielded positive growth (>10^4 cells ml^{-1}) were
452 cryopreserved in duplicate (2 x 500 µL culture and a final concentration of 10% v/v glycerol).
453 Each well with positive growth was assigned an HIMB culture ID and cells from the
454 approximately 18 mL remaining volume of each culture were collected by filtration through a 13
455 mm diameter, 0.03 µm pore-sized polyethersulfone membrane (Sterlitech, Kent, WA, USA),
456 which was then submerged in 250 µL DNA lysis buffer and stored at -80ºC until DNA

457 extraction. The lysis buffer was prepared by adding the following to MilliQ water: 8 mL 1M Tris
458 HCl (pH 8.0), 1.6 ml 0.5M EDTA (pH 8.0), and 4.8 g Triton X, for a final volume of 400 mL,
459 which was then filter sterilized, with lysozyme added to aliquots immediately before use (at a
460 final concentration of 20 mg ml^{-1}).

461 An additional experiment was performed using cryopreserved samples of seawater
462 collected on July 26, 2017, and described previously (Monaghan et al. 2020). Briefly, the
463 cryopreserved sample was enumerated and then diluted to two cell concentrations (2.5 and 52.5
464 cells ml^{-1}), and used to plate 480 and 470 2-ml dilution cultures, respectively. This experiment is
465 referred to as HTC2018. Growth was monitored at 2, 3, and 5 weeks after inoculation with
466 positive growth (>10^4 cells ml^{-1}) from the 2.5 cells ml^{-1} cultures subcultured into 20 ml of sterile
467 seawater growth medium and monitored for growth for up to 10 weeks at 27ºC in the dark.
468 Subcultures were then cryopreserved and cells collected for DNA sequencing as described
469 above. One well from the 52.5 cells ml^{-1} inoculation was directly collected for DNA sequencing
470 without subculturing (Monaghan et al. 2020).

471 **DNA extraction and 16S rRNA gene amplicon sequencing**

472 Genomic DNA (gDNA) from all filtered cultures as well as environmental DNA (eDNA)
473 from STO1 and SB was extracted using the Qiagen DNeasy Blood and Tissue Kit with modified
474 manufacturer's instructions for bacterial cells (Qiagen, Germantown, Maryland, USA). The
475 modifications included the addition of an initial freeze-thaw step (3 cycles of 10 minutes at 65ºC
476 followed by 10 minutes at -80ºC), the addition of 35 µL Proteinase K and 278 µL buffer AL at
477 the appropriate pretreatment step, and finally when eluted the same 200 µL volume was passed
478 through the membrane three times.

479 For the initial identification of all cultures, gDNA was used as template for the
480 polymerase chain reaction (PCR) amplification (Bio Rad C1000 Touch, Bio Rad, Hercules, CA,
481 USA) using barcoded 515F and 926R primers targeting the V4 region of the SSU rRNA gene
482 (Parada et al., 2016) in a reaction volume of 25 µL composed of: 2 µL gDNA, 0.5 µL each
483 forward and reverse primer, 10 µL 5PRIME HotMasterMix (Quantabio, Beverly, MA, USA),
484 and 12 µL of molecular grade H_20 (Monaghan et al. 2020). The reaction was as follows: an
485 initial denaturation step of 3 min at 94ºC, 40 cycles of 45 sec at 94ºC followed by 1 min at 50ºC
486 and 1.5 min at 72ºC, with a final extension of 10 min at 72ºC. The PCR products were prepared
487 for sequencing as previously described (Monaghan et al. 2020) and sequenced on a MiSeq
488 platform by the Oregon State University Center for Genome Research and Biocomputing.

489 **16S rRNA gene sequence analysis**

490 Amplicon sequence data were processed as previously described (Monaghan et al. 2020).
491 Briefly, the data was imported into QIIME2 v2019.4.0, and demultiplexed before being assessed
492 for sequence quality and merged. DADA2 (Callahan et al. 2016) was then used for quality
493 control. Taxonomy was assigned to all reads using a Naïve Bayes classifier trained on the Silva
494 rRNA v132 database (Quast et al. 2013). Cultures were first classified as defined previously
495 (Monaghan et al. 2020), with “monocultures" consisting of more than 90% of reads from a single
496 amplicon sequence variant (ASV), “mixed cultures" with an ASV that was between 50% and
497 90% of the reads, and finally cultures with no dominant members. Any samples with less than
498 1,000 reads were not included in further analyses. We aimed to sequence all strains that included
499 monocultures and mixed cultures of SAR11.

