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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Metagenomics approaches in the study of hypersaline microbial
communities**

A dissertation submitted in partial satisfaction of the
requirements for the degree
Doctor of Philosophy

in

Marine Biology

by

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2014

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Chair

University of California, San Diego

2014

DEDICATION

To two, the loneliest number since the number one.

EPIGRAPH

A beginning is the time for taking the most delicate care that the balances are correct.

—Frank Herbert, *Dune*.

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ABSTRACT OF THE DISSERTATION

**Metagenomics approaches in the study of hypersaline microbial
communities**

by

Juan A. Ugalde Casanova

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2014

Dr. Eric E. Allen, Chair

This dissertation will be abstract.

Chapter 1

Introduction to the thesis

Bacteria and Archaea represent an abundant component of Earth's biomass, with estimates between $9.2\text{-}31.7 \cdot 10^{29}$ cells [38] to $41.8\text{-}64.3 \cdot 10^{29}$ cells [96] globally. Species diversity estimates suggest that there are close to 10^7 different species of Bacteria and Archaea[18], although some authors suggest this number is an over-estimation [77]. Nevertheless, Bacteria and Archaea represent the most diverse group of organisms on Earth, and yet we have scarcely begun to characterize this diversity [101, 73].

Our inability to cultivate most of the microorganisms present in the environment limits our understanding of the phylogenetic and functional diversity of microbial communities. Known as the "Great Plate-Count Anomaly" [84], our current culture collections may not be representative of what can be observed in natural samples by culture-independent methods [3]. Most organisms are currently uncultured, due to our lack of information on their physiology and nutritional requirements [85]. With new and unique cultivation methods, and information derived from genomic surveys [88], we may be able to culture these organisms in the near future.

Over the last decade, the development of advanced technologies and experimental methods has allowed the study of natural microbial communities via culture-independent techniques. Specifically, DNA-based methods, driven by the development of next-generation sequencing [51], have allowed the investigation of natural microbial communities without relying on cultivation. DNA-based surveys

span two broad categories: marker-based analysis (such as the 16S rRNA gene) and whole genome sequencing.

Marker-based analysis provides a characterization, or census, of the phylogenetic diversity present in the community, allowing for estimation of community diversity [14, 71, 13] and identification of community members based on sequence similarity against a reference database [54]. This approach provides only a partial picture of the metabolic repertoire present in natural microbial communities, as it requires extrapolation of genomic data from isolated microorganisms to the entire community. Even strains with very similar 16S rRNA sequences may have different metabolic and genetic properties, which are not captured in the diversity found using a marker gene such as the 16S rRNA gene [35].

Metagenomics, also referred to as community genomics, relies on the direct sequencing of genetic material isolated from a microbial community *en masse* [100], and provides the means to evaluate the phylogenetic and functional diversity of microbial populations directly from the environment. Depending on the complexity of the community under study, not all of the members of the microbial community will be observed in the sequence data, as the most abundant members will dominate the metagenomic analyses [9]. Using metagenomic methods, several authors report studies of microbial communities with low to moderate species diversity (e.g. [89, 69, 22, 87, 12]). Highly diverse communities, such as those found in soils and sediments, require high amounts of sequence information to provide a full picture of the microorganisms that are present [37]. The decrease in sequencing cost, driven by the development of novel sequencing technologies, has enhanced our ability to deeply sample complex communities using metagenomics approaches. Consequently, the challenge will be developing or acquiring the computational resources and algorithms necessary to analyze vast amounts of genetic sequence information [67, 37].

Microbial communities in extreme environments, such as those associated with hypersaline habitats, represent tractable systems that can be studied using metagenomic approaches. Because of their relatively low species diversity [5, 69, 31], it is possible to study the phylogenetic and metabolic diversity that is present in

the community. In this first chapter, I provide an overview of metagenomics, with particular emphasis in assembly-based approximations, followed by an introduction into the microbiology of hypersaline environments and finally an overview of our study site, the hypersaline waters of Lake Tyrrell located in Victoria, Australia.

1.1 Metagenomics

The study of natural microbial communities by analyzing DNA obtained directly from environmental samples was first proposed by Handelsman *et al.* in 1998 [32] to access the genomic information of uncultured environmental microorganisms. The first metagenomic studies were performed by isolating DNA from environmental samples, plasmid-based cloning of this DNA and subsequence sequencing of these clones using the Sanger method [87, 92]. Limited throughput, cloning biases, and the expensive and laborious nature of this process limited the scope of early metagenomic studies, often focused on microbial communities with low to medium species diversity [87, 69], or to studies aimed at describing the general functional and phylogenetic composition of a community [92, 76]. The development of *next generation* sequencing technologies [52], removed some of these limitations, allowing for direct sequencing of DNA samples without cloning, higher throughputs, and a lower cost per base [52]. One of the remaining limitations in current technologies is the length of the sequence reads, which are not yet close to the length of reads generated by Sanger sequencing. Improvements to current methods and the development of further advanced technologies, such as single molecule sequencing [24] and nanopores [10], holds promise to further alleviate these limitations.

Metagenomic studies are driven, in most cases, by discovery, data mining and comparative research, rather than by a specific hypothesis [100]. Accordingly, both the sequence information and the contextual environmental data, or metadata, associated with a sample are of great importance, allowing for both biological and physicochemical context to be applied to the microbial community under study. These data include environmental parameters associated with a sample and

procedures used to process a sample (e.g. filtration procedure, sample processing and library construction). All of these variables become highly important in the bioinformatic analysis of a sample data set[58].

Based on the complexity of the microbial community under study, and the choice of sampling and sequencing methods, two main approaches are used to study microbial communities using metagenomics: gene-centric and assembly-based approaches (Figure 1.1). Gene-centric studies focus on complex communities or projects with a shallow level of sequencing, where assembly of larger contiguous genome fragments from community members is not feasible given the number of reads or size of the data set obtained. . In these cases, the focus is on the phylogenetic and functional diversity as a profile of the community [89, 12], and the comparison of different environments based on these profiles [22, 99, 72]. Gene-centric studies focus on the phylogenetic and functional classification of the metagenomic reads, either by analyzing every read present in the dataset [8, 65, 4], or by using marker-genes as a proxy to create either taxonomic and/or functional profiles of the community [19, 80]. One main drawbacks of this method is that community profiles are limited by the reference databases being used, which can lead to missing unique groups that are present in the microbial community under study.

Assembly-based metagenomics, involves the bioinformatic assembly of the community sequence information, with the goal of recovering larger contiguous genome fragments that can improve the phylogenetic and functional classification of the organisms in the community. This approach allows the recovery of longer fragments, generate better gene models and can identify previously unknown microbial groups [58, 69, 39, 21]. The information obtained from the assembled population genomes can be used to potentially guide cultivation efforts for these organisms [88], in population genetic studies [83, 95, 2], and provide a better picture of the interactions among the members of the microbial community [87, 28]. In addition, the recovery of near-complete genomes from metagenomics datasets allows for the discovery of novel functions that may be missed by viewing the unassembled sequence reads. For example, Chapter 2 of this thesis shows how the

assembly of sequence reads from the Lake Tyrrell metagenome led to the discovery of a previously unknown Phylum of Archaea and to the discovery of a novel family of microbial rhodopsins, called the xenorhodopsins, and evidence that suggests its acquisition through horizontal gene transfer (Chapter 3).

Assembly-based methods are limited by the complexity of the microbial community under study, and the amount of sequencing needed to recover the genomes of the most abundant members of the community. Furthermore, some of the more rare members of the community are inaccessible by sequencing, because the most abundant organisms dominate the metagenomic dataset. For example, estimations of the number of sequences needed to address highly diverse samples, such as from soils, show that billions of sequences are needed to be able to sample some of these most abundant organisms [33]. Even in simpler systems, the main challenge is to classify the assembled genomic fragments into unique phylogenetic bins, each representing a different population. By combining various pieces of information, such as sample characteristics, nucleotide composition, amino acid counts, and library abundance, it is possible to classify these genomic fragments into unique populations, a process known as phylogenetic binning [69, 1]. Larger datasets represent a computational challenge because of the high memory requirements of short-read assembly software programs. In this case, the use of methods for digital normalization may reduce the computational problems [67, 37].

The work presented in this thesis describes a study of the microbial community inhabiting the hypersaline waters of Lake Tyrrell, Australia using an assembly-based approach. The combination of a relatively low-species diversity, driven by the extreme conditions found in this habitat [5], and a deep sequencing approach, allowed for the genomic reconstruction of some of the most abundant members of the community and the discovery of novel microorganisms.

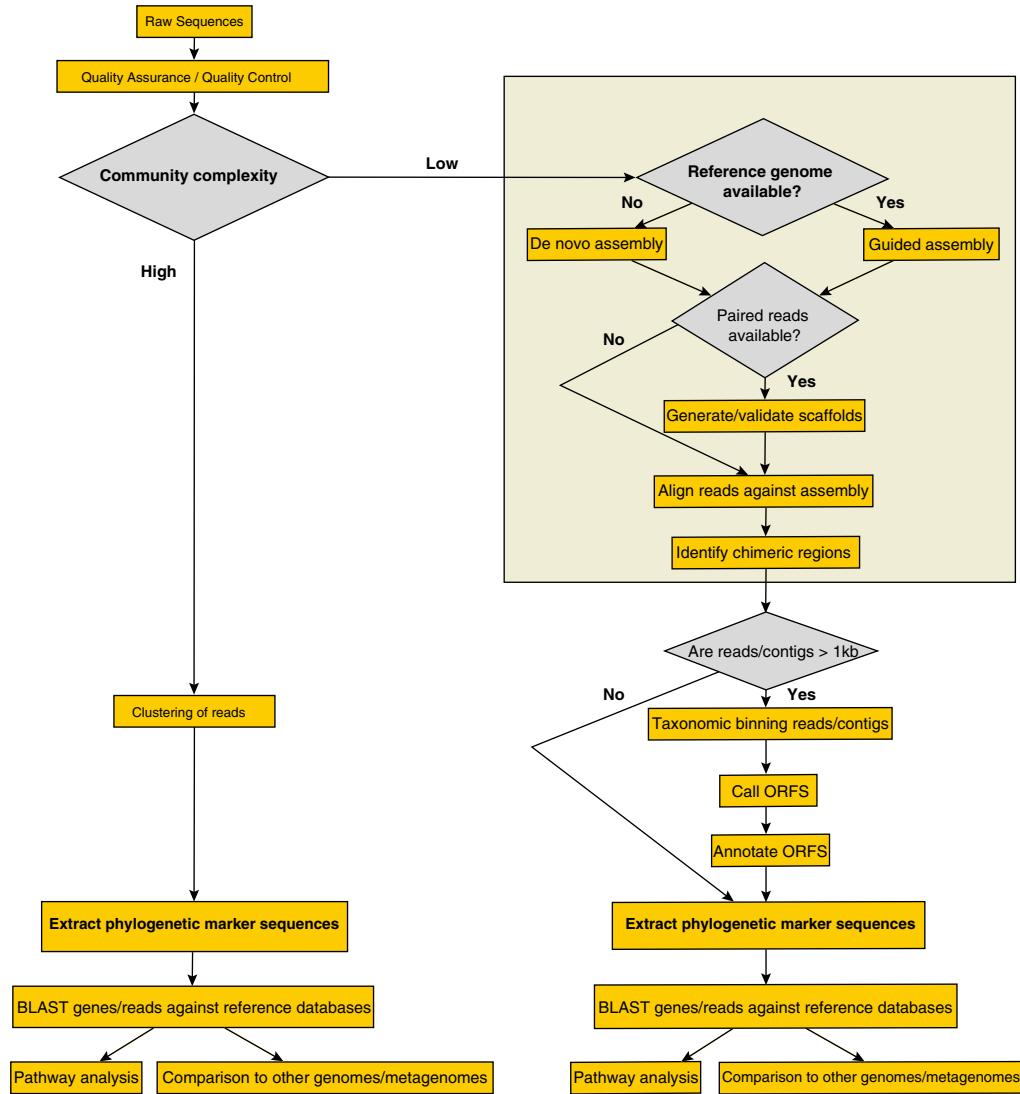


Figure 1.1: Diagram comparing two possible approaches for the analysis of metagenomic data from natural microbial communities. Figure from Bragg and Tyson, 2014 [9].

1.2 Microbial communities in hypersaline environments

Hypersaline habitats are found worldwide, in a variety of natural and man-made environments. Examples include salt lakes, saline soils, salt ats, solar salterns, brine pools, salted foods, and fermented foods [59]. Aquatic hypersaline systems are the most studied, and are either of marine origin (thalassohaline), or formed by the dissolution of mineral salt deposits (athalassohaline).

Within these saline systems, a variety of microbial species are adapted to these environments. Moderate halophiles can beare found between 30-150 g/L NaCl, while extreme halophiles exist in the range of 150-300 g/L NaCl [5]. In addition to the high NaCl concentrations, other salts are also important to consider when measuring the ionic composition of these systems, including Mg²⁺ and Ca²⁺, which can inuence the microbial community in these habitats [55, 68] (Figure 1.4).

Genera from both the Bacteria and the Archaea exist in moderate and extreme saline systems. Within the Archaea, the phylogenetic diversity appears to be limited to the *Euryarchaeota*, specifically in the classes *Halobacteria* and *Methanomicrobia* (Table 1.1) [93]. The discovery of the *Nanohaloarchaea* (described in Chapter 2, and [58]), a novel Class, within the Euryarchaeota,, in globally dispersed hypersaline systems expanded our understanding of the phylogenetic diversity of halophilic Archaea [58]. Recently, a phylogenomic analysis of novel archaeal groups, isolated via single-cell genomics, suggested that the *Nanohaloarchaea* are a new phylum, sister to the *Euryarchaeota* [73], although more work (and more genomes and isolates) is needed to fully resolve the phylogenetic relationships between these groups [97].

Even within the *Halobacteria*, novel taxonomic groups remain unidentified. Our group recently described the genome of a newly isolated halophilic Archaea, *Candidatus Halobonum tyrrellensis* strain G22 [90], which phylogenetic analysis suggests is a new genus within the *Halobacteria* (Appendix A). Analysis of the 16S rRNA gene, and a phylogenomic approach using the markers genes implemented in the software, PhyloPhlan, has further supported this phylogenetic placement

(Figures 1.2 & 1.3).

The phylogenetic breadth of bacterial species found in saline systems is wider than that of Archaea (Table 1.2). In moderate saline environments, Bacteria are more abundant than Archaea [60, 31, 30, 15], but as salinity increases, Archaeal groups become more abundant [31, 15, 57]. One of the most abundant bacterial species found in extreme hypersaline systems is *Salinibacter ruber* [6]. This bacterium shares many phenotypic characteristics with halophilic Archaea [64], and multiple gene clusters appear to have been acquired via horizontal gene transfer from the Archaea [56].

Viruses are also very abundant in hypersaline systems, with reports of counts of at least 10^7 viral-like particles per mL [23]. Viruses could be playing a dual role in these systems; as predators, contributing to the cycling of nutrients through cell lysis; and as a form of information storage, by providing access to an auxiliary gene pool that can be utilized by other microorganisms [47, 98, 34]. Studies of viral dynamics in hypersaline systems have showed that they play a fundamental role in shaping the population structure of microbial communities [74, 75].

Microorganisms that live in hypersaline systems require particular physiological strategies to deal with the high salt concentrations and the potential osmotic pressure gradient that can generate across the cell membrane. Two different osmotic adaptation strategies can be found in halophiles: a salt-in strategy, which involves the accumulation of inorganic ions in the cytoplasm; and a salt-out strategy, that involves pumping ions out the cell and the accumulation of compatible solutes in the cytoplasm [63]. The salt-in strategy is found in the archaeal populations, specifically within the Order *Halobacteriales*, in Bacteria of the Order *Halanaerobiales* and in *S. ruber* [63]. These organisms accumulate inorganic ions, such as K^+ and Cl^- , which requires special enzymatic adaptations reected through protein amino acid compositional changes that favor acidic amino acids, such as glutamate and aspartate. Based on their amino acid composition profiles, it has been suggested that the uncultured members of the Class *Nanohaloarchaea* also utilize this strategy. The salt-out strategy is found in most of the halophilic Bac-

teria and halophilic methanogenic Archaea [63]. These organisms have outward-directed sodium transporters that pump the Na^+ ions out of the cytoplasm, but more importantly, they accumulate a large number of organic solutes to maintain the osmotic potential in the cytoplasm [63].

Halophilic microorganisms show a diverse suite of metabolic capabilities. Several dissimilatory metabolic pathways have been described in halophiles (Figure 1.5) across a wide range of salinity concentrations. The majority of bacterial and archaeal groups isolated from hypersaline sites are aerobic chemoorganotrophs. In addition, as oxygen has a low solubility in salt saturated brines [82], several anaerobic energy pathways are available, including electron acceptor substrates such as nitrate, fumarate, and dimethyl sulfoxide [60, 63]. Primary productivity in saline systems varies according to the salinity. In moderate saline environments (between 100 to 250 g/L), primary productivity occurs in microbial mats dominated by members of the *Cyanobacteria*; in high salt environments, the primary producers are planktonic algae of the genus *Dunaliella* [61].

Another important characteristic of halophilic organisms, particularly among the halophilic Archaea, is the presence of microbial rhodopsins, photoactive proteins found in all three domains of life. Rhodopsins serve as light-driven proton pumps (bacteriorhodopsin), chloride pumps (halorhodopsin), or phototactic and photophobic receptors (sensory rhodopsins) [11]. Halorhodopsins and sensory rhodopsins appear to be limited in their distribution to the *Halobacteria*, with just a few examples found in other organisms [81]. Chapter 3 describes the discovery a novel type of microbial rhodopsin, called the xenorhodopsin, which was found in the genome of the *Nanohaloarchaea*, and that appear to broadly dispersed via horizontal gene transfer between Archaea and Bacteria (although it is not possible yet to establish the directionality of acquisition). Indeed, the closest homolog to the Nanohaloarchaea xenorhodopsin is a putative sensory rhodopsin found in the cyanobacterium *Anabaena* sp. PCC 712 [94, 91].

Even in highly characterized microbial communities [60, 62]], recent studies using culture independent techniques, such as metagenomics and single-cell genomics, recovered novel microbial groups [58, 31, 49]. The limited microbial

diversity, driven by extreme salinity systems, makes them ideal model systems to study microbial diversity using metagenomic methods. We can fully characterize a microbial community using deep-sequencing approaches, with the goal to reconstruct the genomes of each member of the community. Through this approach, we identify not only the functional and phylogenetic diversity of the community, but also the association of such functions to members of the community. Lastly, it allows for the study of population genetics within the community, with the goal of understanding how these organisms adapt and respond to variations in the environment.

It is important to highlight that other saline ecosystems have been studied using metagenomic approaches. Prior hypersaline metagenomic studies have focused on the population genomics of single species, such as *Haloquadratum walsbyi* [44] and *Salinibacter ruber* [66], or on the dynamic changes of the microbial and viral diversity over salinity gradients and over time [99, 74]. Only recent studies have explored the microbial and viral diversity of these systems by using assembly-based metagenomics and single-cell genomics approaches [58, 69, 31, 30, 25, 26].

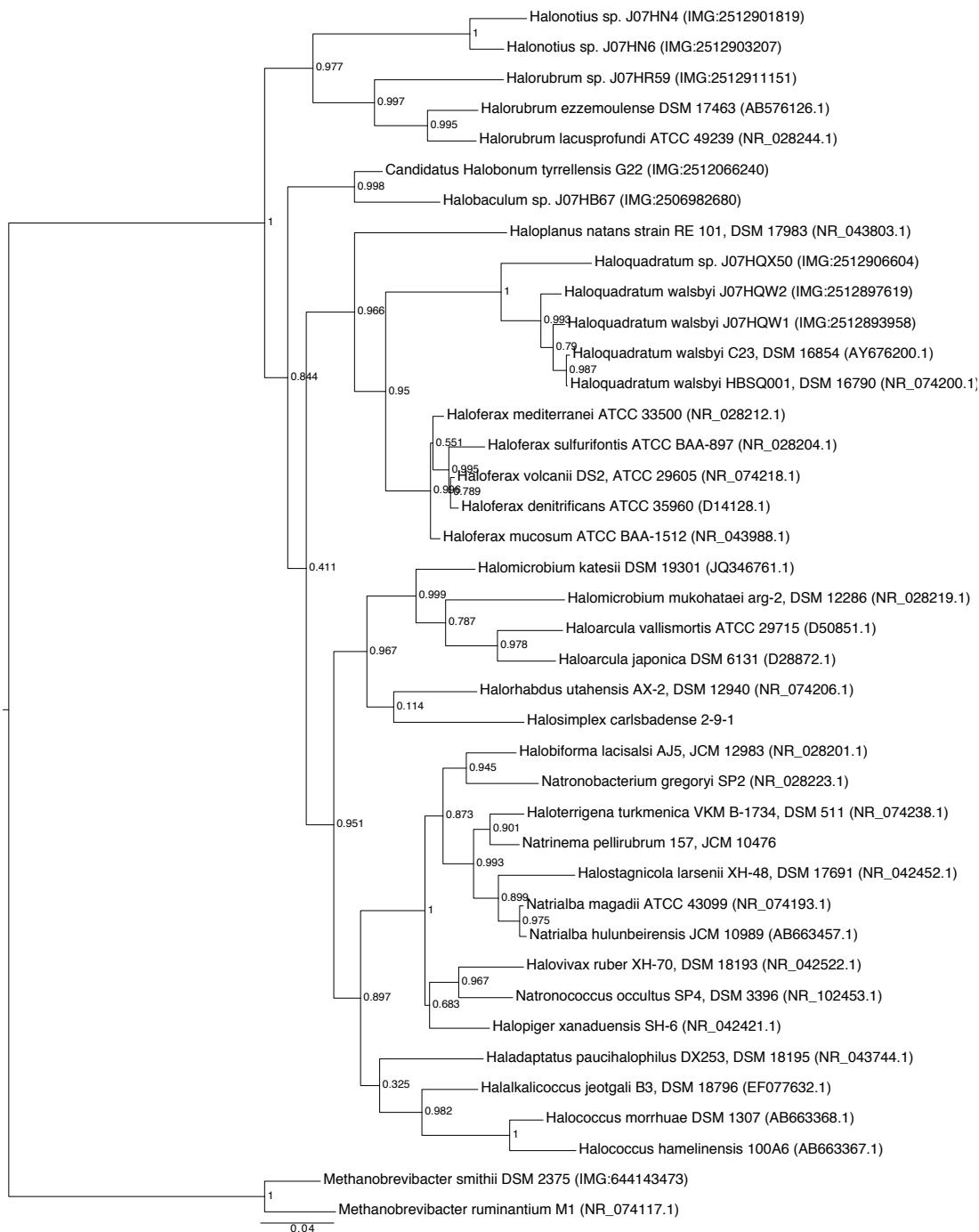


Figure 1.2: Phylogenetic tree of *Candidatus Halobonum* tyrrellensis G22 and related microorganisms, based on 16S rRNA sequences.