500 **Genome sequencing**

501 To prepare samples of interest for whole genome sequencing, all extractions with gDNA
502 concentrations above 0.06 ng μL^{-1}, a total of 10 µL were aliquoted for sequencing. For samples
503 with concentrations below 0.06 ng μL^{-1}, the remaining extraction volume (approximately 175 to
504 185 µL) was concentrated using a SpeedVac (ThermoFisher) to approximately 30 µl and was
505 re-quantified (Qubit 2.0, Invitrogen). From the concentrated samples with a minimum of 0.06 ng
506 μL^{-1}, 10 µL was aliquoted for sequencing. Samples for sequencing were prepared using a
507 Nextera library kit and sequenced on the NextSeq500 platform via a 150 bp paired-end run.

508 Genomes for previously cultured strains HIMB109 and HIMB123 (Brandon 2006) were
509 sequenced by the Joint Genome Institute. Multiple methods were used to sequence these two
510 strains, including directly using 200 µL of cell culture for library prep as well as using multiple
511 volumes (5, 10, or 20 µL) of culture for multiple displacement analysis (MDA) prior to library
512 preparation. The genomes were evaluated based on completeness, length, number of reads, and
513 total contigs post assembly using SPAdes (Bankevich et al. 2012). An additional assembly using
514 all reads generated from various sequencing attempts per genome was also constructed using the
515 same assembly method, the highest quality genomes based on the metrics above were manually
516 curated and used for additional analyses.

517 **Genome assembly and assessment**

518 Short reads were trimmed with Trim Galore!
519 (https://github.com/FelixKrueger/TrimGalore) and assembled using Unicycler (Wick et al. 2017),
520 which acts as a SPAdes (Bankevich et al. 2012) optimizer with Illumina short read data. Once
521 assembled, reference indexes were built, and read mapping was performed using Bowtie2 with

522 default parameters (Langmead and Salzberg 2012). SAMtools (Li et al. 2009) was used to
523 convert the SAM file to a sorted and indexed BAM file. These initial assemblies and BAM files
524 were used to visualize genomes in anvi'o to check for possible contamination (Eren et al. 2015,
525 2021). For genomes with contamination, (determined visually as instances where contigs had
526 anomalous GC content or tetranucleotide frequency), suspicious contigs were removed.
527 Redundancy was also used as a way to flag any genomes that needed further curation. After
528 inspection, curated contigs were exported using the program 'anvi-summarize' and reads were
529 re-mapped to the cleaned version of assemblies. The cleaned genomes were processed again for
530 visualization in anvi'o to ensure no erroneous contigs were included. Mapping quality was
531 inspected visually using the Integrative Genomics Viewer (IGV) (Robinson et al. 2011) and
532 Tablet (Milne et al. 2013) and manual curation was undertaken using mapped read data. Manual
533 inspection was used to determine if a circular genome could be considered closed and complete.
534 All contigs shorter than 1000 bp were removed from the genomes that were not closed after final
535 curation, and anvi'o was used to assess final genome completeness and redundancy (Eren et al.
536 2021).

537 **Phylogenomic analyses**

538 To generate a comprehensive phylogeny of the SAR11 clade, a suite of high-quality
539 genomes were curated. Even with an abundance of metagenomes, the high diversity among
540 SAR11 populations makes constructing reliable MAGs currently unfeasible, so to ensure the
541 phylogeny was as robust as possible, only isolate genomes and SAGs were included. The final
542 set of 493 SAR11 genomes for phylogenetic reconstruction included 81 genomes sequenced in
543 this study, 25 previously published reference genomes, and 387 previously published single

544 amplified genomes (SAGs), in addition to 20 isolate genomes from the family Rhodobacteraceae
545 that were used as an outgroup (Supplemental Table 3). The majority of SAGs included were
546 equal to or greater than 85% complete according to checkM (Parks et al. 2015). However,
547 genomes of lower quality from subclades of SAR11 with no high-quality representatives were
548 included to produce a comprehensive phylogeny, for example SAR11 Ic genomes that ranged
549 from 56.0 to 93.7 percent completion were also included (Thrash et al. 2014) (Supplemental
550 Table 3). Both previously identified subgroup V and IV genomes were excluded from these
551 analyses as subgroup V is not considered to be within SAR11 and the inclusion of subgroup IV
552 has not been rigorously investigated and thus its relationship to the *Pelagibacterales* is
553 questionable (Thrash et al. 2011; Viklund et al. 2013; Haro-Moreno et al. 2020; Muñoz-Gómez
554 et al. 2022).

555 We compared two gene sets to determine the most appropriate genes to use for
556 phylogenetic reconstructions of the SAR11 clade. This included the bac120 gene set utilized by
557 GTDB-Tk to determine the bacteria guide tree, and a curated gene set of marker genes derived
558 from the 200-genes previously demonstrated to be best fit for the *Alphaproteobacteria*
559 (Muñoz-Gómez et al. 2019) (Supplemental Table 4). To curate the second gene set, we generated
560 a custom HMM profile for the 200 *Alphaproteobacteria* genes with a noise cutoff term of
561 1×10^{-20}, ran the HMM profile on all genomes using the anvi'o program `anvi-run-hmms`, and
562 generated a presence-absence matrix of genes in this model across genomes using the program
563 `anvi-script-gen-hmm-hits-matrix-across-genomes`. After evaluating the model hits across the
564 genomes matrix, we removed the genes that occurred in less than 90% of the genomes or those
565 that were redundant in more than 2% of the genomes from the *Alphaproteobacteria* 200-gene

566 collection, which resulted in a new collection with 165 genes, which is referred to as the
567 `SAR11_165` throughout our study (Supplemental Table 4). To generate a concatenated
568 alignment of the genes of interest for downstream phylogenomic analyses, a custom HMM
569 source was generated that encompassed the SAR11_165 genes. The program
570 `anvi-get-sequences-for-hmm-hits` with the custom HMM source was then implemented to
571 extract and align genes of interest. The program trimAL 1.3 (Capella-Gutiérrez et al. 2009) was
572 then used to remove all positions that were missing in more than 50% of the genomes.
573 Phylogenies were generated with IQ-Tree v2.1.2 (Minh et al. 2020) with the best fit model
574 (LG+F+R10) chosen using ModelFinder (Kalyaanamoorthy et al. 2017) and 1,000 ultrafast
575 bootstraps. Phylogenies were rerooted appropriately in FigTree, and exported in NEXUS format
576 with the options selected to "Save as currently displayed" and "Include Annotations (NEXUS &
577 JSON only)". Once exported, phylogenies were then compared using the package phytools
578 (Revell 2024) in R (R Development Core Team 2011).
579 Once the extended phylogeny was established, a subset of SAR11 genomes was used to
580 generate a pruned phylogeny with the SAR_165 gene set. For this, we first used PyANI
581 (Pritchard et al. 2016) to dereplicate all genomes using 95% gANI as a cutoff, then excluded
582 SAGs that did not share at least 90% gANI with a neighboring genome, and finally included 10
583 genomes from the GTDB that spanned 10 families from the order *Rhodospirillales* as an
584 outgroup prior to recomputing the final phylogenomic tree as described above. The 95% ANI
585 dereplication cutoff was chosen to avoid read splitting during competitive read recruitment and
586 for any clusters in which isolate genomes were available, they were chosen as preferred
587 representatives.

588

589 **Classification and nomenclature**

590 The extended phylogeny was used to define cohesive genetic clusters at the distal end of
591 the SAR11 tree. Single genomes that did not share at least 90% ANI with a neighboring genome
592 were not classified into genera.

593 To determine how taxonomic levels across the SAR11 lineage would compare using
594 relative evolutionary distance, we implemented this approach as previously described (Ramfelt et
595 al. 2024). Briefly, a domain-level phylogeny was first constructed using the GTDB-Tk
596 de_novo_workflow (Chaumeil et al. 2019) with SAR11 isolate and SAGs as well as
597 “p__Chloroflexota" as the outgroup. Marker genes were identified from the input genomes using
598 GTDB-Tk `identify`, and then aligned with GTDB-Tk `align` (using the "–skip_gtdb_refs"
599 flag). Finally, a tree was constructed using FastTree v2.1.10 (model WAG+GAMMA) (Price et
600 al. 2010), rooted with the Chloroflexota outgroup. This phylogeny was used as the input for the
601 'scale_tree' program in PhyloRank v0.1.11 (https://github.com/dparks1134/PhyloRank) to
602 convert branch lengths into relative evolutionary distance (RED). RED values of 0.77 and 0.92
603 were used to assess how they would align with family and genus-level lineages, respectively.
604 These values were based on the distribution of internal nodes within the SAR11 clade and values
605 used previously for other family and genus-level lineages (Parks et al. 2018).