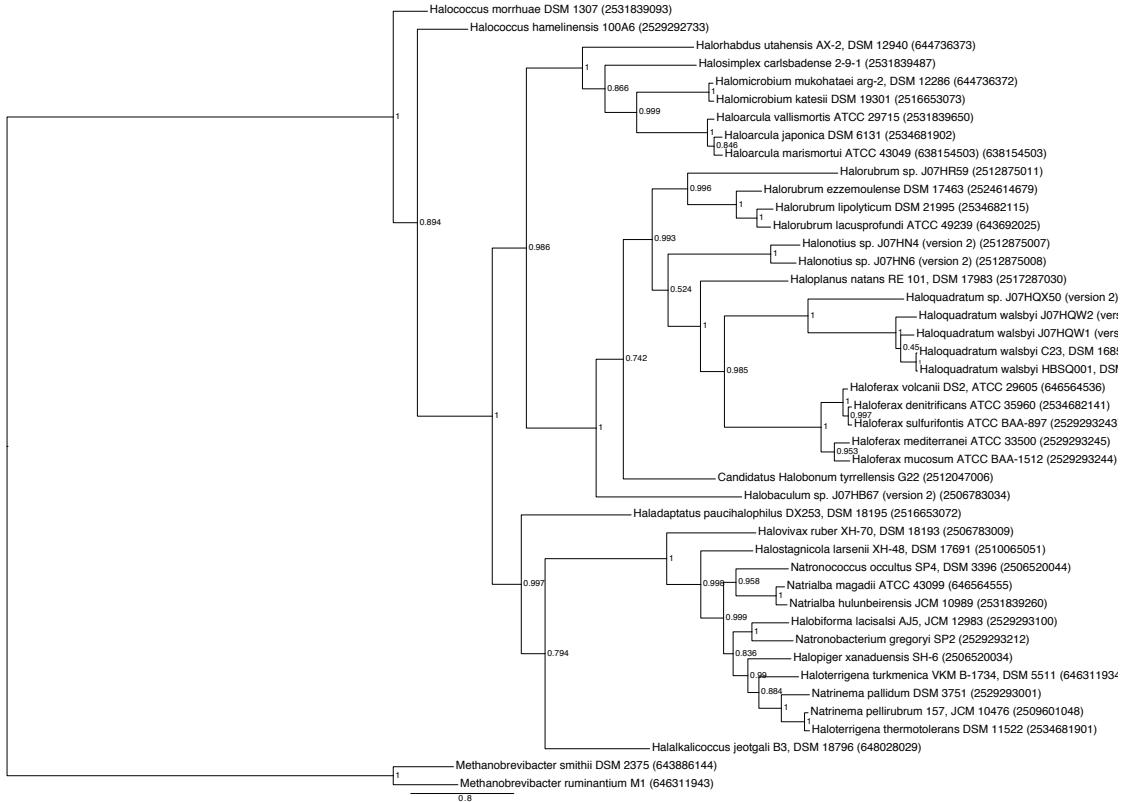


Figure 1.3: Phylogenetic tree of *Candidatus Halobonum tyrrellensis* G22 and related microorganisms, based on phylogenetic marker sequences implemented in the Phylophlan software [79]

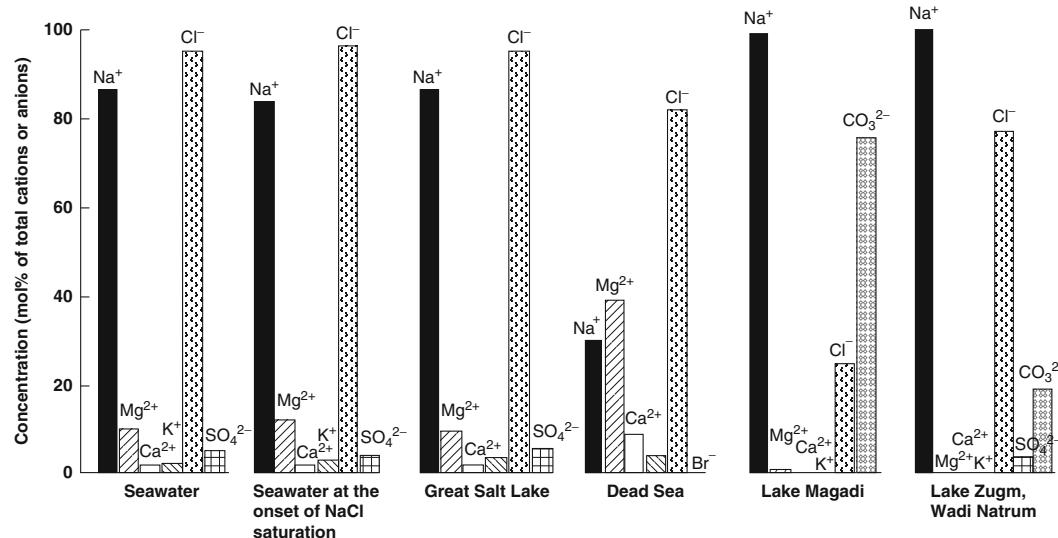


Figure 1.4: Ionic composition of several aquatic systems. Figure from Oren, 2013. [63]

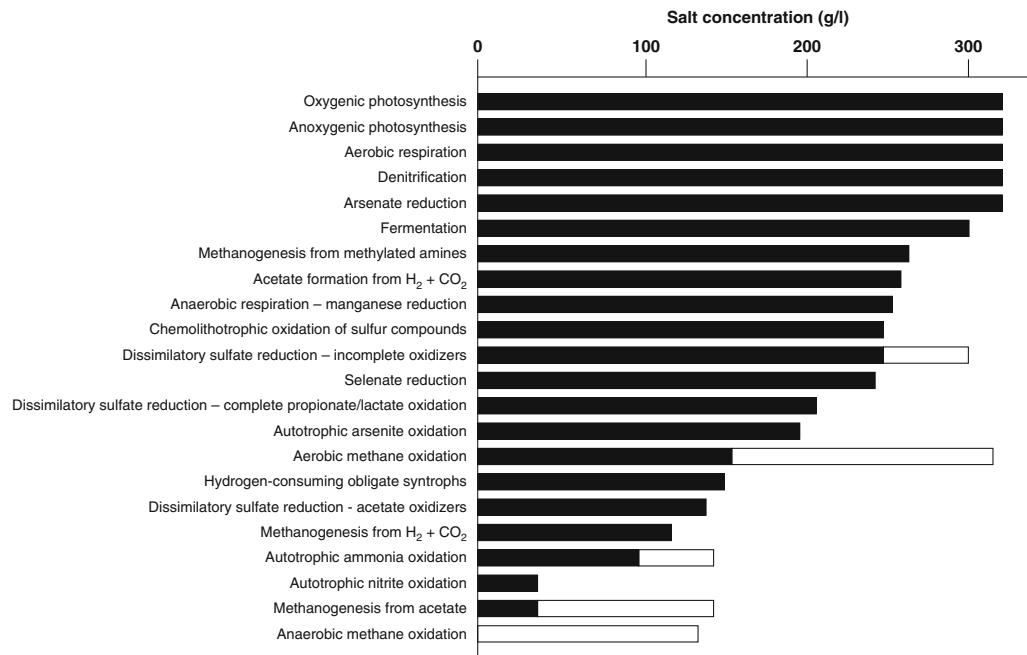


Figure 1.5: Salt concentration limits for some microbial metabolic processes. Black bars indicate information based on laboratory studies, while white bars indicate activities measured in natural microbial communities. Figure from Oren, 2013 [63].

Table 1.1: Halophilic Archaea, modified from [93] to include the recently discovered *Nanohaloarchaea* [58].

Phylum	Class	Genera
<i>Euryarchaeota</i>	<i>Halobacteria</i>	<i>Halobacteriia, Haladaptatus, Halalkalicoccus, Halarchaeum, Haloarcula, Halobaculum, Halobiforma, Halococcus, Haloferax, Halogeometricum, Halogramnum, Halomicrombium, Halonotius, Halopelagius, Halopiger, Haloplanus, Haloquadratum, Halorhabdus, Halorubrum, Halorussus, Halosarcina, Halosimplex, Halostagnicola, Haloterrigena, Halovivax, Natrionalba, Natrinema, Natronoarchaeum, Natronobacterium, Natronococcus, Natronolimnobiust, Natronomonas, Natronorubrum, Salarchaeum</i>
<i>Methanomicrobia</i>		<i>Methanohalobium, Methanocalculus, Methanohalophilus, Methanosalsum</i>
<i>Nanohaloarchaea</i>		<i>Nanosalina, Nanosalinarum</i>

Table 1.2: Halophilic Bacteria, modified from [93].

Phylum	Class	Genera
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinopolyspora, Amycolatopsis, Georgenia, Corynebacterium, Haloactinobacterium, Haloactinopolyspora, Haloechinothrix, Haloglycomyces, Nesterenjonia, Nocardiopsis, Haloactinospora, Streptomonospora, Isoptericola, Prauserella, Saccharomonospora, Saccharopolyspora</i>
<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Anaerophaga</i>
	<i>Flavobacteria</i>	<i>Gramella, Psychroflexus</i>
	<i>Sphingobacteria</i>	<i>Salinibacter, Salisaeta</i>
<i>Cyanobacteria</i>		<i>Rubidibacter, Prochlorococcus, Halospirulina</i>

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Table 1.2 – *Continued from previous page*

Phylum	Class	Genera
<i>Firmicutes</i>	<i>Bacilli</i>	<i>Alkalibacillus</i> , <i>Aquisalibacillus</i> , <i>Bacillus</i> , <i>Filobacillus</i> , <i>Gracilibacillus</i> , <i>Halalkilibacillus</i> , <i>Halolactibacillus</i> , <i>Halobacillus</i> , <i>Jeotgalibacillus</i> , <i>Lentibacillus</i> , <i>Oceanobacillus</i> , <i>Ornithinibacillus</i> , <i>Paraliobacillus</i> , <i>Piscibacillus</i> , <i>Pontibacillus</i> , <i>Salimicrobium</i> , <i>Salinibacillus</i> , <i>Salirhabdus</i> , <i>Salsuginibacillus</i> , <i>Sediminibacillus</i> , <i>Salinicoccus</i> , <i>Tenuibacillus</i> , <i>Thalassobacillus</i> , <i>Virgibacillus</i>
	<i>Clostridia</i>	<i>Acetohalobium</i> , <i>Halanaerobacter</i> , <i>Halanaerobium</i> , <i>Halobacteroides</i> , <i>Halocella</i> , <i>Halona-tronum</i> , <i>Halothermothrix</i> , <i>Natranaerobius</i> , <i>Natronella</i> , <i>Natronovirga</i> , <i>Orenia</i> , <i>Selenihalanaerobacter</i> , <i>Sporohalobacter</i>

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Table 1.2 – *Continued from previous page*

Phylum	Class	Genera
<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Antarctobacter, Citreimonas,</i> <i>Dichotomicrobium, Fodini-</i> <i>curvata, Hwanghaeicola,</i> <i>Hyphomonas, Jannaschia,</i> <i>Maribaculum, Maribius,</i> <i>Marispirillum, Methylarcula,</i> <i>Oceanibulbus, Oceanicola,</i> <i>Palleronia, Paracoccus,</i> <i>Ponticoccus, Rhodobium,</i> <i>Rhodotalassium, Rhodovibrio,</i> <i>Rhodovulum, Roseicitreum,</i> <i>Roseinatronobacter, Roseisali-</i> <i>nus, Roseospira, Roseovarius,</i> <i>Salinihabitans, Salipiger,</i> <i>Sediminimonas, Shimia,</i> <i>Sulfitobacter, Tropicibacter,</i> <i>Woodsholea, Yangia</i>

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Table 1.2 – *Continued from previous page*

Phylum	Class	Genera
	<i>Gammaproteobacteria</i>	<i>Aidingimonas, Alcanivorax,</i> <i>Alkalilimnicola, Alteromonas,</i> <i>Aestuariibacter, Aquisal-</i> <i>imonas, Arhodomonas, Carni-</i> <i>monas, Chromohalobacter,</i> <i>Cobetia, Ectothiorhod-</i> <i>spira, Ectothiorhodosinus,</i> <i>Glaciecola, Gilvamarinus,</i> <i>Haliea, Halochromatium,</i> <i>Halomonas, Halorhodospira,</i> <i>Halospina, Halothiobacil-</i> <i>lus, Idiomarina, Kushneria,</i> <i>Marichromatium, Marinobac-</i> <i>ter, Marinobacterium, Melitea,</i> <i>Methylohalomonas, Microb-</i> <i>ulbifer, Modicisalibacter,</i> <i>Nitrincola, Oleispira, Pseudid-</i> <i>iomarina, Pseudoaltermonas,</i> <i>Psychromonas, Pseudomonas,</i> <i>Saccarospirillum, Salicola,</i> <i>Salinicola, Salinisphaera,</i> <i>Salinivibrio, Thioalkalibacter,</i> <i>Thioalkalivibrio, Thiohalobac-</i> <i>ter, Thiohalorhabdus, Thio-</i> <i>halocapsa, Thiohalomonas,</i> <i>Thiohalophilus, Thiohalospira,</i> <i>Thiomicrospira</i>

Continued on next page

Table 1.2 – *Continued from previous page*

Phylum	Class	Genera
	<i>Delta proteobacteria</i>	<i>Desulfocella, Desulfohalobium, Desulfonatronospira, Desulfosalsimonas, Desulfovermiculus, Desulfovibrio, Desulfurivibrio</i>
	<i>Epsilon proteobacteria</i>	<i>Arcobacter, Sulfurimonas, Sulfurovum</i>
<i>Spirochaetes</i>	<i>Spirochaetes</i>	<i>Spirochaeta</i>
<i>Tenericutes</i>	<i>Mollicutes</i>	<i>Haloplasma</i>
<i>Thermotogae</i>	<i>Thermotogae</i>	<i>Petrotoga</i>

1.3 Lake Tyrrell, Australia, as a model ecosystem

Lake Tyrrell, Victoria, Australia, is located in the center of the Murray Basin Plains (Figure 1.6), in a region with a semi-arid climate, average rainfall of 300 mm/year, and an evaporation rate of 2000 mm/year [50]. The lake is considered an acid-hypersaline system, where low-pH, oxygenated, saline, metal-rich groundwater from springs is evapo-concentrated and mixed with near-neutral pH waters, rich in sulfides [48]. The lake shows seasonal salinity variations. During winter, the salt content is approximately >250 g/L; in summer, due to water evaporation, the residual brines reach concentrations >330 g/L [50].

Lake Tyrrell has been described and studied in detail in terms of its hydrological and geochemical features [48, 50, 36]], making it a great candidate for microbiological characterization. Recent projects have used a diverse array of microbiological techniques to study the microbial diversity of Lake Tyrrell, including Eukaryotes [40], Archaea and Bacteria [69, 58, 90], and Viruses [25, 26]. Future work will combine the metagenomic, proteomic, and available geochemical informa-

tion to provide a more integrative description of the interactions between microbes, viruses, and the environment.

In this thesis, we explore the microbial diversity of the Lake Tyrrell ecosystem, based on the data generated through a metagenomic study. In particular, our study highlights how, by assembling metagenomic data, we obtain a more complete picture of the microbial diversity present in the community, and also how we can exploit this information to obtain a broad picture of the phylogenetic and functional diversity, and to explore in detail the genetic diversity of the members of the microbial community.

Chapter 2 describes how the assembly of metagenomic datasets allowed the recovery and identification of novel microbial groups that are abundant in the hypersaline waters of Lake Tyrrell, and other hypersaline ecosystems. Using additional metadata, such as size fractionation, sequence nucleotide composition, and phylogenetic binning, two near-complete genomes from a novel Class of Archaea, the *Nanohaloarchaea*, were recovered from the metagenomic samples. . This chapter represents work that has already been published [58]. My role, in this study was in the classification of sequences into phylogenetic groups using statistical approaches, such as non-metric multidimensional scaling, and exploring the functional diversity of the two novel genomes.

Chapter 3 corresponds to the bioinformatic analysis and description of a novel type of microbial rhodopsin, xenorhodopsin, identified from the genomes of the Nanohaloarchaea. The results convey how this rhodopsin appears to be a new class of microbial rhodopsin based on phylogenetic analysis and on the presence of unique amino acid signatures. This work has already been published [91], where I was the lead author of the study.

Chapter 4 describes the assembly of several genomes from the Lake Tyrrell metagenome, based on the combination of assembly-based approaches and metadata. These results provide a framework for future analyses of this ecosystem, providing a set of habitat-specific genomes, including phylogenetic, functional, and genetic diversity. This work is already published [69][?]. My role in this study was developing the methods for classification of the assembled scaffolds into different

phylogenetic groups and developing novel visualization and analysis approaches to compare the functional repertoire of community members.

Chapter 5, leverages the assembled habitat-specific genomes, and describes a bioinformatic approach for the analysis of genetic diversity in a metagenome sample. Using this framework, a deep-sequencing approach was used to characterize the genetic heterogeneity present in the microbial members of the Lake Tyrrell community.

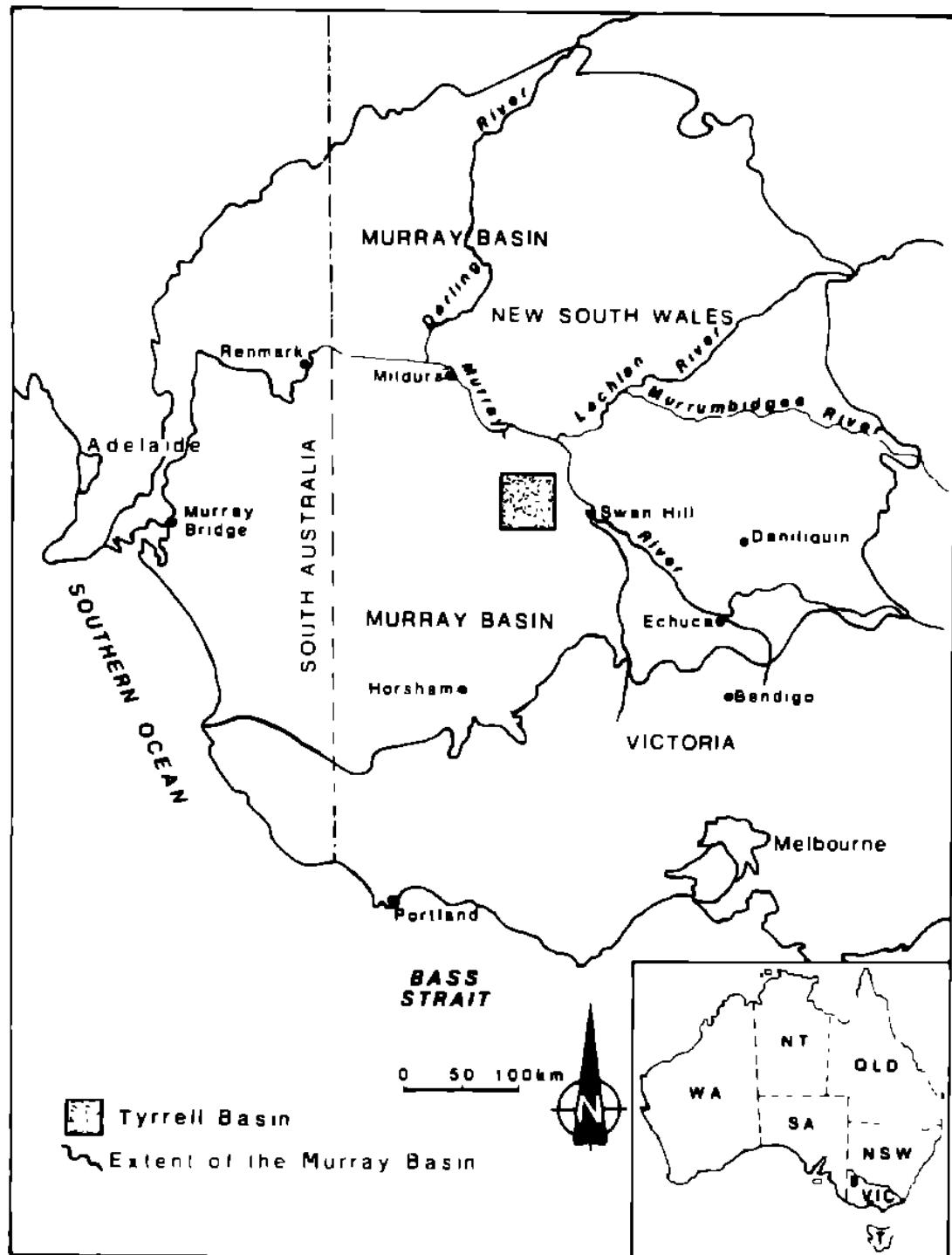


Figure 1.6: Location of Lake Tyrrell in the southeastern region of Australia.
Figure from Macumber, 1992. [50].

Chapter 2

De novo metagenomic assembly
reveals abundant novel major
lineage of Archaea in hypersaline
communities



ORIGINAL ARTICLE

De novo metagenomic assembly reveals abundant novel major lineage of Archaea in hypersaline microbial communities

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This study describes reconstruction of two highly unusual archaeal genomes by *de novo* metagenomic assembly of multiple, deeply sequenced libraries from surface waters of Lake Tyrrell (LT), a hypersaline lake in NW Victoria, Australia. Lineage-specific probes were designed using the assembled genomes to visualize these novel archaea, which were highly abundant in the 0.1–0.8 µm size fraction of lake water samples. Gene content and inferred metabolic capabilities were highly dissimilar to all previously identified hypersaline microbial species. Distinctive characteristics included unique amino acid composition, absence of Gvp gas vesicle proteins, atypical archaeal metabolic pathways and unusually small cell size (approximately 0.6 µm diameter). Multi-locus phylogenetic analyses demonstrated that these organisms belong to a new major euryarchaeal lineage, distantly related to halophilic archaea of class Halobacteria. Consistent with these findings, we propose creation of a new archaeal class, provisionally named ‘Nanohaloarchaea’. In addition to their high abundance in LT surface waters, we report the prevalence of Nanohaloarchaea in other hypersaline environments worldwide. The simultaneous discovery and genome sequencing of a novel yet ubiquitous lineage of uncultivated microorganisms demonstrates that even historically well-characterized environments can reveal unexpected diversity when analyzed by metagenomics, and advances our understanding of the ecology of hypersaline environments and the evolutionary history of the archaea.

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Subject Category: integrated genomics and post-genomics approaches in microbial ecology

Keywords: assembly; halophile; hypersaline; metagenome; Nanohaloarchaea

Introduction

Cultivation-independent molecular ecology techniques currently used to survey environmental microbiota include analysis of phylogenetic marker genes, targeted functional gene inventories and direct sequencing of DNA recovered from environmental samples (reviewed in Hugenholtz and Tyson, 2008; Wooley *et al.*, 2010). Direct metagenomic sequen-

cing is an appealing route for investigating microbial community composition because it provides simultaneous insight into phylogenetic composition and metabolic capabilities of uncultivated populations (Allen and Banfield, 2005; Wilmes *et al.*, 2009). Gene fragments from individual sequencing reads and small assembled contigs can be annotated and assigned to approximate phylogenetic bins based on comparison with databases of known reference genomes (Mavromatis *et al.*, 2007). However, cultivation biases limit the phylogenetic and physiological breadth of available reference genomes (Wu *et al.*, 2009). Single cell genomics can potentially broaden genomic databases, but often provides highly fragmented data because of amplification biases (Lasken, 2007; Woyke *et al.*, 2009). As a result

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of skewed genomic representations in reference data sets, metagenome analysis methods that rely on previously described sequence examples (for example, fragment recruitment approaches) share an inherent potential bias against novel findings. This anti-novelty bias can be overcome by *de novo* sequence assembly, which does not rely on external reference sequences, and can facilitate resolution of phylogeny-to-function linkages for individual community members. Yet *de novo* sequence assembly techniques are rarely applied to metagenomic sequences because of sampling deficiencies and/or computational challenges (Allen and Banfield, 2005; Baker et al., 2010).

Habitats characterized by low diversity microbial communities have proven useful for validating molecular (eco-)systems biology approaches to examine the genetic and functional organization of native microbial consortia (Tyson et al., 2004; Allen and Banfield, 2005; Ram et al., 2005; Lo et al., 2007; Raes and Bork, 2008; Wilmes et al., 2009). High salt-impacted habitats are distributed globally in the form of hypersaline lakes, salt ponds and solar (marine) salters, where evaporative processes result in salt concentrations close to and exceeding saturation. These environments contain microbial communities of intermediate complexity (Oren, 2008), providing excellent model systems for developing scalable analytical techniques applicable to environments with greater species richness and evenness.

The biochemical and physiological challenges faced by extremely halophilic organisms have resulted in unique adaptations to maintain osmotic balance, overcome reduced water activity because of the hygroscopic effects of saturating salt concentrations, and deter DNA damage induced by intense solar irradiation (Bolhuis et al., 2006; Hallsworth et al., 2007). The most extreme halophiles maintain osmotic balance using a 'high salt-in' strategy, which allows intracellular salt concentrations to reach levels approximately isosmotic with the external environment (Oren, 2008). Microorganisms using the salt-in strategy not only endure extreme ionic strength, they require it for growth. Although salt-in adaptation can be energetically more favorable than transporting salt out and the accumulation of compatible solutes (Oren, 1999), it requires significant modifications to the intracellular machinery, including specialized protein amino acid compositions to maintain solubility, structural flexibility, and water availability necessary for enzyme function (Fukuchi et al., 2003; Bolhuis et al., 2008; Paul et al., 2008; Rhodes et al., 2010).

The study of microbial populations in extreme hypersaline environments is well established; the first cultivated halophilic microorganism appeared in Bergey's manual over a century ago (Oren, 2002a). Despite the extreme conditions in salt-saturated habitats, microbial cell densities often exceed 10^7 cells ml^{-1} (Oren, 2002b). Although salt-adapted organisms derive from all three domains of life, most

extreme hypersaline habitats are dominated by halophilic archaea belonging to the monophyletic class Halobacteria (phylum Euryarchaeota), including members of the genera *Haloquadratum*, *Halobacterium*, *Halorubrum* and *Haloarcula* (Oren, 2008). Pure isolates of halophilic archaea currently include >96 species distributed among 27 genera, with genome sequence information available for more than a dozen species (Oren et al., 2009). Numerous cultivation-independent biodiversity surveys have been performed in hypersaline environments using PCR amplification of archaeal and bacterial 16S ribosomal RNA (rRNA) genes, as well as direct metagenomic sequencing of community DNA (Grant et al., 1999; Benloch et al., 2001; Ochsenreiter et al., 2002; Burns et al., 2004; Demergasso et al., 2004; Jiang et al., 2006; Maturano et al., 2006; Mutlu et al., 2008; Pagaling et al., 2009; Sabet et al., 2009; Oh et al., 2010; Rodriguez-Brito et al., 2010). These studies confirm high abundance of a few dominant species with widespread geographical distribution, but the intermittent recovery of atypical, unconfirmed sequence fragments hints at additional, unrecognized diversity among halophilic archaea (Grant et al., 1999; Lopez-Garcia et al., 2001; Pagaling et al., 2009; Oh et al., 2010; Sime-Ngando et al., 2010).

The lure of uncovering biological novelty is a major incentive driving metagenomic investigations in many habitats worldwide. This study demonstrates that even historically well-characterized habitats like extreme hypersaline lakes and solar salters can reveal unexpected genes, metabolic features and entire lineages overlooked previously. The 'assembly-driven' community metagenomic approach applied in the current study has led to the discovery and reconstruction of near-complete genomes for two new archaeal genera representing the first members of a previously undescribed taxonomic class of halophilic archaea. We demonstrate that members of this new archaeal class are present in high abundance and broadly distributed in other hypersaline habitats worldwide.