606

607 **Read recruitment**

608 To assess the distribution of the newly described strains described in this study and put
609 them into context with previously sequenced genomes, we used a read recruitment approach with

610 globally distributed metagenomes. The SAR11 genomes included in this study were grouped into
611 clusters that shared 95% average nucleotide identity (ANI) or greater and representatives from
612 these 95% gANI groups were then used for read recruitment (n = 314, Supplemental Table 12).
613 Results from read recruitment were extrapolated for the other genomes included in each 95%
614 gANI group.

615 Metagenomes used for recruitment included those sequenced in Kāneʻohe Bay (Tucker et
616 al. 2024b), the environment from which the genomes were isolated. Only samples from sites
617 previously categorized as "nearshore" and "offshore" (Tucker et al. 2024b) were used here.
618 Additionally, globally distributed previously published metagenomes were also used including
619 those from TARA Oceans expeditions (Sunagawa et al. 2015), station ALOHA (Mende et al.
620 2017), GEOTRACERS cruises (Biller et al. 2018), the eastern coast of Japan (Kudo et al. 2018;
621 Yoshitake et al. 2021), Monterey Bay (Mueller et al. 2015), and the ocean sampling day program
622 (Kopf et al. 2015) (Supplemental Table 6 for a list of appropriate references and details regarding
623 metagenomes included).

624 Once metagenomes were chosen, raw reads were downloaded using 'prefetch' and
625 'fasterq-dump' in the SRA toolkit. We automated the quality filtering of metagenomes,
626 metagenomic read recruitment, and profiling of recruited reads using the program
627 anvi-run-workflow (Shaiber et al. 2020) with the `--workflow metagenomics` flag, which
628 implements snakemake (Köster and Rahmann 2012) recipes for standard analyses in anvi'o.
629 Briefly, this workflow identified and discarded the noisy sequencing reads in metagenomes using
630 the program `iu-filter-quality-minoche` (Eren et al. 2013b), used SAR11 genomes to
631 competitively recruit short reads from metagenomes using Bowtie2 (Langmead and Salzberg

632 2012) SAMtools (Li et al. 2009) using the program `anvi-profile`, and finally merge individual
633 profiles into an anvi'o merged profile database using the program `anvi-merge`. The resulting
634 anvi'o merged profile database included essential data, including genome coverages and
635 detection statistics across metagenomes, for our downstream analyses. For coverage, we
636 primarily used the 'mean coverage Q2Q3' statistic, which represents the interquartile average of
637 coverage values where, for any given genome, the lowest 25% and the highest 25% of individual
638 coverage values are trimmed prior to calculating the average coverage from the remaining data
639 points, and thus minimizing the impact of biases due to highly conserved or highly variable
640 regions in the final coverage estimates. Visualization of read recruitment data mapped according
641 to the phylogeny constructed was completed using the program `anvi-interactive` with the
642 `--manual` flag.
643

644 **Metagenome profile clustering**

645 We performed a cluster analysis of metagenomes based on genome detection values from
646 the read recruitment step using the k-means algorithm, where we determined the `k` by
647 identifying the elbow of the curve of within-cluster sum of square values for increasing values of
648 `k` using the R code shared by Delmont et al. (2019) at https://merenlab.org/data/sar11-saavs/.
649 The results of the clustering analysis were visualized using anvi'o. To investigate how similar
650 detection patterns of genomes within genome clusters were, in addition to how similar or distinct
651 patterns were between genome-clusters, we performed a non-metric multidimensional scaling
652 (NMDS) analysis using the vegan package in R. Any metagenomes with zero detection across all

genomes were removed. The NMDS results were visualized using ggplot2 and plotly and an interactive plot was generated with ggplotly.

Investigation of C-P lyase pathway

All genomes included in the extended phylogeny (n=X) were searched using `anvi-search-functions` for the key enzyme in the C-P lyase pathway (*phnJ*) to determine the capacity among high-quality SAR11 genomes to utilize the pathway. The genes upstream and downstream of this essential gene were extracted from all 57 genomes and a pangenome was used to compare the presence and absence of other key genes in the pathway as well as the synteny of this region of the genome. The *phnJ* phylogeny (Supplemental Fig. 6) does not reflect the relationships among genomes as demonstrated by the SAR_165 phylogeny (Fig. 1), which is further evidence that this gene is located on a genomic island as previously described (Molina-Pardines et al. 2023).

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Competing interests

The authors declare no competing interests.

Data availability

The assembled sequence data for genomes reported here are available at FigShare at https://doi.org/10.6084/m9.figshare.28087454.v1.