Materials and methods

Sample collection

Surface water samples (0.3 m depth) were collected from Lake Tyrrell (LT), Victoria, Australia and a high salinity crystallizer pond at South Bay Salt Works, Chula Vista (CV) California. Detailed locations, sampling dates, and physical characteristics of the collection sites are provided in Supplementary Figure S1.

Water samples of 20 l each were passed through a 20 μm Nytex prefilter, followed by sequential filtration through a series of polyethersulfone, 142 mm diameter membrane filters (Pall Corporation, Port Washington, NY, USA) of decreasing porosities (3 μm > 0.8 μm > 0.1 μm) using a peristaltic pump. After each stage of filtration, filters were frozen for

future DNA extraction, 16S rRNA gene analysis and metagenomic sequencing. Aliquots of filtered water were fixed with formaldehyde (7% final concentration) overnight at 4°C. Fixed water samples were collected on 0.2 µm polycarbonate GTTP filters (Millipore, Billerica, MA, USA) for fluorescence *in situ* hybridization (FISH) and direct count microscopy.

Library construction and assembly

Genomic DNA was extracted from individual, bar-coded 0.8 and 0.1 µm filters. Filter-specific DNA libraries were constructed with insert sizes of 8–10 kbp and/or 40 kb (fosmids) at the J Craig Venter Institute, as described previously (Goldberg *et al.*, 2006). Details of genomic DNA sequence libraries are provided in Supplementary Table S1.

16S rRNA gene clone libraries were constructed by amplification of LT metagenomic DNA using universal archaeal primer sequences Arc21F and Arc529R (Table 1), as previously described (Bik *et al.*, 2010). A group-specific primer for Nanoarchaea (LT_1215R) was designed using the NCBI primer design tool, and used together with universal archaeal primer Arc21F to amplify both LT and CV community DNA. Amplification products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and sequenced bi-directionally with M13F and M13R primers.

Sanger and pyrosequencing read libraries were assembled both individually and in various combinations, using Celera Assembler software version 5.4 (Myers *et al.*, 2000), in a series of iterative assemblies guided by phylogenetic binning. Detailed genome assembly procedures are provided in Supplementary Information.

Genome annotation

J07AB43 and J07AB56 draft genomes were annotated using the Integrated Microbial Genome Expert Review service of the Joint Genome Institute (Markowitz *et al.*, 2009b). Genome completeness was estimated for the J07AB56 and J07AB43 scaffold groups by comparing genes involved in transcription, translation and replication to those identified as highly conserved in previously sequenced archaeal genomes (Ciccarelli *et al.*, 2006; Wu and Eisen, 2008; Puigbo *et al.*, 2009). Orthologs shared between the J07AB43 and J07AB56 proteomes were detected using the reciprocal smallest distance algorithm (threshold *e*-value = 1e-05; sequence divergence = 0.4) (Wall and Deluca, 2007).

Amino acid composition analysis

Amino acid frequencies in predicted proteins from J07AB56, J07AB43 and 1455 archaeal and bacterial genomes were compared using the Primer 6 software program (Clarke and Gorley, 2006) to perform Non-Metric Multidimensional Scaling (NM-MDS) analysis (Ramette, 2007). For each genome, the frequency of each amino acid for all predicted proteins was calculated using a custom perl script. These values were standardized by Z-score, then used to calculate a Euclidean distance similarity matrix. NM-MDS analysis was performed using default program parameters (25 random starts, Kruskal fit scheme of 1 and a minimum stress value of 0.01). In addition to NM-MDS analysis, a cluster analysis was performed to define groups within the NM-MDS plot using a multidimensional distance parameter of 4%.

Table 1 Primers and probes for detecting 16S rRNA sequences

Use	Target	Name	Sequence (5' to 3')	Reference
PCR	NHA	LT_1215R	ggccgcgtgtatcccgagc	This study
	A	Arc21F	<i>tttCgggtgtatccygc</i> Cga	DeLong (1992)
	A	Arc529R	<i>accgcggcgctgtgc</i>	DasSarma and Fleischman (1995)
	A	ArcP1	<i>atttCgggtgtatccgtc</i>	Ihara <i>et al.</i> (1997)
	A	Arc27Fa	<i>tcygggtgtatccgtsc</i> Gg	Raes and Bork (2008)
	U	Univ151F	<i>tgcacat</i> A ccgggttaa	Lane (1991)
	A	Arc751F	C GA GGGAA GGGrygaa	Baker <i>et al.</i> (2003)
	A	Arc958R	y C GGGAT G Amt C att	DeLong (1992)
	U	Univ1390R	<i>acGgecGgttgttcaat</i>	Brunck and Eis (1998)
	A	UA1406R	<i>acGgecGtewgttcaat</i>	Baker <i>et al.</i> (2003)
FISH	A	Arc1492R	A CCG G TAAC T ttTid C actt	Grant <i>et al.</i> (1999)
	U	Univ1492R	GGT TACCtig T td C actt	Lane (1991)
	A	Arc915	<i>gtgtccccggccaaatct</i>	Amann <i>et al.</i> (1995)
	NHA	Narc_1214	<i>cggcggtgtatcccgagc</i>	This study
	NHA	LT_1198h1	<i>attcggccatactgcac</i>	This study
	NHA	LT_976-h2	<i>ggctctgttagrygrc</i>	This study
	NHA	LT_1237h3	<i>tytsittgthccggccat</i> g	This study
	B	Eub338	<i>gcgtccctccgttaggt</i>	Amann <i>et al.</i> (1990)
	B	Eub338plus	<i>gcwgccacccgttaggt</i>	Daims <i>et al.</i> (1999)

Target specificity abbreviations: A, archaea; B, bacteria; NHA, nanohaloarchaea; U, universal. *PCR primer mismatches are capitalized. Bold indicates primer mismatches to J07AB43 only, underline to J07AB56 only, and boxed to both J07AB43 and J07AB56. Lower case italic letters indicate exact matches to the organisms described in the text. Upper case non-italic letters indicate mismatches of three different types (bold, underline, or boxed).

*These primers were not used in this study; sequences are shown for comparison only.

Phylogenetic analysis

16S rRNA sequences and ribosomal proteins from euryarchaeal genomes in the JGI-IMG database (Markowitz *et al.*, 2009a) and GenBank were compared with metagenomic gene sequences obtained by (i) extraction from assembled scaffolds and (ii) amplification and sequencing of 16S rRNA genes from LT and CV clone libraries. Maximum likelihood trees were constructed using TreeFinder v.10.08 (Jobb *et al.*, 2004) and PhyML v.3.0 (Guindon and Gascuel, 2003). The robustness of each maximum likelihood tree was estimated using non-parametric bootstrap analysis. Details of alignment curation and tree construction are provided in Supplementary Information.

Predicted proteins in assembled genomes were evaluated for phylogenetic relatedness to known sequences in NCBI GenBank nr using the DarkHorse program, version 1.3, with a threshold filter setting of 0.05 (Podell and Gaasterland, 2007; Podell *et al.*, 2008). Minimum quality criteria for match inclusion in the DarkHorse analysis were that BLASTP alignments to GenBank nr sequences cover at least 70% of total query length and have *e*-value scores of 1e-5 or better.

Fluorescence in situ hybridization

Fluorophore-conjugated custom 16S rRNA probes (Table 1) were designed using ARB (Ludwig *et al.*, 2004), screened for specificity *in silico* using ProbeCheck (Loy *et al.*, 2008) and synthesized by Integrated DNA Technologies Inc. (San Diego, CA, USA). FISH was performed on CV and LT water samples collected on 0.2 µm polycarbonate GTTP filters (Millipore) at every stage of filtration (post 20 µm, post 3 µm and post 0.8 µm). The Nanohaloarchaea-specific probe Narc_1214 conjugated with Cy3 along with unlabeled helper probes LT_1198h1, LT_976h2 and LT_127h3 (Fuchs *et al.*, 2000) were used for FISH analysis. Universal probes Arc915 (archaeal) and EubMix (a bacterial probe consisting of an equimolar mixture of Eub338 and Eub338plus) were also used for the purpose of cell counts. Hybridization conditions were optimized at 46 °C for 2 h, as previously described (Pernthaler *et al.*, 2001). Filters were mounted with Vectashield medium (Vector Laboratories, Burlingame, CA, USA), and imaged at 1000 × with a Nikon Eclipse TE-2000U inverted microscope (Nikon Instruments Inc., Irvine, CA, USA). Cell counts were performed on multiple fields per slide, normalizing 16S rRNA-specific probe counts to total number of cells stained with the DNA-binding dye 4',6-diamidino-2-phenylindole.

Accession numbers

16S rRNA gene sequences have been deposited to DDBJ/EMBL/GenBank under accession numbers HQ197754 to HQ197794. Assembled genomes with annotations have been deposited as Whole

Genome Shotgun projects under accession numbers AEIY01000000 (J07AB43) and AEIX01000000 (J07AB56).

Results

Metagenomic assembly

Seven independent DNA sequencing libraries were constructed from size-fractionated surface water samples collected at LT, Australia (Supplementary Figure S1 and Supplementary Table S1). Initial assembly of the combined 632 903 Sanger sequencing reads produced 15 008 scaffolds (maximum length = 2 764 168 bp; scaffold N50 = 29 346 bp). These scaffolds included at least six different relatively abundant microbial populations, each with a distinct nucleotide percent G+C composition. A length-weighted histogram of percent G+C versus total assembled scaffold nucleotides showed peaks corresponding to these populations (Figure 1). The largest peak in this histogram, at 48% G+C, included scaffolds containing 16S rRNA sequences from multiple strains of *Haloquadratum walsbyi*, consistent with previous observations noting the dominance of this species in similar hypersaline environments (Cuadros-Orellana *et al.*, 2007; Oh *et al.*, 2010). Three additional peaks at 60% G+C or higher included scaffolds containing 16S rRNA genes with 89–99% identity to clone sequences annotated as uncharacterized halophilic archaea (class Halobacteria). Microbial populations associated with these peaks are currently under investigation, but fall outside the scope of the present report.

Two groups of scaffolds, with peaks at 43% and 56% G+C, shared an intriguing pattern of unusual characteristics. In addition to distinctive G+C content, >90% of the reads that co-assembled in

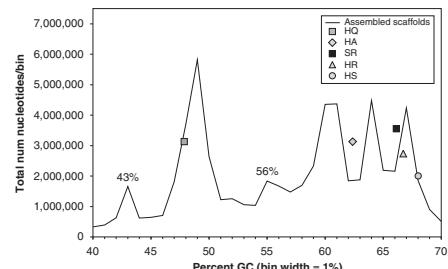


Figure 1 Length-weighted histogram of percent G+C for all scaffolds assembled from the LT community, binned in 1% GC increments. Symbols represent reference control points, indicating where five previously sequenced halophile genomes would have fallen, if they had been present in this data set. Data points are plotted based on total number of nucleotides in each scaffold (y axis) versus average percent GC for the entire scaffold (x axis). HA, *Haloarcula marismortui*; HQ, *Haloquadratum walsbyi*; HR, *Halorubrum lacusprofundi*; HS, *Halobacterium salinarum* R1; SR, *Salinibacter ruber*. Peaks labeled at 43% and 56% GC are the focus of this study.

Table 2 General features of the J07AB43 and J07AB56 draft genomes

	J07AB43	J07AB56
Genome size, bp	1 227 157	1 215 802
G+C percentage	44%	56%
Scaffold number	7	3
rRNA operons	1	1
tRNAs	59	38
Predicted CDSs	1678	1411
CDSs w/func. Pred.	773	719

Abbreviations: CDS, coding sequence; rRNA, ribosomal RNA; tRNA, transfer RNA.

these scaffolds were obtained from microorganisms that had passed through a 0.8 µm filter, but were retained on a 0.1 µm filter, suggesting small cell size. The 16S rRNA gene sequences contained in these scaffolds were <78% identical to any previously known cultured isolate, although they did resemble 16S rRNA gene fragments periodically recovered in culture-independent surveys of microbial diversity in hypersaline waters (Grant *et al.*, 1999; Oh *et al.*, 2010; Sime-Ngando *et al.*, 2010).

To optimize assembly efficiency for these unusual populations, the full set of metagenomic reads were subjected to a series of iterative assemblies guided by phylogenetic binning. The 43% G+C peak was thereby consolidated into seven major scaffolds (J07AB43) and the 56% G+C peak into three major scaffolds (J07AB56) (Supplementary Table S2). The J07AB43 and J07AB56 scaffold groups were subsequently analyzed as draft genomes, each representing the consensus sequence of an individual microbial population. Overall properties of these draft genomes are summarized in Table 2. These properties differ substantially from previously sequenced extreme halophiles in both nucleotide composition, expressed as percent G+C, and total genome size (Markowitz *et al.*, 2009a). With the exception of *H. walsbyi*, at 48% G+C, all other previously described halophilic archaea, as well as the halophilic bacterium *Salinibacter ruber*, have nucleotide compositions of 60% or greater G+C, compared with 43% and 56% for these new organisms. Estimated total genome size and predicted number of coding sequences for J07AB43 and J07AB56 (Table 2) were also considerably smaller than other known extreme halophiles, which currently range from 2.7 to 5.4 Mbp.

Genome completeness

To estimate the extent of genome completeness of J07AB43 and J07AB56, functional annotations for all predicted proteins were searched against a set of 53 housekeeping genes, previously identified as universally present in all archaeal genomes sequenced as of 2009 (Puigbo *et al.*, 2009). These highly conserved genes are physically dispersed throughout the genome (non-clustered) and include ribosomal

proteins, amino acid tRNA synthetases, translation initiation and elongation factors, molecular chaperones and proteins essential for DNA replication and repair. All 53 of the universal archaeal housekeeping proteins were identified in J07AB56 while 44/53 (83%) were found in the J07AB43 draft genome (Supplementary Table S3). The presence of these core proteins, a single rRNA operon and transfer RNAs enabling translation of all 20 amino acids, suggests that both draft genomes are nearly complete.

Community abundance

Community abundance of J07AB43 and J07AB56 was initially assessed by sequencing 16S rRNA gene clone libraries, constructed by amplifying LT community DNA with universal archaeal primers Arc21F and Arc529R (Table 1). Amplified sequences with >91% identity to the J07AB43 and J07AB56 draft genomes were found in 124/315 (39%) of archaeal clones obtained from organisms retained on 0.1 µm pore filters, but only 24/254 (9%) of clones retained on 0.8 µm pore filters. These results are consistent with the observed enrichment of J07AB43 and J07AB56 reads specifically derived from 0.1 µm filter fractions in the assembled data set.

As a second, independent test of community abundance, new lineage-specific 16S rRNA probes were designed to visualize J07AB43 and J07AB56 cells in environmental samples by FISH (Table 1). These probes were used in combination with the DNA-binding dye 4',6-diamidino-2-phenylindole and universal bacterial and archaeal probes to obtain direct cell counts in LT and CV water samples (Figure 2). Cells approximately 0.6 µm in diameter were labeled with lineage-specific probe NArc_1214 in samples from both locations. These results are consistent with size estimates of <0.8 µm but >0.1 µm based on filter-specific composition for both amplified 16S rRNA clones and metagenomic reads. Direct counts of fluorescently labeled cells indicated that the combined abundance of strains matching the new, lineage-specific probes was approximately 14% of all 4',6-diamidino-2-phenylindole-labeled cells in water samples from LT, and 8–11% in samples from CV (Supplementary Table S4).

Community abundance of the organisms responsible for the J07AB43 and J07AB56 draft genomes was further examined using statistical properties of the assembled metagenomic sequence data. The number of reads that co-assembled to create each composite population scaffold group was divided by the total number of reads available and normalized for estimated genome size. Assuming the two new genomes are approximately 1.2 Mbp each, and other microbial species sampled from LT have an average genome size of 3 Mbp, J07AB43 was estimated to represent at least 6.7% of the LT sampled community (17 066 reads) and J07AB56 at least 3.4% (8652 reads), totaling approximately 10% for the two

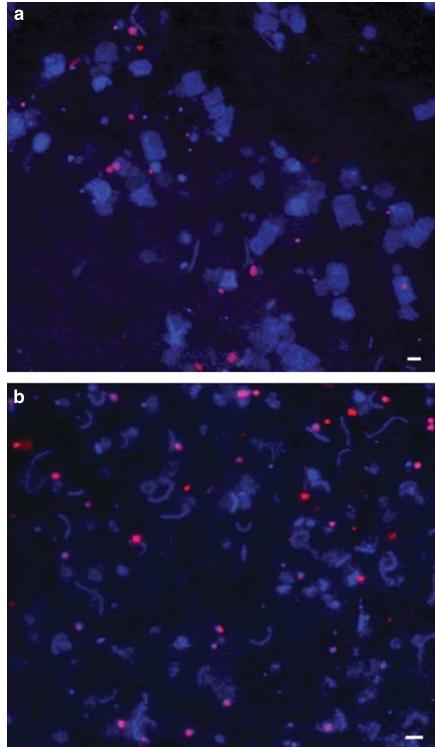


Figure 2 FISH micrographs. (a) LT (0.1 to 3 μm filter fraction), (b) CV South Bay Salt Works (0.1 to 0.8 μm filter fraction). All cells are stained with 4',6-diamidino-2-phenylindole (blue). Nanoarchaea cells shown are stained with lineage-specific Cy3 probe Narc_1214 (red). Scale bar = 2 μm .

populations combined (3.0/1.2*25 718/632 903). Calculations based on metagenomic assembly most likely underestimate true population abundance, because they may exclude closely related polymorphic strains containing DNA sequence variations that were not incorporated into the consensus population assembly.

Taxonomic position of J07AB43 and J07AB56

J07AB43 and J07AB56 16S rRNA shared sequence identities of 68% to 75% with previously sequenced, cultured representatives of class Halobacteria (Supplementary Table S5). An unrooted maximum likelihood phylogenetic tree of euryarchaeotal 16S rRNA gene sequences placed J07AB43 and J07AB56 as a deep sister group of class Halobacteria (Figure 3), with significant bootstrap support.

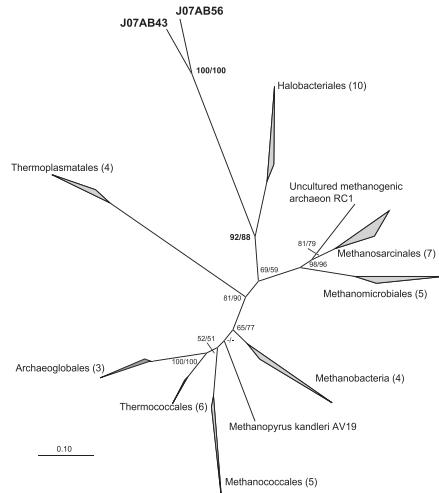


Figure 3 Unrooted maximum-likelihood 16S rRNA gene phylogenetic tree of the Euryarchaeota. Tree is based on 48 sequences, 1275 positions. Numbers of sequences in each collapsed node are indicated in parentheses. Numbers at nodes represent bootstrap values inferred by TreeFinder/PhyML. Bootstrap values <50% are indicated by a '-' sign. Scale bar represents 0.1 substitutions per site. A full, uncollapsed version of this tree is presented in Supplementary Figure S2a.

Concatenated ribosomal protein data sets have been shown to be particularly useful for resolving deep evolutionary relationships (Brochier *et al.*, 2002; Matte-Tailliez *et al.*, 2002; Rokas *et al.*, 2003; Rannala and Yang, 2008). Phylogenetic analysis of 57 ribosomal proteins from the J07AB43 and J07AB56 draft genomes showed, like the 16S rRNA tree, robust placement of these genomes as a deeply branching sister group of class Halobacteria, with bootstrap values of 98% (PhyML) and 74% (TreeFinder). This relationship was corroborated using Dayhoff04 recoding of ribosomal protein alignments (Hrdy *et al.*, 2004; Susko and Roger, 2007), to rule out possible artifacts of biased amino acid composition or fast-evolving lineages (Supplementary Figure S2b). The long branch lengths separating J07AB43 and J07AB56 from members of class Halobacteria indicate that these two sister-lineages are only distantly related, consistent with the average divergence of 35% observed between Halobacteria and J07AB43 and J07AB56 16S rRNA gene sequences (Supplementary Table S5). By contrast, 16S rRNA variability within the Halobacteria is <16%.

Nearly 60% of predicted proteins in J07AB43 and J07AB56 had no GenBank database matches close enough to enable confident phylogenetic assignment. Of those that could be assigned, fewer than 20% matched proteins from members of class

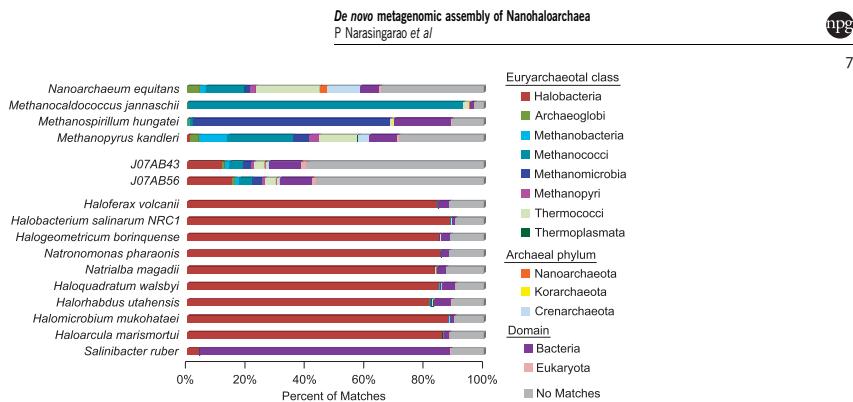


Figure 4 Phylogenetic distribution of non-self-protein BLAST matches for euryarchaeotal genomes. Searches against the GenBank nr database were classified by euryarchaeotal class, archaeal phylum, domain or no match using the DarkHorse algorithm, as described in Materials and methods section.

Halobacteria (Figure 4). In contrast, >80% of predicted proteins in the genomes of previously sequenced Halobacteria had closest non-self matches to other members of their own class, leaving fewer than 20% unmatched. Similar patterns of protein sequence conservation were observed in organisms with many sequenced database relatives, including *Methanocaldococcus janaschii*, *Methanospirillum hungatei* and *Salinibacter ruber*, but not in sparsely sampled species that are only distantly related to other known lineages, such as *Nanoarchaeum equitans* and *Methanopyrus kandleri* (Branciamore *et al.*, 2008).

Genome characteristics of J07AB43 and J07AB56

Although the J07AB43 and J07AB56 genomes are more closely related to each other than to any previously sequenced organisms, gene content analysis identified only 480 (30%) shared protein ortholog pairs between them. Of these, 143 (approximately 10% of each genome) were not found in other halophilic archaea. The majority of these shared lineage-specific sequences were too dissimilar to previously characterized proteins to assign a functional annotation. The remainder was dominated by housekeeping proteins involved in translation and ribosomal structure. Each genome included only one rhodopsin-like gene, compared with multiple paralogs present in the genomes of other extreme halophilic archaea (Ihara *et al.*, 1999), and the extremely halophilic bacterium *Salinibacter ruber* (Mongodin *et al.*, 2005). Notably absent from both genomes were homologs to the highly conserved Gvp family of gas vesicle proteins found in most halophilic archaea, Cyanobacteria and purple photosynthetic bacteria (Walsby, 1994).

Both J07AB43 and J07AB56 have highly unusual amino acid compositions compared with previously sequenced archaeal and bacterial genomes. These unusual compositions appear to support a 'salt-in'

strategy of maintaining osmotic balance, as evidenced by the over-representation of amino acids with negatively charged side chains (aspartic and glutamic acid) and the under-representation of residues with bulky hydrophobic side chains (tryptophan, phenylalanine and isoleucine), to enhance protein structural flexibility and solubility under intracellular conditions of high ionic strength and low water availability. Although a similar salt-in strategy is employed by other extreme halophiles, J07AB43 and J07AB56 use their own, distinct combination of amino acids to achieve this end, preferring glutamic to aspartic acid, serine to threonine, and reduced frequencies of alanine, proline and histidine (Supplementary Table S6). The large number of proteins annotated with 'hypothetical' functions in the J07AB43 and J07AB56 genomes may be at least partially because of their unusual amino acid compositions, which can hinder recognition of database homologs in sequence-based similarity searches.

The peculiar amino acid compositions of J07AB43 and J07AB56 compared with other halophilic archaea are highlighted in a NM-MDS plot of intergenomic distances based on frequencies for all 20 standard amino acids (Figure 5). The data used to construct this matrix included all protein sequences from euryarchaeal genomes used to build the phylogenetic tree in Figure 3, supplemented with four bacterial species found in high salt environments: *Salinibacter ruber* (Bacteroidetes), *Halorhodospira halophila* (Gammaproteobacteria), *Chromohalobacter salexigens* (Gammaproteobacteria) and *Halothermothrix orenii* (Firmicutes).

Although genome percent G+C compositions were not explicitly included as one of the factors in this analysis, there is a trend for microorganisms with lower G+C (denoted with lower label numbers in Figure 5) to be located further to the right along the horizontal axis. This trend is consistent with the known influence of G+C composition on usage

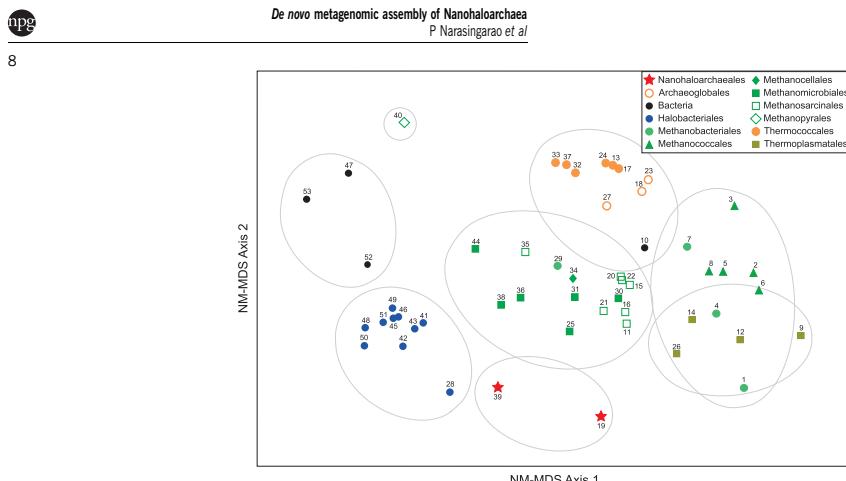


Figure 5 NM-MDS comparison of amino acid compositions. Euryarchaeal genomes were supplemented with four halophilic bacteria genomes. Symbols denote taxonomic classifications. Numbers rank genomes in increasing order of G + C content (1–10: 29–38%, 11–20: 38–43%, 21–30: 43–50%, 31–40: 50–60%, 41–53: 60–67%). Grey circles indicate hierarchical clustering, based on a 4% distance setting to define groups. A complete list of these genomes and their amino acid compositions is presented in Supplementary Table S6.

frequency for some amino acids because of codon bias (Liu *et al.*, 2010). In contrast, position along the vertical axis of Figure 5 was unrelated to percent G+C. Instead, amino acid composition differences captured along this axis appear to correlate more closely with common ancestry and/or shared environmental adaptations. The outlier positions of J07AB43 (#19) and J07AB56 (#39) along the vertical axis of Figure 5 clearly demonstrate their unusual amino acid compositions relative to other archaea. Similar outlier positions were observed for these two genomes when analyzed in the context of a much larger microbial genomic data set, including 1382 bacterial and 73 archaeal species (data not shown).

Inferred metabolic capabilities of the J07AB43 and J07AB56 genomes are consistent with a predominantly aerobic, heterotrophic lifestyle. The absence of identifiable anaerobic terminal reductases suggests they are incapable of anaerobic respiration although the presence of lactate dehydrogenases suggests possible fermentative metabolism under microaerophilic conditions. Both genomes contain enzymes necessary to support glycolysis, as well as operons encoding key enzymes for glycogen synthesis and catabolism. Several of these enzymes, including a glycogen debranching enzyme and predicted alpha-1,6-glucosidase activity, are not present in any other known members of class Halobacteria. However, these enzyme activities are frequently found in archaea from classes Methanococci and Thermoplasmata that utilize starch as an internal storage molecule (König *et al.*, 1985, 1982). This suggests a possible common ancestral origin, with subsequent gene loss in the Halobacteria lineage.

In addition to the Embden-Meyerhoff pathway, genes supporting the entire pentose phosphate

pathway were observed in both genomes, including both oxidative and non-oxidative branches. The presence of a complete pentose phosphate pathway has not been demonstrated previously in any other archaea, by either biochemical or bioinformatic methods (Verhees *et al.*, 2003). The key, rate-limiting enzyme for this pathway is glucose-6-phosphate dehydrogenase, which converts glucose-6-phosphate into 6-phosphoglucono- δ -lactone. Although both J07AB43 and J07AB56 appear to have complete genomic copies of this gene, the closest database relatives to their sequences are all bacterial, suggesting this functionality may have been acquired by ancient horizontal gene transfer. The nearest homolog of the glucose-6-phosphate dehydrogenases in J07AB43 and J07AB56 is from the genome of *Salinibacter ruber*, a common bacterial inhabitant of hypersaline environments believed to have experienced frequent horizontal gene exchange with archaea (Mongodin *et al.*, 2005).

Geographical distribution and diversity

Lineage-specific PCR primer, LT_1215R (Table 1) and general archaeal primer Arc21F were used to construct clone libraries from environmental DNA samples collected from both LT and CV, yielding 43 new 16S rRNA gene sequences. Additional 16S rRNA gene sequences, with >85% identity to J07AB43 and J07AB56, were identified in public databases. These published sequences originated in environmental samples from Africa, Asia and South America, as well as Australia and North America (Supplementary Table S7). The phylogenetic analysis of these 16S rRNA gene sequences reveals at least eight distinct clades with strong bootstrap support

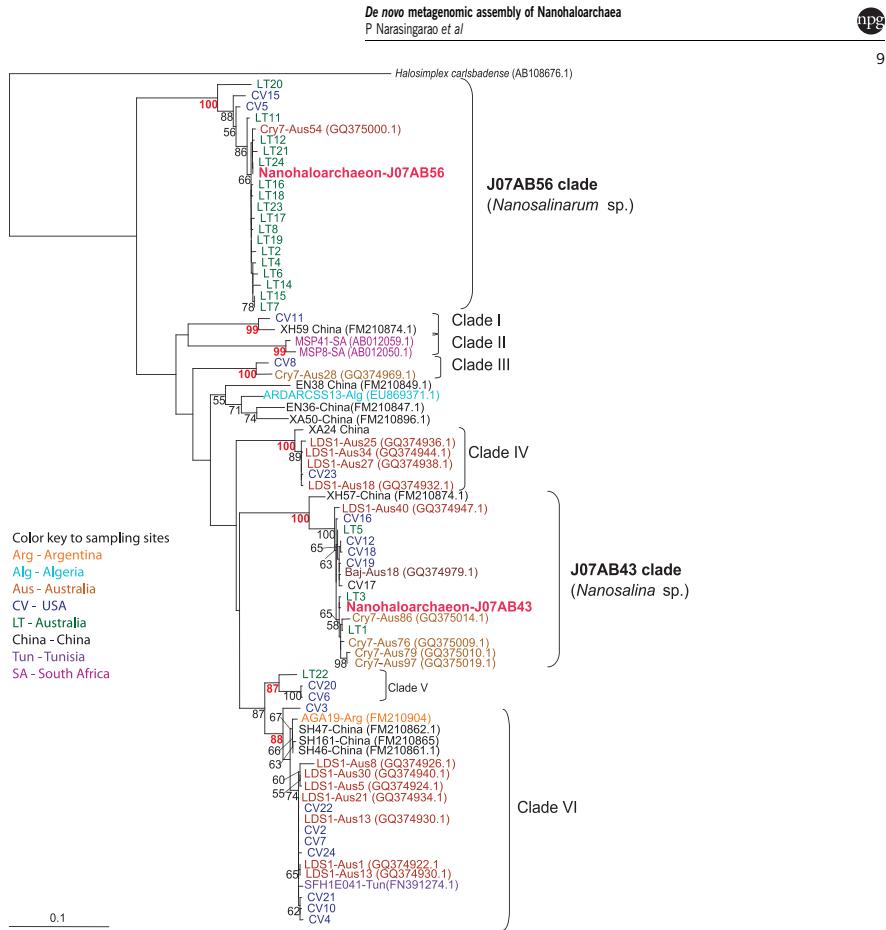


Figure 6 16S rRNA gene maximum likelihood tree of Nanohaloarchaea sequences recovered from worldwide hypersaline habitats. Tree is based on 709 nucleic acid positions in 77 sequences. Numbers at nodes represent bootstrap values (values <50% not shown). Scale bar shows average number of substitutions per site.

(bootstrap values >87%, Figure 6). Based on degree of sequence divergence, each clade most likely represents a new genus or higher taxonomic level. Classification of J07AB43 and J07AB56 into separate genera is strongly supported by tree topology, 16% sequence divergence in the 16S rRNA gene (Supplementary Table S5) and a 13% difference in genomic G + C content.

Discussion

This study has demonstrated that re-examination of a fairly simple, well studied environmental habitat

using a combination of strategic environmental sampling, deep sequencing, and *de novo* metagenomic assembly can reveal significant new information. We have discovered and characterized nearly complete genomes representing a novel archaeal lineage prevalent in hypersaline systems worldwide, yet very different from all previously described members of class Halobacteria.

We propose the creation of a new class 'Nanohaloarchaea' within phylum Euryarchaeota to accommodate this new lineage. We further propose partitioning class Nanohaloarchaea to place J07AB43 and J07AB56 into distinct genera, *Candidatus 'Nanosalina' sp. J07AB43'* and '*Candidatus*

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Nanosalinarum sp. J07AB56'. Evidence supporting these proposals includes: (i) comprehensive euryarchaeotal phylogenetic analyses based on 16S rRNA genes and ribosomal proteins; (ii) lineage-specific features, including numerous genes without previously described close relatives; and (iii) significant intra-lineage diversity and abundance within geographically distinct hypersaline habitats worldwide. Evolutionary distinctness of J07AB43 and J07AB56 from other halophilic archaea is reinforced by taxonomic patterns of BLASTP matches for their predicted proteomes against GenBank nr, as well as amino acid composition-based clustering. The sister-grouping of class Halobacteria and class Nanohaloarchaea reflects probable derivation from an ancient common halophilic ancestor with a 'high salt-in' osmotic regulation strategy, followed by subsequent divergence along separate evolutionary paths.

Lineage-specific characteristics that distinguish '*Candidatus Nanosalina* sp.' and '*Candidatus Nanosalinarum* sp.' from most other extreme halophiles include their small physical size, compact genomes, single-copy rRNA operon, low G+C composition, unique proteome amino acid composition, absence of conserved gas vesicle genes and atypical predicted pathways associated with carbohydrate metabolism. Small compact genomes, as well as single-copy rRNA operons, have been proposed to minimize metabolic costs in habitats where neither broad metabolic repertoire nor high numbers of paralogous proteins are needed to accommodate rapid growth under fluctuating environmental conditions (Klappenbach *et al.*, 2000). Small cell size, which increases surface to volume ratio, could be an adaptation for optimizing nutrient uptake capacity. Alternatively it is possible that small physical size allows Nanohaloarchaea to remain suspended in oxygenated surface waters to support aerobic metabolism, thus eliminating the need for gas vesicles to provide buoyancy.

The low G+C compositions of the two Nanohaloarchaea genomes, especially J07AB43 (43%), are surprising considering their prevalence in high light habitats. In the absence of compensatory mechanisms, lower G+C would be expected to increase susceptibility to ultraviolet-induced DNA damage. One possible explanation is that the low G+C composition of J07AB43 is related to ecological lifestyle. Low G+C composition and genomic streamlining have been associated with decreased nitrogen requirements and a slow-growing, energy-conservative lifestyle in marine bacteria (Giovannoni *et al.*, 2005). However, the habitats from which these Nanohaloarchaea were isolated are not generally considered to be nutrient-limited (Oren, 2002b). Alternatively, it has been proposed that the low G+C composition of *H. walsbyi* (48%) compared with other halophiles is a specific adaptation to counteract the over-stabilizing effect of high magnesium concentrations on DNA structure (Bolhuis

et al., 2006). If extremely high environmental magnesium cannot be adequately excluded from the cell, lower genomic G+C helps maintain DNA structural flexibility and avoids difficulties in strand separation caused by elevated melting temperatures. These same principles could apply to J07AB43, providing a possible selective advantage under high magnesium conditions expected in evaporative high salt environments.

Nanohaloarchaea are estimated to represent at least 10–25% of the total archaeal community in surface water samples from LT, Australia and CV, California, USA. We believe these values are robust, based on agreement of three independent analysis techniques: amplification of environmental 16S rRNA gene sequences; statistical analysis of metagenomic sequencing reads assembled into near-complete draft genomes; and quantitative FISH of cells from natural water samples labeled with lineage-specific probes. Microscopic counts reveal that Nanohaloarchaea are present at cell concentrations exceeding 10^6 cells ml⁻¹ in hypersaline habitats of Australia and North America. The sporadic identification of Nanohaloarchaea in other surveys of hypersaline communities worldwide suggests that Nanohaloarchaea represent a significant yet neglected fraction of the biomass and diversity in these habitats.

The inability of earlier studies to recognize the significant contribution of Nanohaloarchaea to hypersaline community composition is likely due to limitations of the tools routinely used to assess environmental microbial diversity, including laboratory culture, microscopy, amplification of 16S rRNA gene fragments, and sequence database similarity searches for unassembled metagenomic reads. The isolation of cultured strains from environmental habitats is known to exclude many organisms that are highly successful in their native habitats. It is therefore not surprising the 96 hypersaline archaeal isolates described to date do not include any Nanohaloarchaea. Repeated efforts to culture these microorganisms in our own laboratory have also been unsuccessful. Furthermore, cultivation-independent microbial diversity studies based on 16S rRNA gene amplification are known to suffer from primer bias (Sipos *et al.*, 2007). Mismatches between Nanohaloarchaea and many commonly used universal primers may have impeded detection in earlier studies. Primers likely to have been particularly problematic are highlighted in Table 1 (Amann *et al.*, 1990, 1995; Lane, 1991; DeLong, 1992; DasSarma and Fleischman, 1995; Ihara *et al.*, 1997; Brunk and Eis, 1998; Daims *et al.*, 1999; Grant *et al.*, 1999; Baker *et al.*, 2003; Raes and Bork, 2008). The exceptionally small size of Nanohaloarchaea compared with other halophilic microorganisms makes them difficult to visualize by microscopy in the absence of selective enrichment techniques or group-specific probes, and can prevent recovery during sample concentration procedures targeting larger microorganisms or smaller viruses (Rodriguez-

Brito *et al.*, 2010). Similar issues have been noted for other nano-sized archaea, identified solely by 16S rRNA gene sequencing (Casasueva *et al.*, 2008; Gareeb and Setani, 2009).

The presence of ultrasmall, uncultivated novel archaeal lineages in natural environments may be a common occurrence. Nanoarchaea represent the third nano-sized archaeal lineage to be described. However, unlike the thermophilic *Nanoarchaeum equitans* (Huber *et al.*, 2002) or the acidophilic ARMAN lineages (Baker *et al.*, 2006, 2010), members of the Nanoarchaea appear to be free-living based on microscopic observations. The larger genomes of Nanoarchaea (approximately 1.2 Mbp) relative to other symbiotic/parasitic nano-sized archaea (ARMAN, <1 Mbp; *Nanoarchaeum equitans*, <0.5 Mbp) are consistent with a possible non-host associated lifestyle for this group. It is interesting to contemplate the environmental pressures selecting for the evolution of ultrasmall microorganisms with small genomes, and to consider the extent of an ultrasmall microbial biosphere. The realization that ultrasmall populations can comprise a significant fraction of the total microbial community, yet have eluded previous detection, suggests that historical estimates of microbial biomass and numerical abundance in natural environments may be substantially underestimated. This is particularly relevant in non-extreme habitats where the existence of ultrasmall microbial populations have not yet been described or investigated.

Routine metagenomic analysis methods currently rely on the expectation that undiscovered microorganisms will have a certain degree of similarity to those already known, creating a potential bias against novel discoveries. Although this study exposes limitations of commonly used microbial diversity assessment tools in the context of detecting novel archaea in hypersaline lakes, these limitations apply even more emphatically to other more complex microbial communities, which often contain elaborate mixed consortia of Bacteria, Archaea, Eukarya and viruses. This study reinforces the utility of community genomics and *de novo* sequence assembly as important methods for the detection and analysis of biological diversity.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 3

**Xenorhodopsins, an enigmatic
new class of microbial rhodopsins
horizontally transferred between
Archaea and Bacteria**

DISCOVERY NOTES

Open Access

Xenorhodopsins, an enigmatic new class of microbial rhodopsins horizontally transferred between archaea and bacteria

Juan A Ugalde¹, Sheila Podell¹, Priya Narasingarao¹ and Eric E Allen^{1,2*}

Abstract

Based on unique, coherent properties of phylogenetic analysis, key amino acid substitutions and structural modeling, we have identified a new class of unusual microbial rhodopsins related to the *Anabaena* sensory rhodopsin (ASR) protein, including multiple homologs not previously recognized. We propose the name xenorhodopsin for this class, reflecting a taxonomically diverse membership spanning five different Bacterial phyla as well as the Euryarchaeotal class Nanohaloarchaea. The patchy phylogenetic distribution of xenorhodopsin homologs is consistent with historical dissemination through horizontal gene transfer. Shared characteristics of xenorhodopsin-containing microbes include the absence of flagellar motility and isolation from high light habitats. Reviewers: This article was reviewed by Dr. Michael Galperin and Dr. Rob Knight.

Findings

Microbial rhodopsins are a widespread family of photoreactive proteins found in all three domains of life. Based on their functional roles, characterized rhodopsin proteins have been classified into three distinct groups: (i) Proton pumps (bacteriorhodopsins and proteorhodopsins), involved in energy generation, (ii) Chloride pumps (halorhodopsins), involved in the maintenance of osmotic balance, and (iii) Sensory rhodopsins, which direct positive and/or negative phototaxis. Microbial proton pumps have the widest ecological niche distribution, and are found throughout the Bacteria and Archaea in hypersaline, marine, and freshwater habitats [1]. Chloride pumps and sensory rhodopsins are mostly limited to halophilic Archaea of class Halobacteria [1], excepting the few characterized examples in the freshwater cyanobacterium *Anabaena* (*Nostoc*) sp. PCC 7120 [2,3] and eukaryotic green algae including *Chlamydomonas reinhardtii* [4].

The evolutionary history of microbial rhodopsins is complex, showing broad but patchy phylogenetic distribution within and across disparate lineages. It has been suggested that horizontal gene transfer (HGT) has

disseminated photoreceptor and photosensory activities across large evolutionary distances [1]. One salient example is a putative sensory rhodopsin found in the bacterium *Anabaena* (*Nostoc*) sp. PCC 7120 (*Anabaena* sensory rhodopsin, ASR). It has been suggested that this protein was originally acquired from a halophilic archaeon by HGT, and may play a sensory role [1,2]. However, sensory function performance has not yet been demonstrated experimentally, and the ASR protein differs from previously described sensory rhodopsins in: (i) a distinct signaling cascade mechanism that employs a soluble transducer protein, rather than the methyl-accepting taxis transducers (HTR proteins) found in halophilic Archaea [2,5] and (ii) its divergent photochemistry, including unique light-induced *cis/trans* configuration dynamics of the retinal chromophore, providing a possible mechanism for sensing and differentiating specific light qualities [3,6].

In the current study, we report the discovery of several new ASR protein homologs with shared characteristics consistent with the designation of a new class of microbial rhodopsins. ASR homologs were found in *Nanosalina* sp. J07AB43 and *Nanosalinum* sp. J07AB56, the first representatives of a newly described major lineage of Archaea (class Nanohaloarchaea) within phylum Euryarchaeota [7]. The *Nanosalina* sp. and *Nanosalinum* sp. rhodopsin proteins are highly similar

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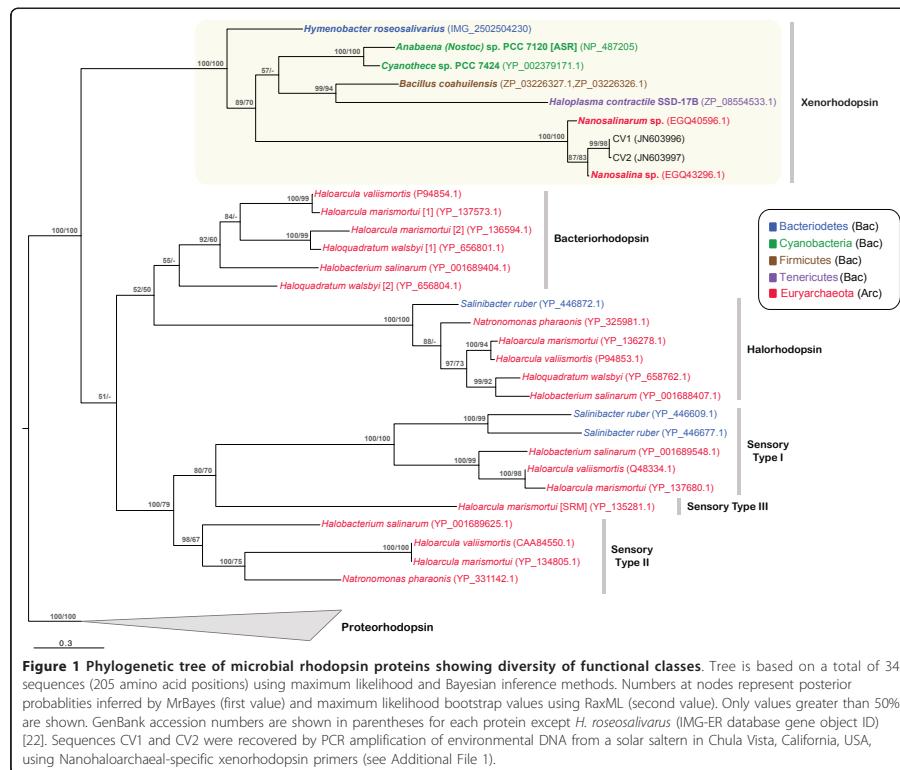
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to each other (89% amino acid identity) and are present in both genomes as single copy genes. Surprisingly, these two Nanohalarchaeal proteins most closely resemble rhodopsins in taxonomically distant *Cyanothecce* sp. PCC 7424 and *Anabaena* (*Nostoc*) sp. PCC 7120, at 31 and 34% amino acid identity respectively. No homologs were identified in other members of the Euryarchaeota, although related proteins were detected at 30–31% amino acid identity in *Bacillus coahuilensis* m4-4 (phylum Firmicutes), a sporulating halophilic bacterium isolated from a desiccation lagoon [8], the psychrophilic bacterium *Hymenobacter roseosalivarius* AA-718 (phylum Bacteroidetes), and the halophilic bacterium *Haloplasma contractile* SSD-17B (phylum Tenericutes) [9,10].

Figure 1 shows a phylogenetic analysis using maximum likelihood and Bayesian inference methods for the ASR homologs, together with a set of representative

protein sequences from all previously recognized functional microbial rhodopsin classes. Methods and experimental procedures are provided in Additional File 1. The phylogenetic tree also includes additional sequences we obtained by PCR amplification using primers specifically targeting Nanohalarchaeal rhodopsin genes. These sequences were recovered from a hypersaline environment (South Bay Salt Works, Chula Vista, California, USA) that is geographically distant from the original isolation site of the Nanohalarchaeal genomes (Lake Tyrrell, Victoria, Australia). Tree topology shows robust clustering of all ASR homologs as a single clade, distinct from other rhodopsin types. We propose the name “xenorhodopsins” to describe this class of rhodopsin proteins, articulating the wide taxonomic diversity of its members.

The patchy distribution and topology of the xenorhodopsin clade is consistent with HGT events between



domains and involving five disparate bacterial phyla. The large numbers of currently sequenced Firmicute (873), Bacteriodetes (169), Cyanobacteria (68), and Haloarchaea genomes (18) lacking xenorhodopsin homologs make it unlikely that the gene/species tree incongruencies shown in Figure 1 could be explained by independent gene loss among multiple species. Sufficiency of taxon sampling and information content in our 205-position trimmed amino acid sequence alignment (Supplementary File 2) are well supported by significant bootstrap values (Figure 1), and corroborated by complete topological agreement between trees constructed using Bayesian and maximum likelihood methods. Additionally, the new xenorhodopsin sequences identified here do not change overall tree topologies of other microbial rhodopsin sequences previously reported in the literature [11].

To supplement HGT analysis based on phylogenetic incongruencies, DNA signature patterns were analyzed for individual xenorhodopsin proteins relative to the genomes in which they were found, based on percent G +C, codon usage patterns, and Interpolated Variable Order Motifs [12] (Additional File 1: Table S1). By all of these criteria, xenorhodopsin genes in *Nanosalinarum* J07AB56, *Cyanothece* PCC 7424, *Nostoc* PCC 7120, *Hymenobacter roseosalivarius*, and *Haloplasma contractile* closely resemble other loci within their respective genomes. These data support the likelihood that the observed incongruencies between xenorhodopsin protein and species trees for these genomes represent ancient rather than recent HGT events, with subsequent amelioration of foreign DNA signatures over time. A different pattern was observed for xenorhodopsin proteins in the *Bacillus coahuilensis* and *Nanosalina* J07AB43 genomes, where atypical codon usage suggests that HGT events may have occurred more recently (Additional File 1: Table S1).

The absence of xenorhodopsin genes in all Euryarchaeota other than members of class Nanohaloarchaea suggests that these genes were acquired subsequent to divergence of Nanohaloarchaea from other Euryarchaeotal classes. The high degree of similarity among xenorhodopsin proteins obtained from two different Nanohaloarchaeal genera, as well as environmental sequences from a distant geographical location (North America versus Australia), is consistent with inheritance from a common ancestral source, coupled with strong selective pressure for amino acid sequence conservation. The discrepancy between ancestral inheritance and the atypical codon usage pattern observed in the *Nanosalina* J07AB43 protein may be explained by relatively recent secondary exchange with other Nanohaloarchaea, as multiple genera of this lineage are known to coexist in shared habitats [7].