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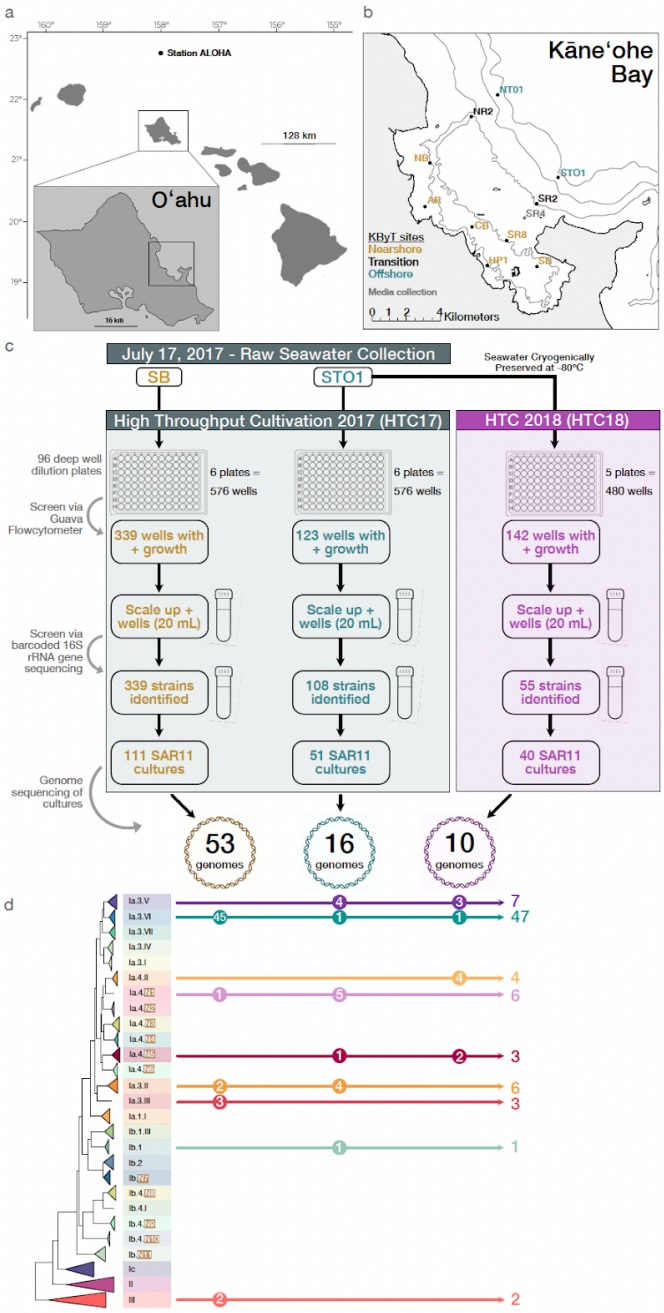
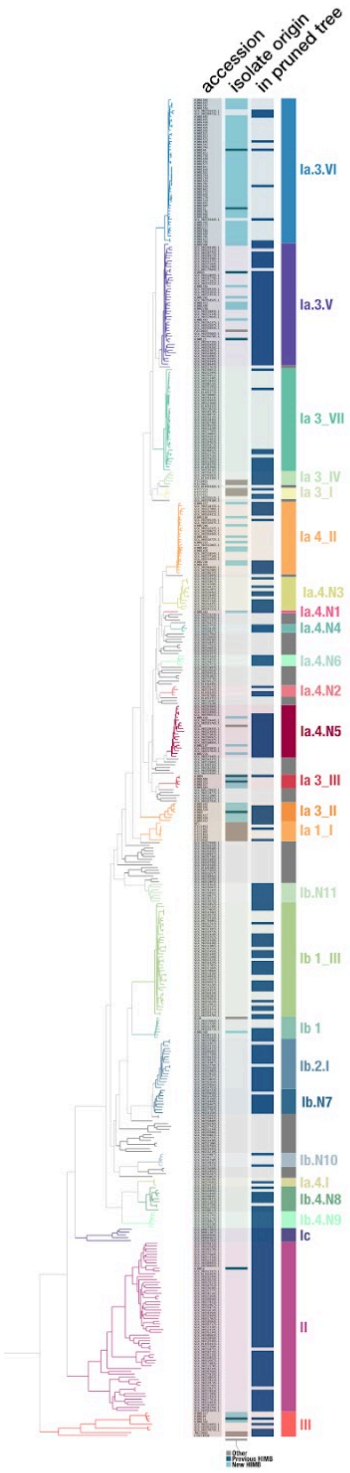
Supplemental Figures

All supplemental figures are available on FigShare at:

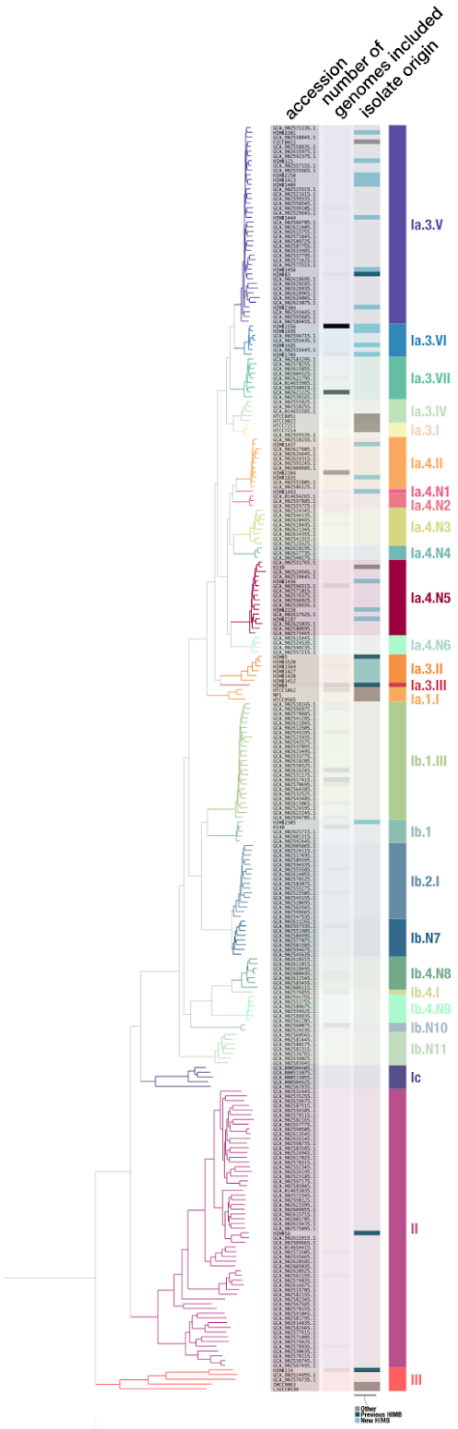
https://doi.org/10.6084/m9.figshare.28087760.



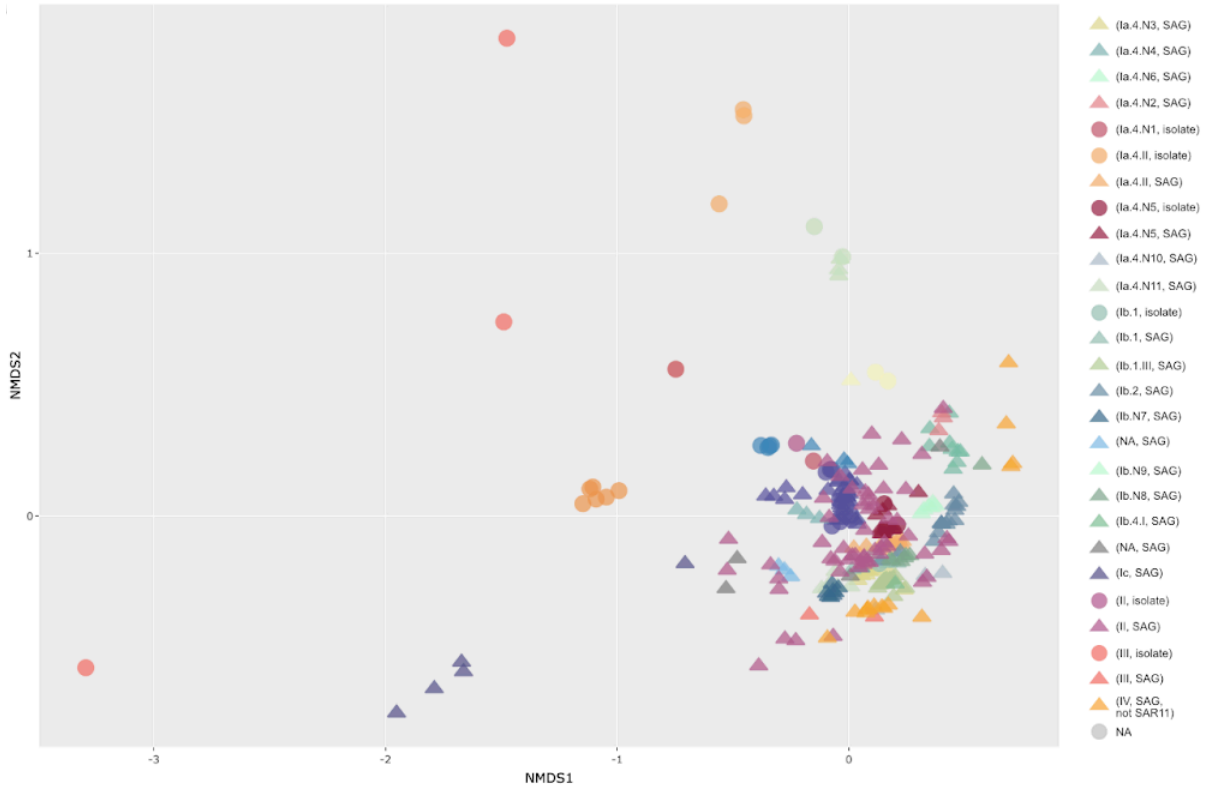
Supplemental Figure 1. **Sampling sites used for high-throughput culturing experiments. (a)** Location of O'ahu in the Hawaiian archipelago in relations to Station ALOHA approximately 100 km north. **(b)** Map of the embayment on the windward side of O'ahu with sites included in the Kāne'ohe Bay Time-series with sites classified as 'nearshore' (orange text), 'transition' (black text), or 'offshore' (turquoise text). Site SR4 in gray from which seawater media was collected for the cultivation experiments is also indicated. Bathymetry lines are approximate. **(c)** Flowchart outlining the high throughput cultivation (HTC) experiments conducted in 2017 and 2018 leading to the isolation of hundreds of SAR11 cultures and 79 new SAR11 isolate genomes. **(d)** Schematic SAR11 phylogeny to indicate which samples harbored genomes from which subgroups.