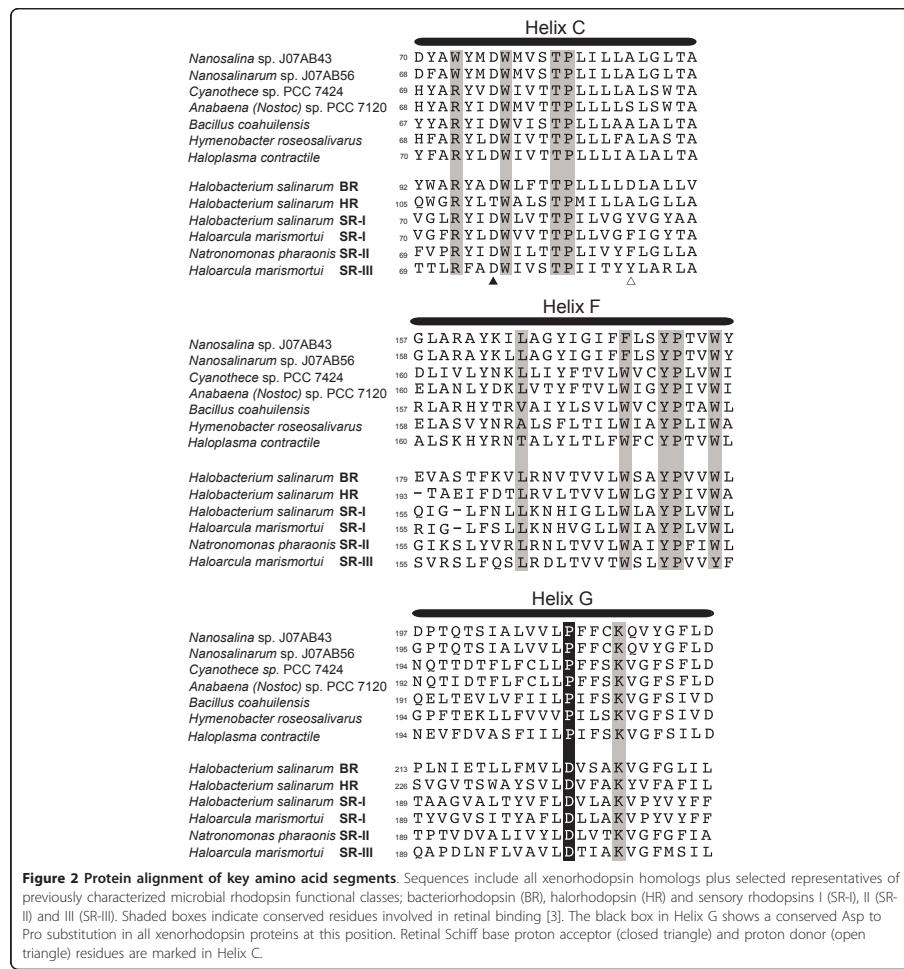
The phylogenetic tree presented in Figure 1 includes only known, modern representatives of lineages that may have incorporated multiple HGT events between extinct ancestors and/or serial exchanges with unknown species whose genomes have not yet been sequenced. Although the complexity of these relationships precludes confident reconstruction of the exact timing, direction, and order of individual gene transfer events, cross-domain and cross-phylum gene acquisition through HGT provides the most parsimonious explanation for the data.

Amino acid alignments of residues known to determine function for previously characterized microbial rhodopsins are inconsistent with proton or chloride transporting activity for xenorhodopsins, suggesting a possible sensory role (see Additional File 2 for full alignment). Figure 2 shows that residues required to bind the retinal chromophore molecule are conserved across all xenorhodopsin group members. Ion transporting rhodopsins can be distinguished from sensory rhodopsins by comparing the residues that serve as the retinal Schiff base proton donor and proton acceptor during the photocycle [2,13]. These residues correspond to Asp98 (acceptor) and Asp109 (donor) in the *H. salinarum* bacteriorhodopsin (Helix C). Consistent with previously described sensory rhodopsins, ASR and all other xenorhodopsin homologs lack the canonical Asp residue at the donor position, a hallmark of proton translocating rhodopsins. Likewise, known sensory rhodopsins and xenorhodopsins both lack the Thr (acceptor) and Ala (donor) configuration diagnostic of chloride pumps (Figure 2).

Despite the insights provided by these results, it is not possible to predict functional activity based on sequence alignment alone. The structural sensitivity of microbial rhodopsins is highlighted by the ability to engineer aberrant functional properties in these proteins. A single amino acid substitution, Asp217 to Glu, has been shown to confer inward proton pumping activity to the ASR protein [14] and a single amino acid substitution is sufficient to convert a bacteriorhodopsin proton pump into a chloride pump [15].

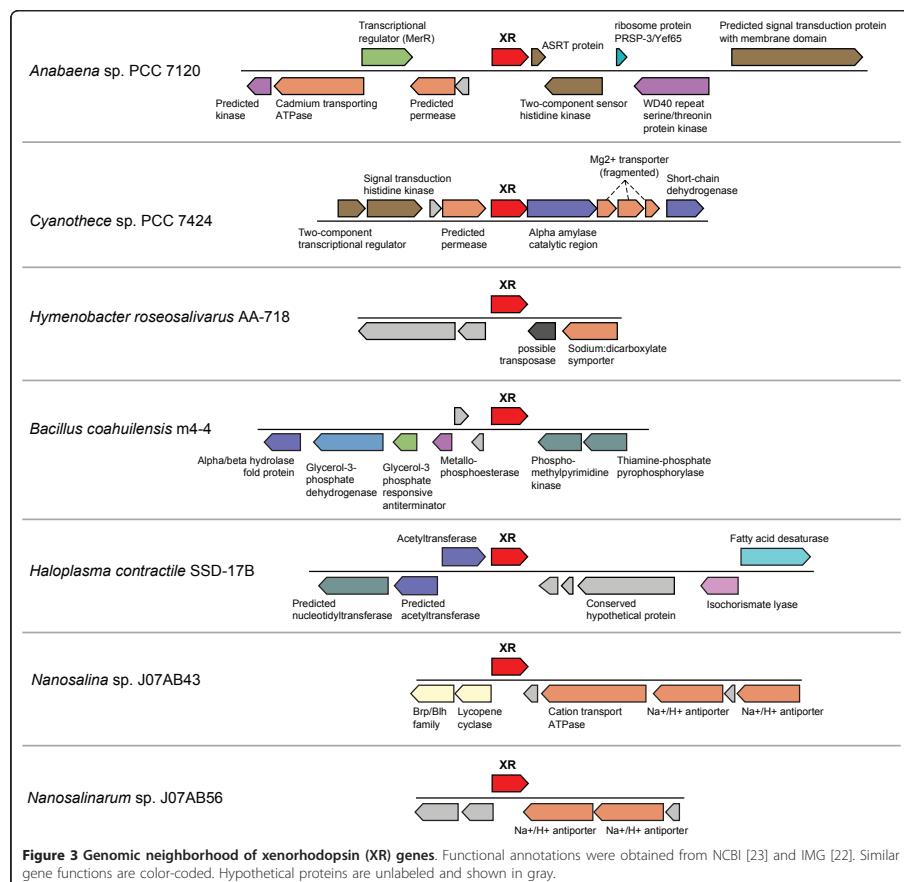
One prominent difference between the xenorhodopsins and all other microbial rhodopsin proteins is a universal Pro to Asp substitution (Helix G), a substitution noted previously in the *Anabaena* (*Nostoc*) sp. PCC 7120 and *B. coahuilensis* homologs [8,16]. The shared position of this residue in all xenorhodopsins discovered to date suggests that it may provide a useful diagnostic for this protein class.

Sequence conservation and phylogenetic analysis of xenorhodopsin proteins is strongly supported by comparative 3-dimensional protein structure modeling. This similarity is illustrated in Additional File 3, showing a



SWISS-MODEL [17] prediction of the *Nanosalina* sp. rhodopsin structure using ASR as a template. The modeled structure demonstrates high congruence in residues that form the retinal binding pocket, as well as similar truncations in loop motifs (Additional File 3). The conserved primary and tertiary structure of xenorhodopsins combined with their distinct phylogenetic clustering supports their classification as a coherent, highly conserved group.

An important element of previously characterized sensory rhodopsins in halophilic Archaea is the presence of a signal transduction mechanism, most often genetically encoded in a genomic position adjacent to the rhodopsin gene [18]. In *Anabaena* (*Nostoc*) sp. PCC 7120, the proposed soluble transducer protein ASRT (*Anabaena* sensory rhodopsin transducer) is encoded by a gene in the same operon as ASR [18] (Figure 3). Consistent with its putative role in light-activated sensory transduction,



the ASRT protein has been shown to bind DNA, specifically the promoter region of genes involved in the synthesis of light-harvesting accessory pigments [19]. However, no homologs of ASRT were identified in other genomes containing a xenorhodopsin gene, suggesting the ASR-ASRT association is a specific feature of *Anabaena* (*Nostoc*) sp. PCC 7120. Moreover, the identification of ASRT homologs in numerous bacterial and archaeal genomes that lack an ASR (xenorhodopsin) homolog suggests the ASRT protein family is not specific to photosensory signal transduction processes.

The lack of identifiable common transducer elements suggests possible plasticity in the transducer component

(s) modulating possible xenorhodopsin-mediated photosensory activity. For example, *Cyanothece* sp. PCC 7424 has genes encoding a two-component regulatory system within the same genomic neighborhood as the xenorhodopsin gene (Figure 3). The two Nanohaloarchaeal genomes (*Nanosalina* sp. and *Nanosalinum* sp.) have genes encoding a putative Na⁺/H⁺ antiporter system adjacent to the rhodopsin gene. The high sequence identity shared between these transporter sequences along with their conserved genomic location is atypical for these two archaeal genomes, representing different genera, which are generally non-syntenic [7]. It is tempting to speculate that genes in this local region of

conservation could be related to rhodopsin function in these organisms.

Despite highly diverse taxonomic origins, the seven species possessing a xenorhodopsin protein share a number of common characteristics, including the absence of flagellar motility, relatively low genomic percent G+C content and isolation from habitats with a high incidence of UV light (Additional File 1: Table S2). The lack of flagellar motility is noteworthy because it eliminates the potential usefulness of previously characterized sensory rhodopsin classes which act by influencing the rotational state of the flagellar motor for phototaxis. The particularly low G+C compositions of *Nanosalina* sp. (43%), *Anabaena* (*Nostoc*) sp. PCC 7120 (41%), *Cyanothece* sp. PCC 7424 (38%), *Bacillus coahuilensis* (38%) and *H. contractile* (33.6%) are atypical for unicellular inhabitants of high light environments, rendering them especially sensitive to potential UV damage via the formation of thymidine dimers. The isolation of *H. contractile* from deep marine sediments, where light is not a factor, may be an anomaly, since closely related 16S rRNA gene sequences have also been found in high-light solar salt-enriched environments [9].

Consistent with these observations, one intriguing hypothesis is that xenorhodopsins may play a role in pre-emptive photoprotection by inducing light-dependent changes in the expression of photoprotective pigments, a role proposed for the ASR protein due to its photochromic properties [3,6]. Alternatively, these proteins could be linked to expression of DNA repair mechanisms. However, these speculations must be tempered by the caveat that no sensory or ion transport function has yet been experimentally validated for ASR, or any other xenorhodopsin protein. Future work on the biochemistry, photochemistry, and molecular genetic characterization of the xenorhodopsin class of proteins will undoubtedly provide fascinating insights into their physiological function in light-induced biological processes.

Reviewers' comments

Reviewer 1

Dr. Michael Y. Galperin, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD, USA

I agree with the authors' conclusion that *Anabaena* sensory rhodopsin (ASR) and closely related proteins form a separate family of rhodopsins. However, I believe that the current version of the paper would need a substantial revision to become acceptable for *Biology Direct*.

The notion that ASR comprises a new type of sensory rhodopsins is not new and should not be presented as

such. Spudich and colleagues described the uniqueness of ASR in their early papers [2,16] and unequivocally stated that ASR belongs to a separate family [6]. This does not diminish the contribution of this work, which describes six new members of that family, but the text of the Abstract and the tone of the whole paper must be changed.

Author's response

We thank the reviewer for bringing to our attention these deficiencies in our original summary of previous work recognizing the uniqueness of the ASR protein. We have modified the manuscript to address these issues by changing the title, the abstract, and the interpretational emphasis of our text. We believe these revisions clarify the significance of our findings in discovering that the ASR protein is not a single, isolated anomaly, but rather part of a large, cohesive family of related proteins with an unusual taxonomic distribution. To further emphasize this point, we propose the name "xenorhodopsin" to describe the members of this group, rather than calling them ASR-like (or Sensory Rhodopsin-IV) proteins.

Although the name "Anabaena sensory rhodopsin" is being widely used in the literature, it is important to note that there has been no experimental proof that this protein actually performs sensory function. Indeed, ASR has been shown not to function as a proton pump and it has been reasoned that it is unlikely to work as a chloride pump. Nevertheless, there remains a distinct possibility that ASR functions as a membrane pump for some other ion, for example, sodium. This proposal is hardly more speculative than the suggestion of the sensory function and is supported by at least three lines of evidence:

- 1) the adjacency of genes coding for ASR homologs and Na^+/H^+ antiporters, noted by the authors themselves.
- 2) the observation of Kawanabe *et al.* [14] that a single amino acid change converts ASR into an inward proton pump; and 3) the observation of De Souza *et al.* [20] that so-called ASR transducer is found in variety of genomes that do not encode ASR and is likely to bind sugars. Further, the previously overlooked absence of the ASRT gene in the complete genome of *Cyanothece* sp. PCC7424 and its recently reported ability to bind DNA [19] strongly suggest that the putative ASR-ASRT signaling cascade is a specific feature of *Anabaena* sp. PCC7120. The authors correctly point out the absence of flagellar motility in the ASR-carrying organisms; this argument, however, is weakened by the chemotactic ability of both *Anabaena* sp. PCC7120 and *Cyanothece* sp. PCC7424, owing to the presence of 3 and 9 methyl-accepting chemotaxis sensors, respectively [21]. In the absence of direct experimental data, the authors should

discuss possible alternative functions of the ASR-like family and should be more careful in describing this new rhodopsin family as sensory rhodopsins.

Author's response

We have expanded the text to include a discussion of possible alternative functions for the xenorhodopsin family, including how lack of experimental evidence for ASR sensory function affects interpretation of conserved amino acid sequences, the importance of mutational experiments demonstrating gain of inward proton pumping function, and the apparent species-specific nature of the ASR/ASRT interactions.

I would also suggest moving the Supplementary Figure S1 (Genomic neighborhood of SR-IV genes) to the main text.

Author's response

The previously presented Supplementary Figure S1 is now Figure 3 in the main text.

Reviewer 2

Dr. Rob Knight, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, USA

In this manuscript, the authors analyze a set of microbial rhodopsin sequences (including some that they amplified for this study from an environmental sample), and demonstrate that there is a new clade of sensory rhodopsins that is basal, with high bootstrap support, and that includes sequences from a surprisingly broad phylogenetic range (including one archaeal and three bacterial phyla). This distribution is interesting because previous studies of sensory rhodopsins have found them primarily in the Euryarchaeota and in the Bacteroidetes.

The methods are generally sound except that the taxonomy of the sister groups to the new clade is poorly resolved (i.e. non-significant bootstraps), and it would be reassuring if the split were confirmed using other phylogenetic methods besides likelihood (e.g. distance or Bayesian methods) before the new set of sequences was claimed as distinct.

Author's response

We have supplemented our original phylogenetic analysis with Bayesian and distance-based methods, and find that all agree in supporting identical tree topologies. We have revised Figure 1 and the text to clarify the fact that the topologies agree and that bootstrap values supporting branches relevant to the new clade are highly significant using all methods.

Additionally, although the patchy phylogenetic distribution is suggestive of horizontal gene transfer, formal methods (of which several exist) should be used to confirm HGT as opposed to other factors that can lead to gene/species tree incongruence.

Author's response

Although many methods of HGT detection have been proposed in the literature, their lack of consistency and potential unreliability in the face of complex, real world data have long been a matter of controversy and debate. Phylogenetic tree incongruency is currently considered the gold standard by which all other HGT prediction methods are judged, and this is the primary technique we have used to reach conclusions presented in the manuscript, which we believe are compelling.

To supplement the phylogenetic analyses, we have performed several additional HGT analyses using methods based on DNA signature patterns, included these results as Supplementary Table S1, and expanded discussion of HGT in the text to include interpretation of these additional results.

Additional material

Additional File 1: Supplementary Methods and Tables.

Additional File 2: Trimmed amino acid alignment file of microbial rhodopsin sequences.

Additional File 3: SWISS-MODEL 3-dimensional protein structure model of *Nanosalina* sp. xenorhodopsin using ASR as a template.

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Authors' contributions

All authors conceived the study. JAU and SP performed sequence analysis. PN performed experiments. JAU, SP and EEA wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Chapter 4

Assembly-driven community genomics of a hypersaline microbial ecosystem

Assembly-Driven Community Genomics of a Hypersaline Microbial Ecosystem

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Abstract

Microbial populations inhabiting a natural hypersaline lake ecosystem in Lake Tyrrell, Victoria, Australia, have been characterized using deep metagenomic sampling, iterative *de novo* assembly, and multidimensional phylogenetic binning. Composite genomes representing habitat-specific microbial populations were reconstructed for eleven different archaea and one bacterium, comprising between 0.6 and 14.1% of the planktonic community. Eight of the eleven archaeal genomes were from microbial species without previously cultured representatives. These new genomes provide habitat-specific reference sequences enabling detailed, lineage-specific compartmentalization of predicted functional capabilities and cellular properties associated with both dominant and less abundant community members, including organisms previously known only by their 16S rRNA sequences. Together, these data provide a comprehensive, culture-independent genomic blueprint for ecosystem-wide analysis of protein functions, population structure, and lifestyles of co-existing, co-evolving microbial groups within the same natural habitat. The “assembly-driven” community genomic approach demonstrated in this study advances our ability to push beyond single gene investigations, and promotes genome-scale reconstructions as a tangible goal in the quest to define the metabolic, ecological, and evolutionary dynamics that underpin environmental microbial diversity.

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Introduction

Microbial diversity studies based on 16S rRNA gene amplification have identified large numbers of uncultured, uncharacterized organisms whose metabolic capabilities, lifestyle strategies, and ecosystem contributions remain largely unknown. Conversely, the subset of cultured microbial species from any particular habitat often fails to include even some of the most abundant members of the community. Efforts to bring these unknown organisms into laboratory culture are confounded by our limited understanding of the metabolic specializations of environmental microorganisms, the interdependencies of intra-/inter-species interactions, and the physicochemical conditions that promote or diminish microbial survival and population structure in natural environments.

Direct metagenomic sequencing of environmental samples can potentially provide functional information missing from 16S rRNA gene surveys and circumvent the constrained diversity found in representative cultured isolates. Composite genomes have been assembled from several environmental data sets [1,2,3,4,5,6], however comprehensive characterization of the genetic diversity of most naturally occurring microbial communities remains a significant challenge. Environmental sampling of predicted met-

abolic functions as a simple “bag of genes” via metagenomic read-based analysis cannot fully capture the genetic and metabolic potential of individual populations, and may overlook the significance of community-wide processes involving cooperative interactions between multiple species [7,8,9].

Reference genomes from cultured isolates and/or single-cell projects can greatly assist in taxonomic assignment of genes encoded on short metagenomic DNA fragments. However, with the recent exception of the human microbiome project [10], the time, effort, and expense required to develop reference resources of sufficient breadth to adequately represent the full diversity of most ecosystems using these methods are currently prohibitive, and the vast majority of environmentally identified species remain uncharacterized.

The issue of inadequate database representation is particularly relevant for microbial communities in extreme hypersaline aquatic environments, which are dominated by archaeal populations [11]. These environments provide an attractive model for studying microbial ecology, because the demands of surviving such extreme conditions limit taxonomic diversity, yet cell densities frequently exceed $10^7\text{--}10^8$ per mL [12]. The aquatic milieu allows convenient large-scale sampling and fractionation of discrete

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populations in particular size ranges, simplifying many types of analysis. These ecosystems have been well-studied historically using culture dependent methods, 16S rRNA gene surveys and, more recently, single-cell genomics and metagenomics (reviewed in [11]). Despite these advances, the number of available sequenced genomes relevant to microbial communities in this specific habitat remains very small, and is not representative of the *in situ* diversity present in a natural microbial assemblage.

The extreme hypersaline habitat of Lake Tyrrell, Australia has recently been used to demonstrate the utility of *de novo* metagenomic assembly for characterizing organisms previously known only by their 16S rRNA gene sequences, including representatives of a globally distributed new class of Archaea, the Nanohaloarchaea [13,14]. In the current study, we extend this previous work, combining cell size-fractionated sample collection, deep metagenomic sequencing, multidimensional phylogenetic binning, and iterative *de novo* assembly to reconstruct ten additional population genomes. These new genomes provide a comprehensive, culture-independent genomic blueprint for ecosystem-wide analysis of protein functions, population structure, and lifestyles linked to specific microbial strains co-existing and co-evolving within the same natural habitat.

Materials and Methods

Sample Collection, Library Construction and Sequencing

Surface water samples collected from Lake Tyrrell, Victoria, Australia at 0.3 m depth were passed through filters of decreasing porosities ($20\text{ }\mu\text{m} > 3\text{ }\mu\text{m} > 0.8\text{ }\mu\text{m} > 0.1\text{ }\mu\text{m}$) to obtain fractions enriched by cellular size [13]. Physical properties of the collection site are summarized in **Table S1**. Sanger sequencing libraries were constructed at the J. Craig Venter Institute using DNA from $0.8\text{ }\mu\text{m}$ and $0.1\text{ }\mu\text{m}$ filters [15], and sequenced using both paired-end Sanger sequencing and Roche 454 Titanium pyrosequencing (**Table S2**). 16S rRNA gene clone libraries were constructed from the same DNA samples used for sequencing, using archaeal primer sequences Arc21F ($5'\text{-TTCCGGTTGATCCTGCCGGA-3'}$) and Arc529R ($5'\text{-ACCGCGGGCKGCTGGC-3'}$) and bacterial primer sequences 27F ($5'\text{-AGAGTTTGATCCTGGCTCAG-3'}$) and 1391R ($5'\text{-GACGGGCRGTGWGTRCA-3'}$) [16].

Lake Tyrrell Metagenome Assembly

Assemblies were performed using Celera Assembler software version 5.4 [17]. Read sizes, library sources, and the assembled positions of reads in contigs and scaffolds were extracted from the Celera Assembler ACE output file into a local MySQL database using custom perl scripts. Numbers of scaffold nucleotides, percentages of reads obtained from different libraries, and local coverage depth for specific scaffold subregions were calculated from SQL database queries.

Figure S1 summarizes the bioinformatic assembly pipeline. All trimmed Sanger reads were combined into a composite pool for initial assembly. Scaffolds from this assembly were classified into groups using the phylogenetic binning procedures described below, then used to construct a custom reference library for PhymmBL version 3.2 [18], to assign unassembled 454 Titanium reads to taxonomic bins.

After an initial composite assembly of total community DNA, iterative rounds of *de novo* assembly were performed on taxonomic subgroups identified by scaffolds sharing common signatures based on multiple independent properties, to optimize assembly fidelity for each group individually. Each taxonomic subgroup was assembled independently using a previously described subtractive enrichment strategy based on iterative scaffold binning [13].

Scaffolds were re-binned and subsequently deconstructed into their component reads after each assembly iteration. Reads associated with scaffolds having properties characteristic of a subgroup other than the one currently being targeted were removed prior to the next round of assembly. To avoid over-pruning, singletons and reads associated with unclassified scaffolds were retained in successive rounds of assembly.

Taxonomic binning, subtractive enrichment, read deconstruction, and re-assembly steps were repeated for each taxonomic subgroup until no misassemblies were detected and no improvement was observed in completeness of conserved marker genes, maximum contig length, number and size of scaffold gaps, or uniformity of binning parameters for scaffolds >50 Kb. Assembly quality was confirmed by visual inspection using Hawkeye [19] to assess mate-pair consistency and read depth uniformity.

Archaeal genome assembly completeness was evaluated based on 53 transcription, translation, and replication genes nearly universally conserved in Archaea [20,21,22]. Bacterial draft genome completeness was assessed using the Core Gene Evaluation Script developed for the Human Microbiome Project [23]. Metagenomic sequence data has been deposited at DDBJ/EMBL/GenBank under the accession APHM00000000, NCBI BioProject number PRJNA59457. Assembled genome sequences have been deposited in the JGI-Integrated Microbial Genome resource [24] under taxon-oid numbers 2502082092 (J07HX64), 2506783034 (J07HB67), 2512875005 (J07HQW1), 2512875006 (J07HQW2), 2512875007 (J07HN4), 2512875008 (J07HN6), 2512875009 (J07HQX50), 2512875010 (J07HX5), 2512875011 (J07HR59), and 2513020022 (J07SB67).

Phylogenetic Binning and Scaffold Annotation

Raw metagenomic reads and assembled scaffolds containing 16S rRNA gene sequences were identified by BLASTN search against the GreenGenes reference database [25], requiring a minimum alignment length of 200 nucleotides and e-value of 1e-7 or better. Scaffold genes were predicted and annotated using the Integrated Microbial Genomes Expert Review (IMG/ER and IMG/MER) systems [24]. Averaged amino acid frequencies for all predicted proteins on each scaffold were calculated using a custom perl script. Taxonomic associations of predicted protein matches to GenBank nr reference sequences were tallied using DarkHorse version 1.4 [26].