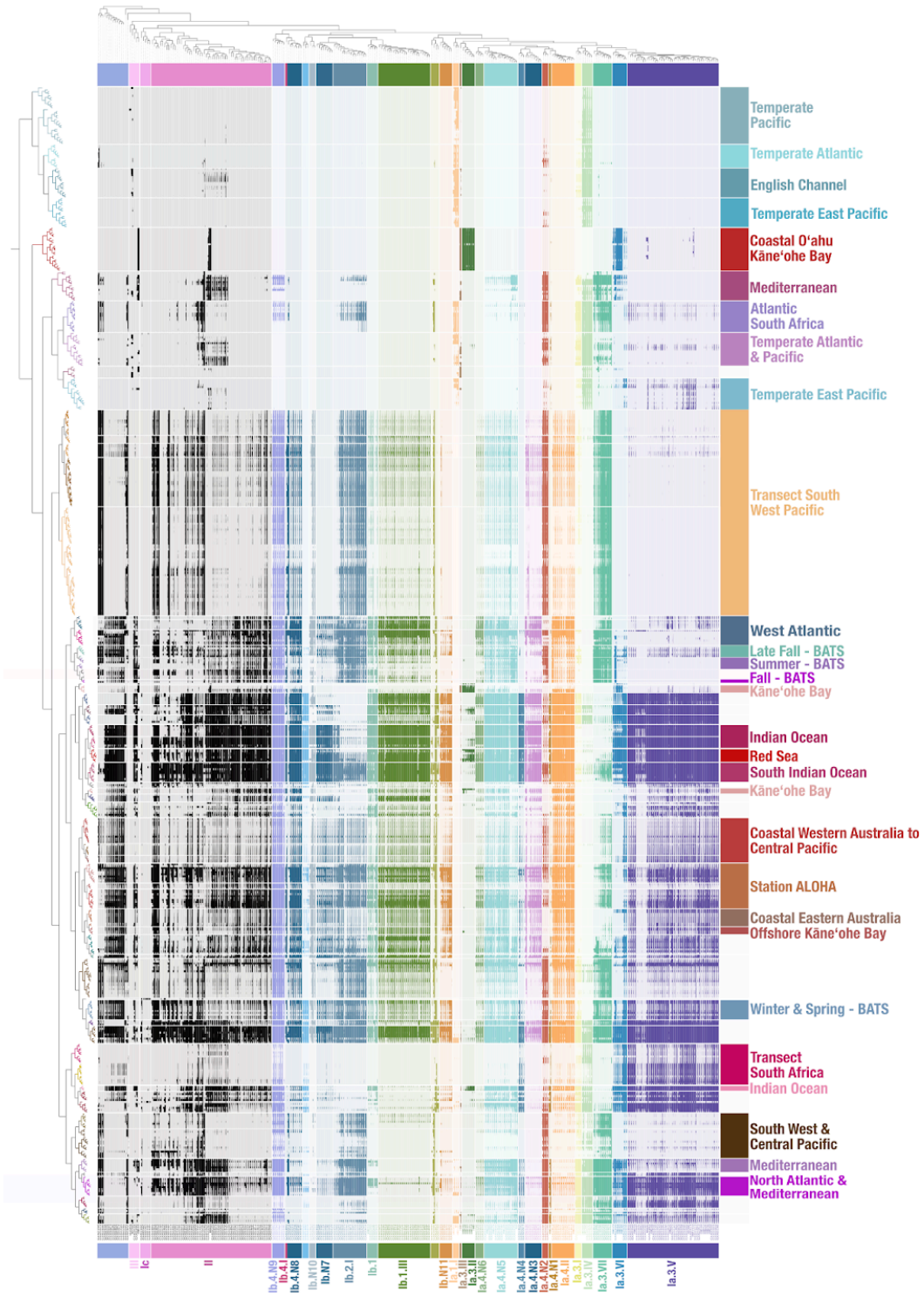
Supplemental Figure 2. Phylogenomic tree of all 481 *Pelagibacterales* genomes initially included. Of the 481 SAR11 genomes 106 were isolates and 375 SAGs and the phylogeny is based on a curated SAR11-specific set of 165 genes. Isolate origin is indicated and indicates if the genome was from this study, a previous isolate from Kāneʻohe Bay, or from another source. Genomes included in the pruned tree are also indicated.