Non-metric multidimensional scaling (MDS) analysis was performed on scaffolds of 5000 nucleotides or longer containing $<50\%$ gap residues using Primer version 6.1.2 [27]. Scaffold input properties included nucleotide percent G+C; read depth; percent of reads from $0.1\text{ }\mu\text{m}$ filters; percentages of lysine, arginine, threonine, glutamic acid, aspartic acid, alanine, valine and isoleucine in predicted proteins; and percent of proteins with DarkHorse-filtered best matches to Eukaryota, Bacteria, Viruses, Nanohaloarchaea, and the genera *Halococcus*, *Halorubidus*, *Halococcus*, *Halorubrum*, *Haloflexax*, *Halogeometricum*, and *Salinibacter*. Scaffolds sharing a common signature based on these metrics were placed in the same taxonomic bin.

Unassigned scaffolds were searched against Lake Tyrrell-specific genome assemblies using BLASTN to identify potential variant sequences associated with strain level heterogeneity present in the natural population but not captured by targeted *de novo* assembly. Unassigned scaffolds matching a composite reference genome at 85% or higher average nucleotide identity (ANI) over $>40\%$ of their length were classified in the same “population group” as the matched genome [28]. Scaffolds matching at 95% or higher ANI were assigned to the same species. Total numbers of nucleotides for binned scaffolds in each population group, including species-

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level classifications, were calculated using SQL queries from assembly-specific MySQL databases, and converted to a proportional treemap graph using the TreeMap package in R, version 2.14.1 [29].

Construction of Phylogenetic Trees

The Greengenes alignment tool NAST [25] was used to construct a reference alignment of 16S rRNA genes from assembled scaffolds, cultured isolate reference genomes, and closely related environmental sequences. Maximum likelihood reference trees were constructed using RaxML version 7.2.7 [30] and FastTree version 2.1.1 [31]. Partial 16S rRNA gene sequences from unamplified metagenomic reads and Lake Tyrrell PCR amplified clone libraries were inserted into reference trees using placere version 1.1 [32] and visualized using Archaeopteryx version 0.968 [33]. Amplified 16S rRNA sequences from Lake Tyrrell community DNA have been submitted to NCBI under accession numbers JX880413–JX81179 (archaeal) and JX881180–JX885105 (bacterial).

Clustering of Predicted Proteins

Predicted proteins were clustered into families using an unsupervised Markov Clustering algorithm (MCL software version 10–201), with BLASTP e-value cutoff 1e-5 and inflation parameter setting 1.4 [34]. Protein family diversity was estimated using MOTHUR version 1.23.1 [35]. Assembled genomes were clustered together based on their profiles of shared protein families using the modularity analysis function of Gephi, version 0.8.1 [36].

Results

Community Sequence Assembly

Metagenomic sequence assembly effectiveness for combined Sanger libraries was assessed statistically (**Table S3**), and visualized by comparing histograms of nucleotide composition (percent G+C) for unassembled reads versus assembled scaffolds and population genomes (**Figure 1**). Raw metagenomic sequencing reads prior to assembly have a broad, biphasic nucleotide distribution, reflecting their heterogeneous origin. The percent G+C distribution of assembled scaffolds is more tightly focused into discrete peaks because the assembly process consolidates multiple overlapping reads into longer, consensus sequences with uniform properties. The length-weighted nucleotide distribution for scaffolds thus reveals overall patterns that are hidden by random noise in the shorter read sequences.

Because the percent G+C content of individual microorganisms tends to be relatively uniform when averaged over long stretches of DNA, consolidated scaffold peaks in a length-weighted G+C histogram like **Figure 1** are useful in surveying diversity of dominant microbial populations within a mixed community. Prominent scaffold peaks at 43, 49, 56, 60–62, 64, and 67% GC suggested that the Lake Tyrrell microbial community contains at least 6 different abundant genomic populations. This observation was confirmed by the reconstruction of one or more composite genomes from each major peak (**Table 1**), including multiple archaeal populations with similar G+C compositions within broader peaks at 47–50%, 59–61%, and 63–64% G+C, and both archaeal and bacterial populations within the 67% G+C peak.

16S rRNA diversity

Assembled sequences contained 34 distinct 16S rRNA gene sequences of 450 nt or longer, including 27 longer than 700 nt (**Table S4**). One scaffold contained a full-length 16S rRNA sequence that was 97% identical to cultured isolates of the

halophilic bacterium *Salinibacter ruber*. The remaining 16S rRNA sequences were all archaeal, based on both BLAST searches against the Greengenes database and phylogenetic placement relative to characterized 16S rRNA gene sequences in a maximum-likelihood phylogenetic tree (**Figure 2**). Assembled archaeal 16S rRNA genes were distributed among seven broad phylogenetic groups, including class Nanohaloarchaea and relatives of previously sequenced isolates from Halobacterial genera *Halococcus*, *Halomonas*, *Halorubrum*, *Halobaculum*, *Halorhabdus*, and *Halococcus*. Nearly all assembled 16S rRNA gene sequences had closer matches among uncharacterized environmental clones than sequenced isolate genomes.

A composite phylogenetic tree comparing archaeal 16S rRNA sequences from assembled scaffolds with the shorter, unassembled fragments (>350 nt) present in raw reads, placed >99% (1187/1202) of the unassembled read sequences into branches that were either basal, adjacent or identical to sequences represented by assembled scaffolds (**Figure S2a**). Assignment of basal positions to some of the shorter sequences present in unassembled reads reflects the unavailability of sufficient information to accurately resolve the placement of these 16S gene fragments. Several low-abundance clusters found in raw reads were not detected among the assembled scaffolds. These sequences were placed on branches adjacent to *Halococcus ruber*, *Haladaptatus paucihalophilus* and *Halobacter salinarum*.

A similar, but less complete pattern of extended archaeal microdiversity was observed in archaeal PCR products when compared with assembled scaffold sequences (**Figure S2b**). A number of lineages present in both assembled scaffolds and raw metagenomic reads were missing from the PCR-generated 16S rRNA sequences. This result is consistent with previously described cases of universal archaeal primer bias preventing detection of novel archaeal taxa via PCR amplification [13,37].

Eighty-five percent of the sequences amplified with archaeal primers matched assembled metagenomic scaffold sequences at 97% or greater sequence identity, suggesting membership in the same species. An additional 5% of the archaeal amplicons matched assembled sequences at 95–97% identity, most likely representing different species of the same genus. Eighty-eight percent of the 16S rRNA amplicons obtained using bacterial primers matched cultured isolates of *Salinibacter ruber* at 97% or higher identity, confirming the dominance of this lineage among the bacterial community that was also observed in the assembled scaffolds.

Scaffold Binning and Targeted Genome Reconstruction

Eleven distinctive scaffold clusters were identified by applying the technique of Non-Metric Multidimensional Scaling to scaffold properties used for phylogenetic binning (**Figure S3**, **Table S5**). Each cluster was subjected to targeted iterative assembly yielding twelve genomes, eleven archaeal and one bacterial (**Table 1**). Each of these genomes represents the composite sampling of multiple individuals belonging to a genetically-similar population of closely related cells (species), approximating the dominant genotype extracted from a larger, polymorphic pool of closely related variants (strains). The treemap illustration presented in **Figure 3** shows the relative abundances of these populations in the context of all assembled scaffolds, organized according to taxonomically related population groups. This figure highlights the fact that each major population group contained multiple scaffold groups that could be identified as closely related to each other, but not necessarily assigned to specific genomes.

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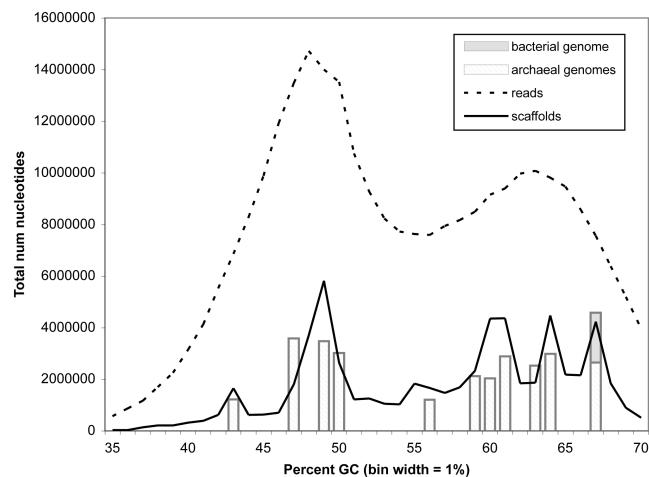


Figure 1. Length-weighted %G+C nucleotide composition of unassembled reads, assembled scaffolds, and composite population genomes. Genomes were constructed by targeted assembly of scaffolds with a uniform signature of phylogenetic binning properties, as described in Materials and Methods. Genome names, percent G+C, and other general properties of assembled genomes are shown in Table 1.
doi:10.1371/journal.pone.0061692.g001

Taxonomic Groups in Assembled Scaffolds

Haloquadratum-related populations J07HQW1, J07HQW2, and J07HQX50. Microbial populations related to cultured isolates of *Haloquadratum walsbyi* comprised 38% of the assembled Lake Tyrrell community sequences. Three distinct population genomes were reconstructed, named J07HQW1, J07HQW2, and J07HQX50. Based on 16S rRNA sequence identity, J07HQW1 (99%) was more closely related to *H. walsbyi* cultured isolates than J07HQW2 (97%) or J07HQX50 (93%). These relationships were

confirmed by adjacency in a maximum-likelihood phylogenetic tree (Figure 2). Mean assembly depths of coverage for both J07HQW1 and J07HQW2 (8.8-fold) were more than three-fold higher than for J07HQX50 (2.5-fold), suggesting considerably greater environmental abundance (Figure S4).

Authenticity of assembled 16S rRNA gene sequences from groups J07HQW1, J07HQW2, and J07HQX50 were corroborated by the presence of identical sequences in independent PCR clone libraries, as well as near-exact matches (>99% identity) in

Table 1. Consensus population genome properties.

Genome name	Length (nt)	G+C pct	num scf	rRNA operons	tRNAs	predicted CDS	pct complete marker genes§
<i>Haloquadratum walsbyi</i> str J07HQW1	3,594,539	47	1	2	47	3,584	100
<i>Haloquadratum walsbyi</i> str J07HQW2	3,475,501	49	1	2	52	3,856	98
<i>Haloquadratum</i> sp. J07HQX50	3,019,909	50	2	1(2)*	39	2,872	91
<i>Nanosalinum</i> sp. J07AB56	1,215,802	56	3	1	38	1,454	100
<i>Nanosalina</i> sp. J07AB43	1,227,157	43	7	1	59	1,739	83
<i>Halonotius</i> sp. J07HN4	2,888,659	61	2	1	52	3,230	100
<i>Halonotius</i> sp. J07HN6	2,529,000	63	6	1	47	2,914	100
uncultured archaeon sp. J07HX64	2,982,938	64	1	1	43	3,095	92
uncultured archaeon sp. J07HX5	2,040,945	60	1	1(2)*	24	2,139	53
<i>Halobaculum</i> sp. J07HB67	2,649,547	67	3	1	37	2,707	94
<i>Halorubrum</i> sp. J07HR59	2,120,805	59	7	1(3)*	26	1,841	83
<i>Salinibacter</i> sp. J07SB67	1,931,021	67	443	nd	13	1,641	39

§Marker gene detection details are shown in Table S6.

*Parenthetical values indicate cases where locally elevated depth of coverage suggests that assembly software may have compressed multiple 16S gene copies into a single locus.

doi:10.1371/journal.pone.0061692.t001

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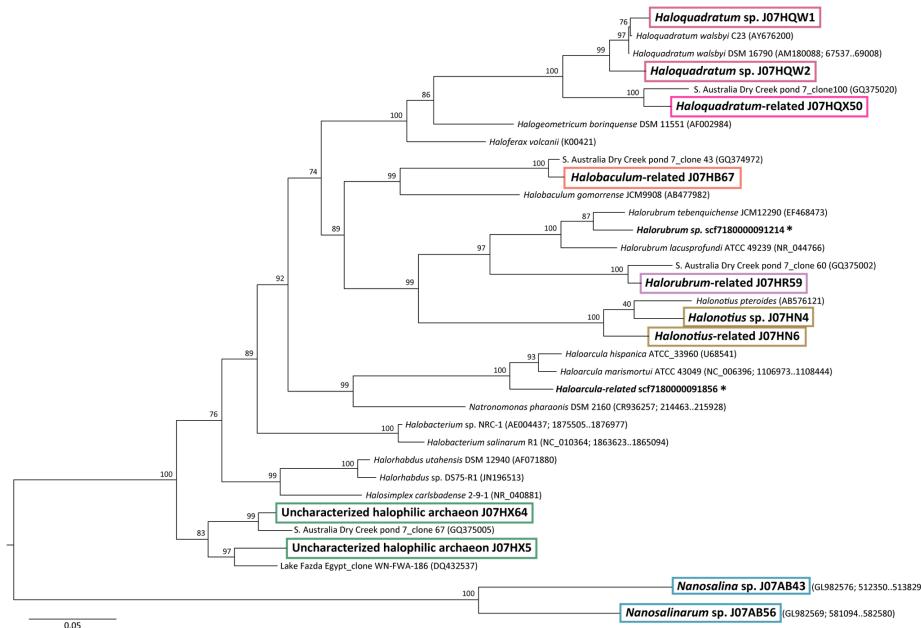


Figure 2. Phylogenetic distribution of archaeal 16S rRNA gene sequences in assembled scaffolds and population genomes. Names in bold indicate new 16S rRNA sequences identified in this study. Boxed names indicate sequences contained within Lake Tyrrell-specific population genomes. Asterisks indicate isolated individual sequences found on small scaffolds that were not associated with any assembled population genome.
doi:10.1371/journal.pone.0061692.g002

16S rRNA sequences amplified by other investigators studying a different Australian hypersaline habitat [38]. In that study, sequences most closely matching J07HQX50 (phylogroup 2) were suggested to represent a separate genus from *H. walsbyi* strains C23 and DSM 16790. BLASTP analysis of predicted proteins in all three *Haloquadratum*-related genomes against Genbank nr reinforced taxonomic the relationships observed with 16S rRNA genes (Figure 4). The J07HQW1, J07HQW2, and J07HQX50 genomes all included a significant number of core gene matches to *H. walsbyi* cultured isolates. However, the overall percentage of predicted proteins with best matches to previously sequenced *Haloquadratum* genomes was less than half in J07HQX50 (28%) compared to J07HQW1 (58%), consistent with evolutionary diversification as a separate genus.

For populations like J07HQX50, where no physical data is available, distribution of scaffold reads between libraries obtained from 0.1 versus 0.8 µm filters can be used to obtain rough estimate of cell size. Although it is not possible to determine exact cell size from read library distributions, high and low ends of the microbial size range sampled in Lake Tyrrell can be bracketed based on microscopically observed diameters of approximately 2 µm for the square cells of cultured *Haloquadratum* isolates (80% on 0.8 µm filters) and 0.6 µm for coccus-shaped environmental Nanohaloarchaea (<10% on 0.8 µm filters) [13].

Eighty percent of reads from scaffolds in all three *Haloquadratum*-related genomes were isolated from 0.8 µm pore filters, making

them the largest cells in the current study. Finding 20% of the reads on 0.1 µm pore filters was initially unanticipated, based on the diameter of cultured *Haloquadratum* isolates and their known propensity to form multicellular aggregates. However, cultured *Haloquadratum* cells contain especially fragile internal gas vesicles, susceptible to collapse under pressures experienced during cellular concentration by filtration [39,40]. In addition, nominal pore sizes reported for fiber-based filters are average values for a non-uniform size distribution that covers a wider range, explaining why some cells, especially those with flexible and/or asymmetric shapes, can routinely pass through filters with smaller than expected pore sizes.

Nanohaloarchaea populations J07AB43 and J07AB56. Sequences from archaeal class Nanohaloarchaea accounted for approximately 17% of the assembled microbial community, forming the second most abundant microbial group. Taxonomic binning of scaffolds from this group was facilitated by their significant divergence from other microbial groups in nucleotide G+C compositions, 16S rRNA gene sequences, predicted amino acid frequencies, and filter size distribution of reads [13]. Finding greater than 90% of the J07AB43 and J07AB56 reads in 0.1 µm pore filters agrees with previously reported cell diameters of approximately 0.6 microns, and suggests that they are the smallest cells whose genomes were assembled from the Lake Tyrrell environmental sequences.

Hypersaline Habitat-Specific Genome Assembly

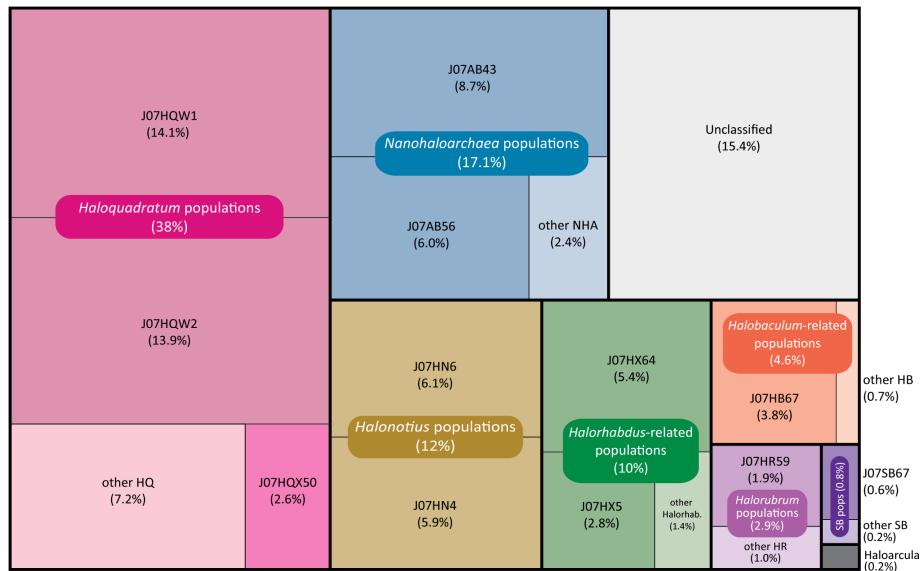


Figure 3. Relative abundance of microbial population groups. Colors correspond to taxonomically related microbial populations, including both assembled genome sequences and non-genomic scaffolds containing less abundant variant sequences. Percentage calculations include total number of assembled nucleotides in reads associated with each group, normalized for the group's average genome size. Percentage of unclassified sequences was calculated using an estimated genome size of 3 MB, the approximate abundance-weighted average for all other groups. Known viral and plasmid sequences, representing approximately 0.2% of assembled nucleotides, have been excluded from these calculations.

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Halonotius-related populations J07HN4 and J07HN6. The next most abundant population group, comprising approximately 12% of the community, contained two population genomes, J07HN4 and J07HN6. 16S rRNA gene sequences from these populations were 95–97% identical to *Halonotius pteroides*, a cultured isolate for which no genome sequence is currently available [38,41]. Despite differences in nucleotide composition between the two Lake Tyrrell *Halonotius*-like populations (63% versus 61% G+C), both shared similar amino acid composition profiles and taxonomic distributions of database matches for predicted proteins (Figure 4).

Based on scaffold read library distribution between 0.1 and 0.8 μm size fractions, *Halonotius*-like populations have the next smallest cells after Nanohaloarchae in the Lake Tyrrell community. The percentage of 0.1 μm filter reads in J07HN6 (80%) was much higher than J07HN4 (50%) suggesting smaller cellular diameter in J07HN6. Although *Halonotius* cells have not been observed to undergo significant aggregation in culture, no data is currently available on whether this behavior might occur under natural conditions. Neither of the *Halonotius*-related genomes contain gas vesicle protein (gvp) synthesis genes, but both contain flagellar synthesis genes. Small flagellated cells and the absence of gas vesicles are consistent with light and electron micrograph observations of *H. pteroides* isolates in culture, which have cell diameters ranging between 0.7–1.5 μm and variable morphologies including cocci, elongated rods and airfoil-like shapes [41].

Halorhabdus-related populations J07HX64 and J07HX5. Approximately 10% of assembled scaffold sequences

formed a group most closely related to the genus *Halorhabdus*. The J07HX5 and J07HX64 genomes differed by 4% G+C, with 16S rRNA genes that were 96% identical to each other. J07HX64 matched an environmental 16S rRNA gene cloned from an Australian salt crystallizer (GQ375005) at 98% identity [38]. The closest environmental match to J07HX5 was to a 16S rRNA gene cloned from an Egyptian hypersaline lake (DQ432537), at 96% identity [42].

Predicted proteins from J07HX5 and J07HX64 shared similar amino acid composition signatures (Table S5) and similar taxonomic patterns of reference database BLASTP matches (Figure 4). *Halorhabdus* was the single most frequently matched genus at 15%, although several other Haloarchaeal genera matched at frequencies of 5–8%. Percentages of 0.1 μm pore filter reads comprising the J07HX5 (21%) and J07HX64 (24%) genome scaffolds suggest an effective cell size similar to *Halorhabdus*.

Halobaculum-related population J07HB67. Approximately 5% of the assembled Lake Tyrrell sequences were associated with a scaffold group named J07HB67. These scaffolds contain a 16S rRNA gene matching the genome of cultured isolate *Halobaculum gomorrense* at 92% identity. The J07HB67 16S rRNA gene is 99% identical to Australian salt crystallizer environmental clone GQ374998 (phylotype 7) [38]. Approximately 33% of reads associated with J07HB67 populations were isolated from 0.1 μm pore filters, suggesting that cells from this population are larger than those of the *Halonotius* group, but smaller than *Halorubrum*,

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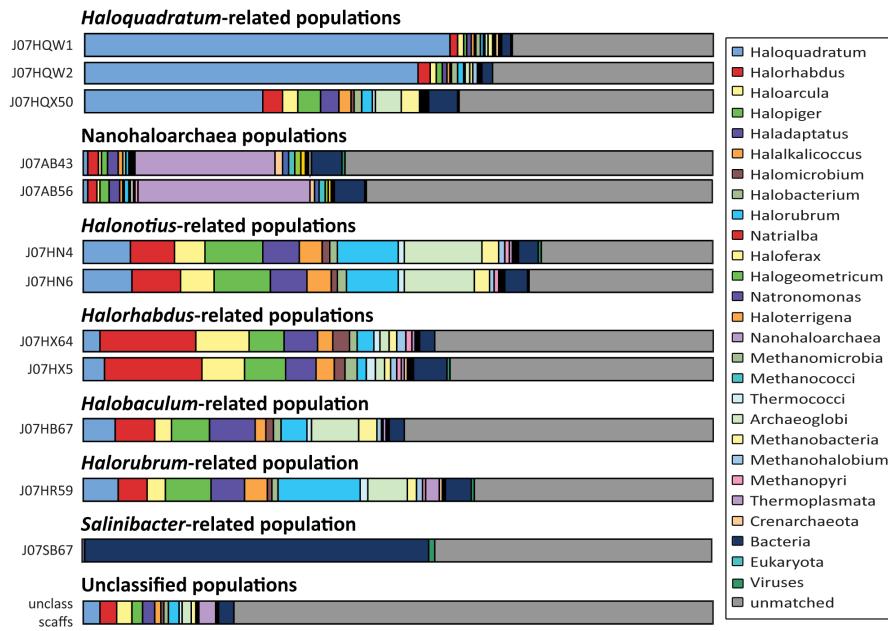


Figure 4. Phylogenetic distribution of protein BLAST matches for assembled population genomes and unclassified scaffolds. Taxonomic distribution of non-self matches versus the Genbank nr database were calculated using the DarkHorse algorithm at a filter threshold setting of 0.05, including only alignments covering at least 70% of both query and target sequences with an e-value of 1e-5 or better.
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J07HX5 and J07HX64. This finding is consistent with microscopic observations of *H. gomorrense*, whose rod-shaped cells measure 0.5–1 µm wide by 5–10 µm long [43].

Halorubrum-related populations. Assembled scaffolds from at least two *Halorubrum*-related populations, representing approximately 3% of the Lake Tyrrell microbial community, were linked by a common pattern of filter size distribution, percent G+C, amino acid sequence composition, and taxonomic classification of BLASTP hits against GenBank nr, in which *Halorubrum* was the most frequently matched genus. Two different *Halorubrum*-related 16S rRNA sequences were observed in assembled scaffolds, 90% identical to each other. Only one of these scaffold groups (J07HR59), representing approximately 2% of the assembled microbial community, was sufficiently abundant for population genome assembly. The J07HR59 16S rRNA sequence matched an environmental clone (GQ374972) described as *Halorubrum*-related phylotype 4 at 97.4% identity [38], but J07HR59 and GQ374972 form a separate, independent branch from previously cultured isolate *Halorubrum* genomes (Figure 2). The other *Halorubrum*-related Lake Tyrrell population, representing approximately 1.0% of the assembled community, claded with previously cultured isolates, matching the *Halorubrum tebenguiquichense* 16S rRNA gene at 96% identity.

Haloarcula and other low abundance archaeal populations. Several small scaffolds containing solely archaeal 16S rRNA gene sequences were identified from populations with

minimal genomic sampling (Table S4). These included two 16S rRNA sequences similar to cultured isolates of genus *Haloarcula*, at 3–4X depth of coverage. However, other scaffolds identifiable as *Haloarcula*-related were assembled at a coverage of 1.2 fold or less. *Haloarcula*-related 16S rRNA genes may have been more completely assembled than other loci from the same population due to multiple co-assembling gene copies; sequenced *Haloarcula* isolate genomes typically contain three 16S rRNA copies. Based on an estimated genome size of 3.9 Mbp, *Haloarcula*-related populations comprised approximately 0.2% of the assembled community, consistent with the lower depth of coverage of non-16S rRNA containing scaffolds.

Salinibacter population J07SB67. The only bacterial 16S rRNA sequence obtained from Lake Tyrrell metagenomic assembly matched cultured isolates of *Salinibacter ruber* at 98% identity, consistent with the observation that 3,480/3,958 (88%) of 16S rRNA sequences independently amplified using universal bacterial PCR primers matched cultured *Salinibacter* at 97% or higher identity. The assembled *Salinibacter* 16S rRNA gene was located on a small, 2,795 nucleotide scaffold, adjacent to a single predicted hypothetical protein. However, more than 400 additional scaffolds, ranging in size from 1,000–19,000 nucleotides, shared patterns of BLAST match taxonomy, nucleotide composition, and predicted amino acid composition consistent with assignment to a *Salinibacter*-related species.

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Targeted assembly of the *Salinibacter*-related scaffold group yielded an incomplete genome of only 1.2 Mbp, versus 3.6 MB for previously sequenced *Salinibacter* isolates (33.3% genome coverage) [44]. Thirty-nine percent of highly conserved bacterial core proteins present in both cultured *Salinibacter* isolate genomes were recovered, consistent with total genome length. Depth of coverage for *Salinibacter*-related scaffolds averaged 1.5 fold, corresponding to a nucleotide abundance of approximately 0.6 percent of the microbial community.

Viral and “Plasmidome” community sampling. Despite the use of sample preparation methods designed to capture only cells between 0.1 and 3 μm in diameter, a group of 142 small scaffolds, representing approximately 0.2% of assembled nucleotides, contained DNA fragments that appear viral in origin. These fragments ranged in size from 1,000 to 25,000 nucleotides in length, with compositions varying between 35–71% G+C. Most of these putative viral scaffolds were reconstructed exclusively from 0.1 μm filter reads. These results are consistent with non-specific retention of viral particles on filter surfaces and/or recovery of phage genomes from infected cells during sample preparation. Predicted proteins in these scaffolds included BLAST matches to viral groups previously shown to be abundant in hypersaline waters, including BJ1-like Siphoviridae and PhiCh-like Myoviridae [45,46,47,48,49,50]. Recovered data were insufficient to determine whether or not these sequences were integrated as prophage in microbial genomes.

Forty scaffolds ranging in size from 1–50 kbp, comprising approximately 0.2% of assembled nucleotides, contained genes encoding p4 plasmid primase, suggesting that they may be archaical plasmid sequences. Two additional scaffolds contained matches to the *Salinibacter ruber* plasmid protein init_Rep_3. Nucleotide composition of putative plasmid scaffolds ranged from 49–66% G+C, at 1.1–12.8 fold depth of coverage, suggesting association with both dominant and rare community members. However, most putative plasmid scaffolds could not be confidently assigned to a specific host organism, and contained few predicted proteins similar to previously sequenced database representatives. Plasmid numbers in cultured halophilic Archaea and Bacteria vary between zero (e.g. *Halococcus walshii* DSM 16790) and seven (e.g. *Halococcus marismortui* ATCC 43049), with sizes ranging from <2 Kbp (*Halobacterium salinarum*, NC_002121) to >600 Kbp (*Halofreya volcanii* DS2, NC_013966). This extremely wide variability makes it difficult to determine the extent to which the plasmid scaffolds we observed represent partial versus complete sequences.

Unclassified Sequences. Approximately 15% of assembled scaffold sequences could not be confidently assigned to any of the groups described above. The low assembly coverage and short sequence lengths in these scaffolds most likely encompass not only less abundant members of the community, but also partial, incomplete fragments corresponding to polymorphic insertions, deletions, mutations, and rearrangements between related strains. Seventy-six percent of predicted protein sequences in the unclassified scaffold group failed to match any sequences in Genbank nr. Database matches were predominately archaical in origin, including the same reference organisms as assembled consensus population genomes (Figure 4).

To estimate the extent to which unclassified scaffolds might represent uncaptured functional diversity within the community, all predicted proteins from the original composite assembly, including both classified and unclassified sequences, were screened for matches to PFAM, COG, and KEGG protein database patterns. At least one pattern was found in 31,696 of 62,918 predicted proteins. Even though unclassified scaffolds comprise

15% of total assembled nucleotides, they contained only 326 patterns absent from the classified data set, corresponding to 7.5% of the overall pool. Classified scaffolds contained 92.5% of all protein patterns detected (5,197 proteins). Protein domain patterns unique to the unclassified scaffolds included a large number of viral-related functional elements, as well as low complexity short repeats characteristic of incomplete protein fragments, suggesting that this group contains an over-representation of partial genes and viral fragments.

To eliminate potential bias due to the highly conserved nature of COG, KEGG, and PFAM patterns, unsupervised Markov algorithm clustering was also performed on all 62,918 predicted proteins in the initial combined assembly. Based on frequencies of these unsupervised clusters, Chao and Ace estimators indicate that assembled scaffolds include greater than >90% of the expected functional diversity in the sampled community. Classified scaffolds contained 4,432 of the 5,242 clusters observed, with only 810 clusters occurring uniquely in the unclassified scaffold set. Close agreement between the percentage of protein clusters (84.6%) and total nucleotides incorporated in assembled scaffolds (84.5%) supports use of the classified data set as a representative sample of functional diversity within the community.

Population Distribution of Community Functions

Markov algorithm clustering was applied to all 31,062 predicted proteins from the twelve Lake Tyrrell genomes, generating 6,591 protein families. Protein family clusters shared between different populations were plotted as connections in a network representation (Figure 5). Highly interconnected clusters, converging at the center of the diagram, include both universal housekeeping genes and habitat-specific adaptive capabilities. Functions broadly shared among all taxonomic groups suggest a common aerobic, heterotrophic lifestyle. Protein families conserved in all 11 archaical populations also include UV damage repair endonucleases, peroxiredoxins and thioredoxins, halocyanins, $\text{Ca}^{2+}/\text{Na}^+$ antiporters, and type IS605 OrfB family transposases.

Population-specific protein families located at the periphery of Figure 5 capture functional novelty of both individual genomes and closely related taxonomic groups relative to the rest of the community. Table 2 compares numbers of unique clusters found in each of the twelve consensus genomes. The population with the greatest number of unshared protein families is *Salinibacter*, the only bacterium in the group, even though the assembled genome was less than 40% complete. The two Nanohaloarchaeal genomes J07AB43 and J07AB56 also contained many unique clusters, both individually and shared between them.

Although each of the three *Halococcus* genomes had more than 350 unique clusters, these numbers were similar to other Lake Tyrrell Haloarchaeal populations when normalized for genome size. Numbers of novel clusters found in *Halococcus*-related populations suggest more diverse protein functions than other community members, but most likely also include a higher number of pseudogenes, as observed previously in *Halococcus* isolate genomes [51,52]. In contrast to *Halococcus*, *Halomonas* populations J07HN4 and J07HN6 contain surprisingly few novel protein clusters in each individual genome, despite 16S rRNA sequences that are more divergent from each other than the J07HQW1 and J07HQW2 genomes. Unique functional properties of the *Halomonas* group are captured instead in clusters shared between J07HN4 and J07HN6.

Many protein families shared between different community members contain only sequences whose function cannot be predicted from bioinformatic inference. Proteins of unknown function are more abundant among population-specific protein

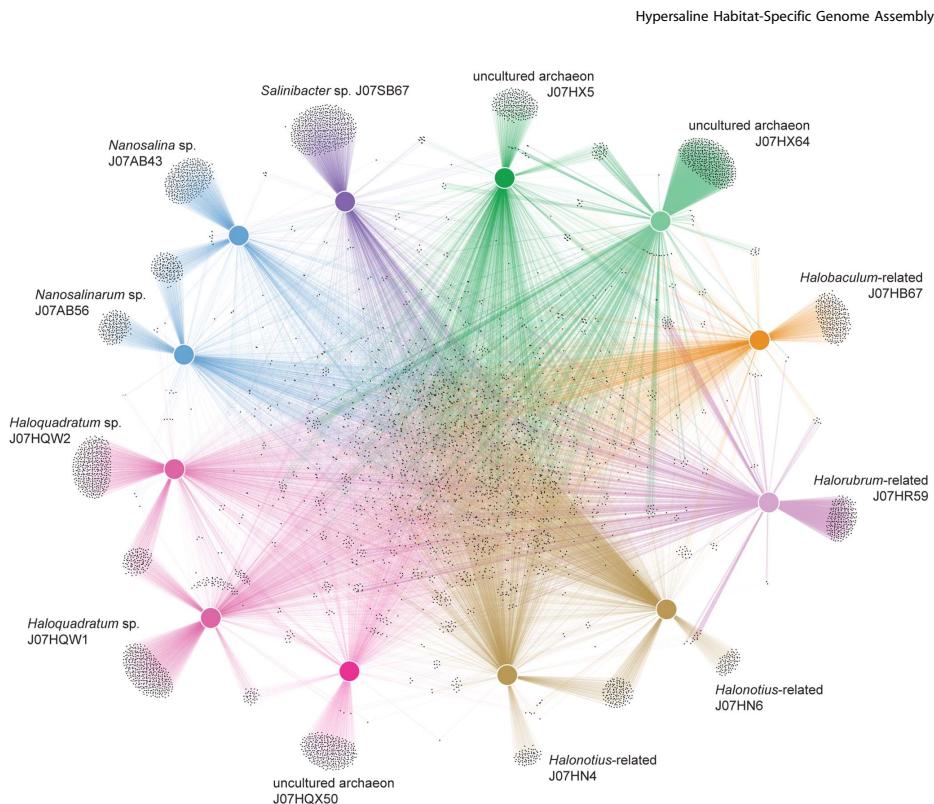


Figure 5. Metabolic connectivity graph showing community distribution of protein family clusters. Cohesive populations are shown as similarly colored nodes and vectors according to numbers of shared features, based on unsupervised protein family clustering of 12 habitat-specific genomes.
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families than in more widely distributed clusters. However, even confidently annotated proteins sometimes generate multiple clusters with similar descriptions, and may correspond to protein isoform variants with unknown but possibly significant differences in biological activity.

Discussion

In this study we have captured the taxonomic diversity, population abundance, and functional properties associated with both broad phylogenetic groups and individual microbial populations in a mixed, natural ecosystem community. Reconstruction of 12 habitat-specific population genomes from a single pool of metagenomic sequencing reads demonstrates the value of combining *de novo* assembly with iterative, multi-dimensional phylogenetic binning. This approach proved particularly useful in characterizing previously undescribed novel organisms, avoiding problematic issues of amplification primer bias and variable 16S rRNA gene copy number in divergent populations. Eight reconstructed genomes represented species with no previously cultured isolates, including populations comprising 2–14% of the

sampled microbial community. Ten of the twelve genomes were nearly complete, in assemblies of seven or fewer scaffolds.

Each of these population genomes represents a composite sequence constructed from multiple, closely related individual cells, providing a set of core gene models and operon structures common to most members of the population. These genomes do not include peripheral pan-genomic content that is unique to individual strains. Regions of significant population divergence (intra-species heterogeneity) are incorporated as gaps in larger scaffolds and/or separate shorter overlapping scaffolds with lower read coverage. The composite sequences we have obtained by community metagenomic assembly cannot be expected to furnish the same level of detail and accuracy as the closed, finished genome of an individual isolate; yet their ability to deliver full length genes in cellular context has provided important new insights into community structure, novel taxa, and compartmentalized protein functional associations that could not be obtained from unassembled reads alone.

Although Sanger technology was the primary source of reads for this study, the subtractive taxonomic enrichment strategies we

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Table 2. Population-unique protein family clusters.

Genome name	num unique clusters	total num genome clusters	pct. unique clusters
<i>Salinibacter</i> sp. J07SB67	581	1,639	35%
<i>Nanosalina</i> sp. J07AB43	366	1,678	22%
uncultured archaeon sp. J07HX64	441	3,047	14%
<i>Nanosalinarum</i> sp. J07AB56	184	1,410	13%
<i>Halorubrum</i> sp. J07HR59	232	1,839	13%
<i>Haloquadratum</i> sp. J07HQX50	351	2,872	12%
uncultured archaea sp. J07HX5	258	2,139	12%
<i>Haloquadratum</i> walsbyi str. J07HQW1	403	3,584	11%
<i>Haloquadratum</i> walsbyi str. J07HQW2	433	3,855	11%
<i>Halobaculum</i> sp. J07HB67	296	2,846	10%
<i>Halonotius</i> sp. J07HN6	90	2,913	3%
<i>Halonotius</i> sp. J07HN4	81	3,229	3%
total	3,716	31,051	12%

doi:10.1371/journal.pone.0061692.t002

have developed could also be applied to metagenomic assemblies using paired-end reads obtained by more contemporary platforms such as Illumina. Our *de novo* assembly procedures were especially effective in facilitating genome recovery for populations (species) with no closely related sequenced relatives. Assembly quality was improved as data complexity was reduced and the accuracy of read binning enhanced by iterative, scaffold-based read selection using multiple, independent parameters. These parameters included uniform nucleotide composition, depth of coverage, taxonomic distribution of BLASTP database matches, and amino acid composition of predicted proteins. Read distribution frequencies from overlapping libraries obtained using different filter pore sizes provided an additional source of independent information to help distinguish difficult-to-separate groups and verify assembly fidelity, as well as offering a novel opportunity to estimate physical cell size of uncharacterized organisms relative to other members of the community.

Archaea greatly outnumbered Bacteria in the Lake Tyrrell hypersaline ecosystem, as previously reported for other extreme hypersaline environments [14,53]. Although relatives of *Haloquadratum walsbyi* were the most abundant taxonomic group, comprising approximately 38% of the community, nearly 47% of the assembled sequences were derived from a combination of Nanohaloarchaea (17%) and relatives of the Halarchaeal genera *Halonotius* (12%), *Halorhabdus* (10%), *Halobaculum* (4.6%), *Halorubrum* (2.9%), and *Haloarcula* (0.2%). Based on historical accounts of other hypersaline habitats [52,54,55], diversity within the *Haloquadratum*-related population was higher than expected, including at least three different species from two different genera.

The 62,918 environmental genes recovered from the assembled metagenomic sequences were estimated to encompass more than 90% of the functional diversity present in the community. The construction of multiple habitat-specific Lake Tyrrell population genomes has enabled genome-wide assignment of functional activities to specific individual organisms of known abundance in the community. These assignments provide new opportunities to begin comparing shared and novel protein families across related and divergent co-occurring populations adapted to the same environmental conditions with a level of organism-specific context that would not be possible with unassembled reads alone.

The relatively constrained metabolic repertoire of broadly shared protein functional families in the Lake Tyrrell community may be linked to physicochemical uniformity in the shallow, aquatic hypersaline environment from which organisms were sampled. The common evolutionary history of halophilic Archaea adapted to extreme salinity may also play a role. It has been speculated that abundances of different microbial populations under these conditions might be driven more by top down forcing dynamics, for example protozoan predation and/or viral infections, rather than nutrient availability [56]. The current study does not include seasonal fluctuations in temperature, salinity and nutrient inputs, which might reveal greater diversity over longer time scales. The availability of new habitat-specific reference genomes from the Lake Tyrrell ecosystem provides new reference data to track these populations over time and space at the level of both genes and genomes.

Functional genes and metabolic processes unique to individual populations may also provide information useful in designing cultivation methods for previously uncultured organisms, including the possibility of mixed co-cultures to accommodate natural symbiotic or co-dependent trophic relationships. The potential utility of this approach is illustrated by the observation that strains of *Haloquadratum walsbyi*, notoriously difficult to grow in isolate culture, form significantly larger colonies in the presence of *Salinibacter ruber* [57]. Although *Salinibacter*-related populations comprise only a small percentage of the ecosystem described here, *Haloquadratum* abundance could be driven by similar nutritional complementation provided by alternative members of the community.

The new genomes described in this study expand opportunities to identify novel phylogenetic groups in other environments, providing new templates for fragment recruitment and assembly, as well as group-specific probes for *in situ* quantitation. Organisms previously identified by 16S rRNA gene sequences alone can now be prioritized as targets for more detailed investigations based on functional, as well as taxonomic information. Furthermore, the assembly of habitat-specific genomes provides an important foundation to decipher genotype-phenotype relationships based on metatranscriptomic and metaproteomic investigations in similar environments. The simultaneous interrogation and synthesis of composite data from multiple microbial populations in

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natural ecosystems will provide the comprehensive level of genotypic and phenotypic data necessary to model synergistic activities of community members, while contributing to an enhanced understanding of the ecology and evolution of environmental microbial species.

Supporting Information

Table S1 Water chemistry of Lake Tyrrell sampling site. Located at 35°19'12.24S 142°48'00.45E. (PDF)

Table S2 Summary of metagenomic sequencing libraries used in this study. Average read length is shown \pm standard deviation. (PDF)

Table S3 Assembly statistics for combined Sanger metagenomic libraries using Celera Assembler version 5.4. Assembly parameters used were as follows: utgErrorRate = 0.10; ovfErrorRate = 0.10; cnsErrorRate = 0.10; cgwErrorRate = 0.12; utgBubblePopping = 0; utgGenomeSize = 500000; merSize = 15; doFragmentCorrection = 0; doExtendClearRanges = 1; doResolveSurrogates = 1; Unitigger parameter -j = -20. (PDF)

Table S4 Assembled 16S rRNA sequences and their closest database matches to environmental clones and cultured isolates. Matches were required to have BLAST alignments to previously identified 16S rRNA genes of 450 nt or longer, with e-value $<1e-7$ and 80% or greater sequence identity between query and subject. Part A shows 16S rRNA gene sequences obtained in targeted genomic assemblies. Part B shows additional 16S rRNA gene sequences observed in scaffolds obtained by composite assembly of all Sanger reads. (PDF)

Table S5 Distinctive properties of major scaffold clusters. Percentages are based on taxonomic classifications of all predicted protein tophit matches to Genbank nr, as determined using the DarkHorse algorithm at a filter threshold setting of 0.05, including only alignments covering at least 70% of both query and target sequences with an e-value of 1e-5 or better. (PDF)

Table S6 Estimated genome completeness. Based on presence/absence of 53 conserved genes in assembled archaeal composite population genomes. (PDF)

Figure S1 Bioinformatic Analysis Pipeline. (PDF)

Figure S2 Phylogenetic trees showing abundance of clustered archaeal 16S rRNA sequences from (A) unas-

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Chapter 5

Microbial population structure and genetic heterogeneity in a hypersaline environment

5.1 Abstract

5.2 Introduction

The increasing number of microbial genomes being sequenced over the last few years, due to the drastic price reduction on the cost of sequencing a genome, has allow for comparisons among the genomes of closely related microbial species and even more important strains from the same microbial species (REF). These comparisons have showed that even closely related strains of microorganisms, have enough differences in their genomic sequences that could explain their different functional behavior (REF), and is a reflection of the different evolutionary histories of each strain (REF). This has been studied in detail specially in medical applications, where . [Multiple studies and genomes, selective pressure]. In non-medical studies, a recent study by (Science, Shapiro), studied multiple *Vibrio* genomes, comparison, etc.

With the development of community genomics approaches, we have known

started to comprehend the level of genetic variation that can be found in natural microbial population (multiple REFs). In particular, the use of assembly-based approaches, allows us to reconstruct genomes (consensus o poulation genomes) from the members of the microbial community under study, and then using bioinformatic approaches, we can assest the level of genetic diversity that is present in a microbial population (REF). Most of the current studies have focus on very simple systems (REF, ISMEJ, enrichemnts) or only on a few members of the population under study (REF, Jill). In contrast, one of the studies that used a great amount of available data was based on metagenomic sequenecs obtained from the human microbiome, but in this case the genomes used were not necessary obtained from the same samples where the metagenomic sequences were obtaied. In this work, we used as a foundation the available information that was recovered from a assembly-based metagenomic study in the Lake Tyrreell hypersaline ecosystem, using the information as a reference, and then using deep-sequencing approaches, using Illumina sequencing, to assest and study the level of genetic variation that is present in a natural microbial community. Based on this information we can assest the level of genetic variation between populations, and also start to comprehend the effect of natural selection in these populations. (REFs, check microbiome paper).

we can observe the current status of the community, and ask questions later by sampling other times (establish a baseline for future studies).

5.3 Material and Methods

5.3.1 Sample collection and sequencing

Surface water samples from Lake Tyrrell were collected in 2007, at two different seasons, summer (January) and winder (August), with with two days of difference in each season (January 23 and 25, August 7 and 9). Each water sample was filtered directly into an Sterivex cartridge (Milipore, Bedford, MA, USA) ($0.22\text{ }\mu\text{m}$) using a peristaltic pump. For DNA extraction each Sterivex was processed according to the following protocol:

- Addition of Proteinase K to a final concentration of 0.5 mg/ml⁻¹ and SDS to a final concentration of 1%.
- Incubation at 55°C for 25 minutes, followed by incubation at 70°C for 5 minutes.
- Transfer of the lysate from the Sterivex, to a clean Eppendorf tube.
- Nucleic acid extraction with two steps of phenol:chloroform extraction.

For all four samples, the sequencing library construction was performed by the UC San Diego IGM Genomics Center. The four libraries were multiplexed and run on a single lane on an Illumina HiSeq machine, on high-throughput mode (2X100 PE reads).

After demultiplexing using the Illumina software, all the reads were processed using Nesoni 0.117 (<http://www.vicbioinformatics.com/software.nesoni.shtml>), to remove adapters, trim low quality positions and remove low quality reads from the datasets. For trimming a minimum quality score of 20 was used, and all reads shorter than 70 nucleotides (after trimming) were removed.

5.3.2 Read mapping

The trimmed reads were mapped against a set of habitat-specific genomes (Table 5.1 generated by the assembly of metagenomic information of the Lake Tyrrell microbial community [58, 69, 68]. In addition, an archaeal isolate, *Candidatus Halobonum tyrrellensis* [90], obtained from samples collected in August of 2007, was also included in the analysis. Each sample was mapped independently to the set of reference genomes, using Bowtie 2.2.1 [43] with the *very-sensitive* alignment option, and adjusting the N-ceiling function to 0,0.01 to reduce the number of ambiguous characters present in the alignment. Several tools were used in the analysis of the resulting files, including SAMtools 0.1.19[45], BEDtools 2.17 [70] and BCFtools 0.2.0 (<http://samtools.github.io/bcftools/bcftools.html>)

5.3.3 Taxonomic classification

The mapped and unmapped reads were classified using Phylosift 1.0.1 [19], using the provided set of marker genes. This set included all of the January 2007 genomes assembled from the Lake Tyrrell community [58, 69], but did not include the newest one from the August 2007 community [68].

5.3.4 Variation analysis

The mapped reads were processed using Picard Tools 1.99 (<http://picard.sourceforge.net>), and then GATK XXX ([20] was used to realign indel regions to the reference genomes. The corrected files were processed with Freebayes v9.9.2-29-g9ed353c [29] (ploidy:1, minimum base quality: 20, minimum mapping quality: 30). Quantification of the type of variations was done using SNPeff [17], and custom Python scripts.

Calculation of the pN/pS values was done using custom Python scripts (available in XXX), based on the approach used in Tai *et al.* [86]. For each gene the pN/pS value was calculated as:

$$\frac{pN}{pS} = \frac{\frac{\text{Observed non synonymous mutations}}{\text{Number of non synonymous sites}}}{\frac{\text{Observed synonymous mutations}}{\text{Number of synonymous sites}}}$$

5.3.5 Computational resources and data availability

All of the analysis were carried out using a large cluster instance (c3.8xlarge: 32 Intel Xeon E5-2680 v2 cores, 60 Gb RAM) on the elastic cloud computing resource at Amazon. Plotting and calculations were carried out using custom developed Python scripts. The majority of the code and preliminary analyses are available on a Github repository (XXX).

Table 5.1: List of the Lake Tyrrell habitat-specific genomes used for read mapping

Genome name (abbrv)		Length	G+C pct	N	scaf-folds	Reference
<i>Haloquadratum</i>	<i>walsbyi</i>	3,549,539	47	1		[69]
J0HQW1						
<i>Haloquadratum</i>	<i>walsbyi</i>	3,475,501	49	1		[69]
J0HQW2						
<i>Haloquadratum</i>	sp.	3,019,909	50	2		[69]
J07HQX50						
<i>Nanosalinarum</i>	sp.	1,215,802	56	3		[58]
J07AB56						
<i>Nanosalinarum</i>	sp.	1,277,157	43	7		[58]
J07AB43						
<i>Halonotius</i> sp. J07HN4		2,888,659	61	2		[69]
<i>Halonotius</i> sp. J07HN6		2,529,000	63	6		[69]
uncultured archaeon	sp.	2,982,938	64	1		[69]
J07HX64						
uncultured archaeon	sp.	2,040,945	60	1		[69]
J07HX5						
<i>Halobaculum</i> sp. J07HB67		2,649,547	67	3		[69]
<i>Halorubrum</i> sp. J07HR59		2,120,805	59	7		[69]
<i>Salinibacter</i> sp. J07SB67		1,931,021	67	443		[69]
<i>Halorubrum</i> sp. A07HR60		2,876,249	59	14		[68]
<i>Halonotius</i> sp. A07HN63		2,392,686	63	37		[68]
<i>Halorubrum</i> sp. A07HR67		2,890,468	67	16		[68]
uncultured archaeon		2,389,822	71	15		[68]
A07HB70						
<i>Candidatus</i> Halobonum		3,675,087	70	72		[90]
tyrellensis G22						

5.4 Results and Discussion

5.4.1 Overview of the Illumina dataset for January and August of 2007

Between 71-74% of the original reads were retained after trimming and quality filtering (Table 5.2). An approach to easily visualize the community composition and identify broad differences in population composition between samples, is to quantify the G+C content of the reads and plot the abundance by bins (usually of size 1%) [69, 30, 68]. Figure 5.1, shows the differences between the four libraries, and highlights the previously assembled genomes from the Lake Tyrrell microbial community [58, 69, 68]. The plot allows to identify the different populations present in the January versus the August libraries, in particular where the January community is dominated by organisms with lower G+C content, compared to the August community. This agrees with previous observations in the same microbial community [68], where the main driver of these differences was suggested to be the ionic composition of the water column, in particular magnesium (Table 5.3). Microorganisms like *Haloquadratum* (J07HQW1, J07HQW2 and J07HQX50) are more dominant in the January samples, compared to the August samples.