Supplemental Figure 3. Pruned phylogenomic tree with 268 *Pelagibacterales* genomes. Of the 268 genomes 50 were isolates and 218 SAGs based on a curated SAR11-specific set of 165 genes. Isolate origin is highlighted on the tree and indicates if the genome was from this study, a previous isolate from Kāneʻohe Bay, or from another source. Number of additional genomes in the same 95% gANI cluster are indicated as well by intensity of the bar which range from 0 to 45.



Supplemental Figure 4. NMDS of genomes with detection data. All of the genomes included in the analysis are included here, with distinction between isolate genome (circle) or SAG (triangle) indicated. Genomes not designated a subgroup noted as NA.



Supplemental Figure 5. Read recruitment data including all metagenomes included in cluster analysis of metagenomes based on genome detection values using the k-means algorithm. Subgroups are indicated at the bottom of the figure with labels indicating the metagenome groups along the right hand side of the figure.

Supplemental Figure 6. Phylogeny of the *phnJ* gene for all *Pelagibacterales* genomes in the pruned phylogeny data set.

Supplemental Table Legends

All supplemental tables are available on FigShare at:

https://doi.org/10.6084/m9.figshare.28087490.v1.

Supplemental Table 1. Summary of the 16S rRNA gene amplicon data from HTC17 and HTC18.

Supplemental Table 2. Detailed information for the isolate genomes reported in this study. The genome summary information originated from checkM (v1.1.2). *Indicates the genome has been manually verified to be completely closed.

Supplemental Table 3. Summary statistics for all genomes used for analyses in this study. This includes isolates reported here, previously published isolate genomes, and high-quality single amplified genomes (SAGs) used in the extended SAR11 phylogeny. The genomes used in read-recruitment are indicated. *Indicates the accession is the IMG Genome ID not the NCBI Accession.

Supplemental Table 4. Gene sets evaluated for use in SAR11 phylogenetics. The sets evaluated include the bac120 (Parks et al., 2018) and a subset of 165 of the genes (SAR11_165) delineated for the Alphaproteobacteria (Wang and Wu 2013; Muñoz-Gómez, 2019).

Supplemental Table 5. Summary of average genome statistics for the 23 genera established in the *Pelagibacteraceae* as well as the Ic, II, and III families.

Supplemental Table 6. Studies from which metagenomes were sourced.

Supplemental Table 7. List of all metagenomes used for read recruitment and accession numbers.

Supplemental Table 8. Detection values across genomes from all metagenomes used in read recruitment.

Supplemental Table 9. Average detection across genome cluster for bins in Fig 3.

Supplemental Table 10. Coverage values across genomes from all metagenomes used in read recruitment.

Supplemental Table 11. Type genomes and classification hierarchy for the *Pelagibacterales* including proposed naming schemes.

Supplemental Table 12. All of the final 95% ANI clusters defined including the cluster number, the final representative genome for that cluster and the list of other genomes in the same cluster.