Another interesting observation that emerge from this G+C plot, is that the August samples have similar compositions based on the G+C content, while the January samples show differences. This could be possibly attributed to the differences in magnesium concentrations (Table 5.3) between the two days in January, which could explain the increase in the *Haloquadratum* populations on the January 25th sample, and in general of populations with a lower G+C content. Looking at possible explanations for this, we found on the weather records, that a storm occurred previous to the January 23th sampling, suggesting that the input of freshwater diluted the salt concentrations in the water column, reducing the magnesium concentration. After two days, due to water evaporation and other environmental factors, the magnesium concentration raised, explaining the difference that we see between the two days.

Table 5.2: Summary of the Illumina HiSeq libraries for each of the four samples.

Library name	Total reads	Read-pairs after QC	Unpaired reads after QC	Total bases (Gb)
January 23	49,963,357	37,016,243	7,679,004	7,978.18
January 25	39,400,015	29,444,267	5,894,815	6333.12
August 7	46,472,319	33,485,834	7,659,231	7266.38
August 9	40,256,946	28,843,346	6,812,171	6276.12

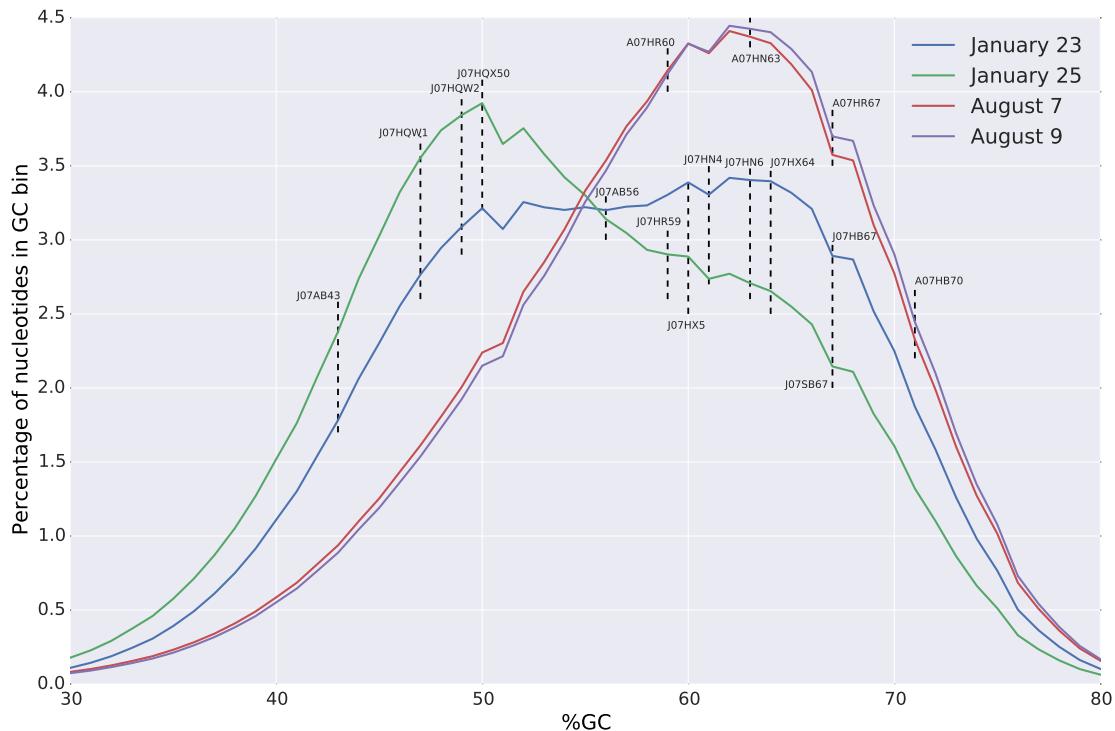
**Figure 5.1:** GC plot of libraries

Table 5.3 (*next page*): **Table 5.3:** Physical and chemical composition of the Lake Tyrrell water samples. Concentrations are given in units of mmol L⁻¹

Sample	Temp °C	Total ionic strength	Na	K	Mg	Ca	C1	C1	SO42
<i>Jan 23</i>	21.6	pH	5,721	4,338	32	298	10	5,345	123.6
<i>Jan 25</i>	27.9	7.09	5,950	4,163	43	419	11	5,291	170.5
<i>Aug 6</i>	9.9	7.00	4,403	3,724	19	126	15	4,298	50
<i>Aug 8</i>	11.5	7.01	4,060	3,557	18	117	14	3,830	47

5.4.2 Genome abundance and community structure, based on read mapping

All of the reads that passed the quality filters were mapped against the set of reference genomes (Table 5.1). The summary of this mapping (Table 5.3), shows the difference in the number of reads that mapped to each genome. In both January samples, the *Haloquadratum* J07HWQ1 and J07HWQ2 genomes, recruited the largest number of reads, which agrees with previous estimation of their abundance in the Lake Tyrrell community [69]. It is important to highlight that number of mapped reads to each genome, do not reflect the real abundance of each organism in the community. Given the strict criteria used for mapping, it is likely that we are missing information on some of these genomes (more on this later), and also several of these genomes represent closely related species, sharing the same DNA sequences. In addition, variations due to genome size and even genome copy number need to be taken in consideration. This explains, for example, why the *Nanohaloarchaea* genomes (J07AB56 and J07AB43) do not recruit a large percentage of the reads, in contrast with their estimated high abundance in this community [69]. This stringent criteria, also explain why the genome of *Candidatus Halobonum tyrrellensis* (G22), recruits a low number of reads from the dataset. This particular organisms, is an interesting situation, as it was isolated from surface waters collected in August of 2007, and by 16S showed to be similar to the *Halobaculum* genomes assembled from the January and August samples (J07HB67 and A07HB70). Further analysis using the complete sequence, showed that it was a different population, and possibly a new Genus within the Haloarchaea [90]. This is a clear example on how in many situations, isolates from an environment are not representative of the most abundant members that can be found living in that environment.

A visualization of the numbers of mapped reads at different identity levels (Figure 5.2), shows that for all the genomes (with the exception of G22), the majority of the reads mapped at a 100% identity, and in none case was lower than 85%. This reflects the stringency of the parameters used for mapping, and also allows to be confident that the reads are derived from that particular population.

Looking more in detail to each of the individual genomes, these plots already show some information on the possible strain diversity present in each of the populations. In the case of the *Haloquadratum* genomes, most of the reads mapped at identities of 95% or higher, reflecting a low level of population heterogeneity among these populations. On the other extreme, in the case of the *Salinibacter* populations, we can observe a range of identities going from 88% and higher.

Given the deep coverage of the community in this four libraries (an average of 4.3 billion nucleotides in each one), this dataset represent not only the most abundant members of the community, represented in the reference genomes used for mapping, but also allows access to less abundant organisms. To quickly evaluate the differences between the mapped and unmapped reads, we used a taxonomic classification approach to estimate difference between the mapped and unmapped reads on each dataset. For example, for the mapped reads in the January 23 dataset (Figure ??), as expected the majority of the mapped reads were classified as *Haloquadratum*, followed by the *Nanohaloarchaea*. In comparison, the unmapped reads for this library (Figure ??), shows a diversity of groups, including a high percentage of Bacterial reads. This suggests that there is still novel groups (not represented in the current set of genomes used for mapping) that are present in the community, and that could be recovered with further analysis. In the present work, I will focus only on the genomes and its mapped reads, as this genomes provide an already validated set of habitat-specific genomes that can be used for the analysis of diversity in this microbial community.

The

Plot of the read recruitment Phylogenetic composition of the unmapped reads (Phylosift?) Comparison between samples, based on guppy in phylosift

Low abundances compared to Plos paper - ζ read recruitment, level of stringency

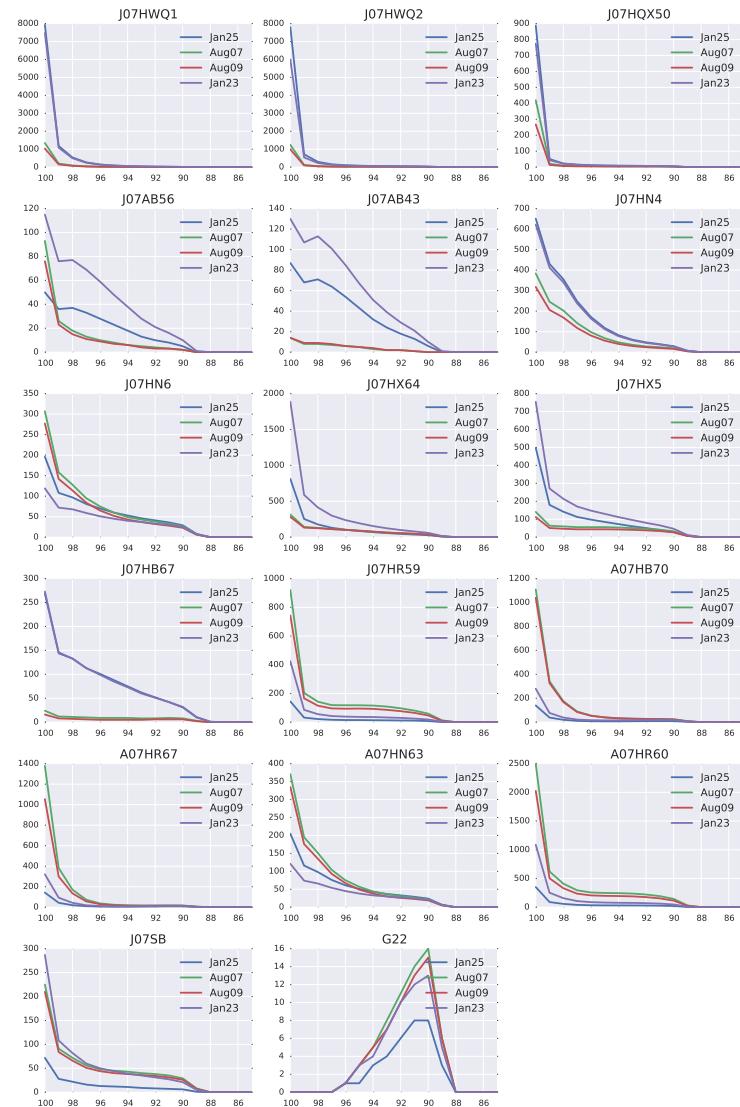
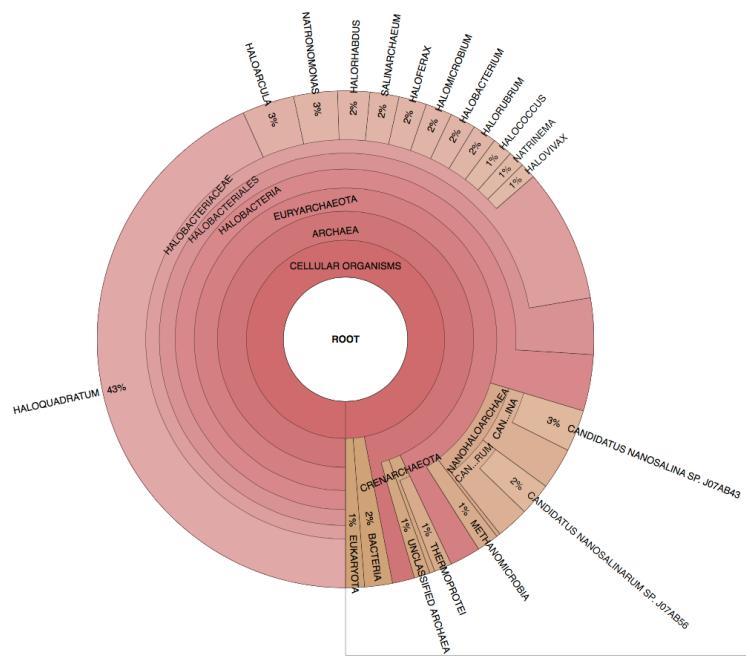
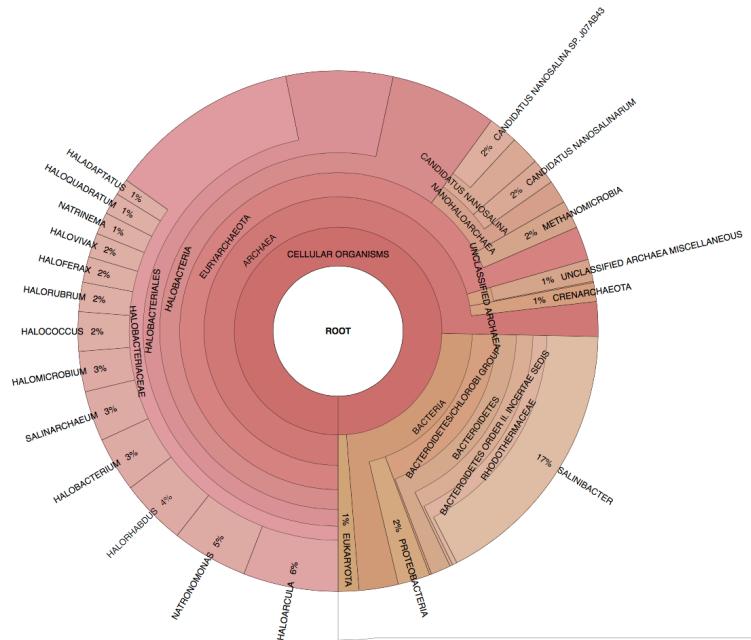


Figure 5.2: Total number of recruited reads, grouped by identity. The X axis shows the identity of the read to the reference genome (%), while the Y axis shows the number of reads recruited at that identity (thousands of reads).



(a) Mapped reads



(b) Unmapped reads

Figure 5.3: Phylosift classification of the mapped and unmapped reads of the January 23 sample

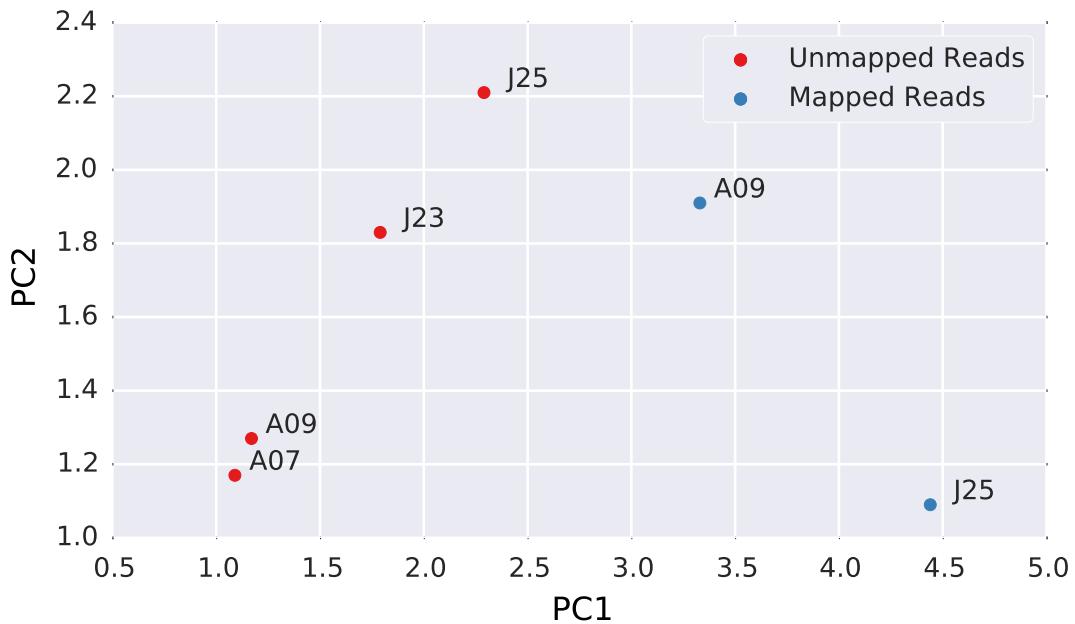


Figure 5.4: EPCA phylosift results

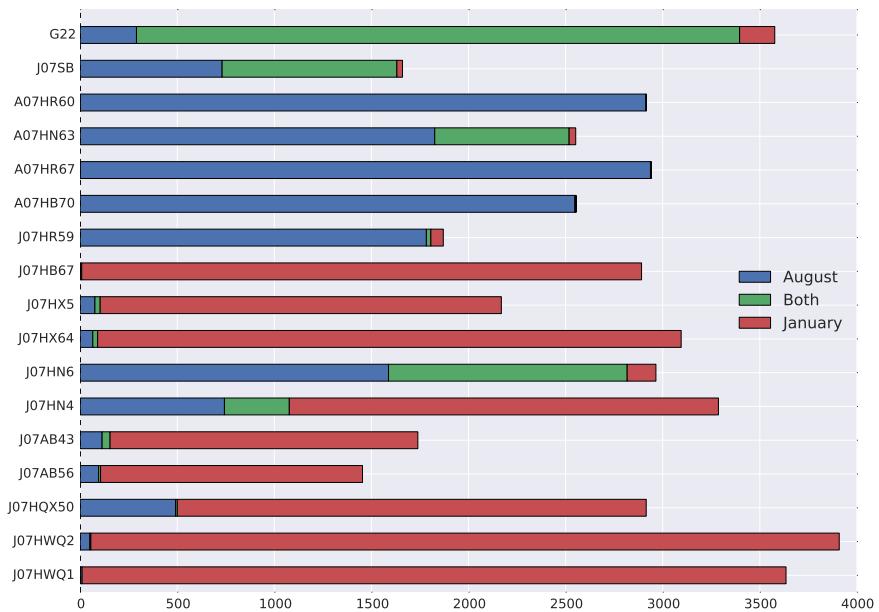


Figure 5.5: Gene significant by seasons

Table 5.3: Number of reads from each library, recruited to the Lake Tyrrell reference genomes

Genome	Jan 23	Jan 25	Aug 07	Aug 09
<i>J07HWQ1</i>	9,712,976	10,347,084	1,802,421	1,385,337
<i>J07HWQ2</i>	7,311,175	9,428,490	1,628,137	1,301,141
<i>J07HQX50</i>	922,138	1,041,326	501,477	330,307
<i>J07AB56</i>	565,197	266,831	194,445	165,278
<i>J07AB43</i>	760,203	486,360	63,209	64295
<i>J07HN4</i>	2,149,204	2,249,692	1,306,287	1,089,673
<i>J07HN6</i>	592,818	831,367	1,027,472	911,341
<i>J07HX64</i>	4,167,113	1,819,023	1,202,103	1,144,206
<i>J07HX5</i>	2,106,559	1,382,371	673,972	539,843
<i>J07HB67</i>	1,124,816	1,128,191	125,973	84,643
<i>J07HR59</i>	839,856	310,496	2,105,598	1,693,772
<i>A07HB70</i>	550,429	277,030	1,970,106	1,866,967
<i>A07HR67</i>	563,043	270,602	2,166,129	1,680,150
<i>A07HN63</i>	547,808	786,856	1,126,032	1,003,322
<i>A07HR60</i>	2,126,700	758,549	5,405,933	4,362,857
<i>G22</i>	62,983	39,696	72,261	66,778
<i>J07SB</i>	797,957	211,306	737,471	673,630
<i>Unmapped</i>	45,344,829	31,913,858	51,012,461	44,849,506

5.4.3 SNPs

Results of the snps (??). Low number of SNPs for G22, so I'll leave out of the analysis. Reasons? Comments about the trends?

Compariosn of the rate of SNPs (independent of the type). DONE (Table)

Venn diagram of the SNPs, can we collapse January samples and August samples. DONE

Genes with most of the SNPs Genes with no SNPs Use BEDTOOLS ANNOTATE FOR THIS

Dn/DS network diagram of DN/ds and selection, january versus august

Table 5.4: Change rate for SNPs

Genome	Jan 23	Jan 25	Aug 07	Aug 09
<i>J07HWQ1</i>	261	262	267	270
<i>J07HWQ2</i>	398	397	305	407
<i>J07HQX50</i>	695	693	706	718
<i>J07AB56</i>	99	103	202	192
<i>J07AB43</i>	93	94	137	139
<i>J07HN4</i>	110	110	111	111
<i>J07HN6</i>	348	383	351	356
<i>J07HX64</i>	105	106	107	108
<i>J07HX5</i>	108	109	110	111
<i>J07HB67</i>	110	109	155	191
<i>J07HR59</i>	738	872	651	663
<i>A07HB70</i>	139	142	137	137
<i>A07HR67</i>	171	175	169	169
<i>A07HN63</i>	581	504	471	476
<i>A07HR60</i>	264	271	256	257
<i>G22</i>	24,126	33,241	29,898	31,227
<i>J07SB</i>	165	177	164	164

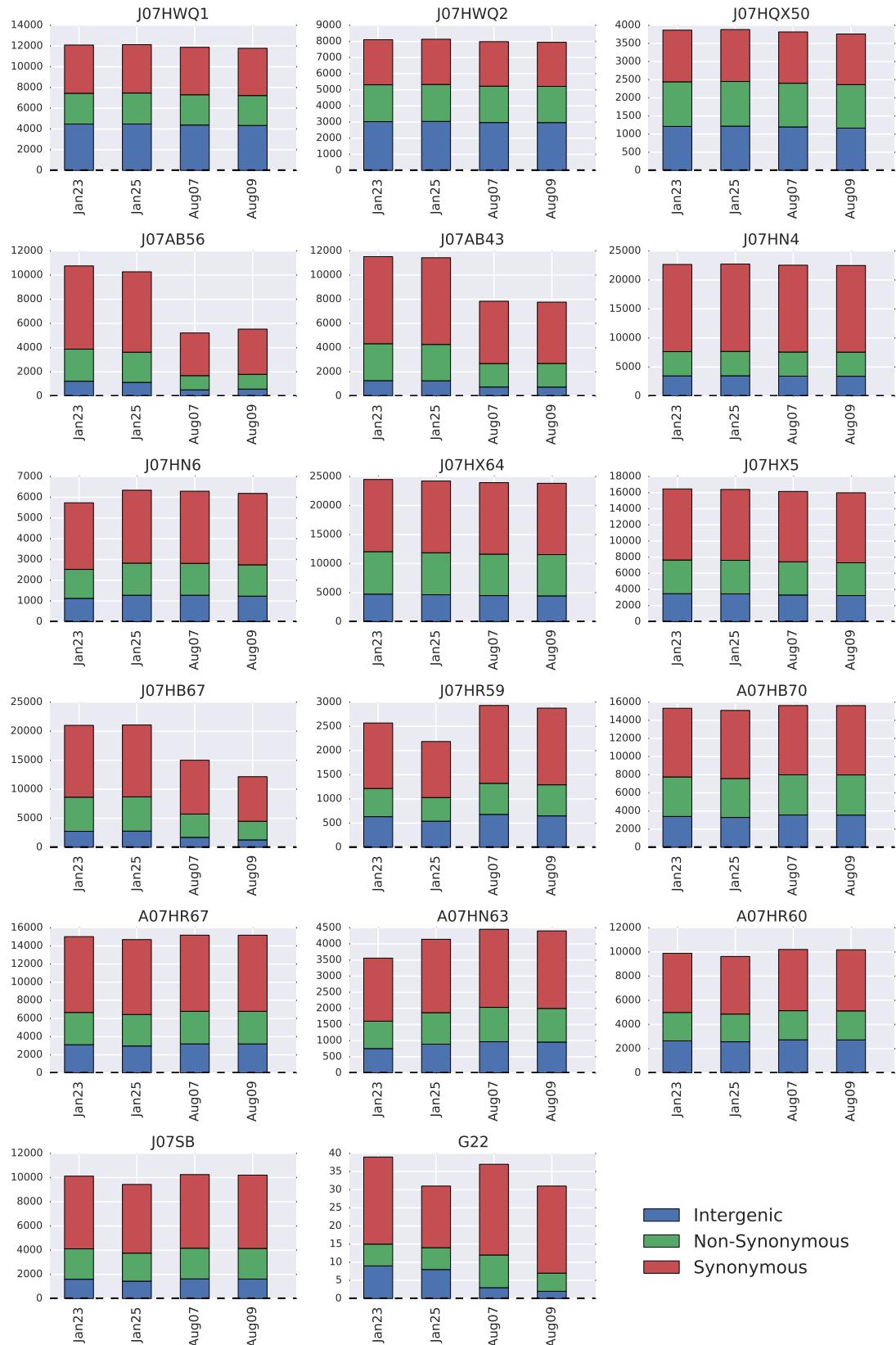


Figure 5.6: SNPsummary

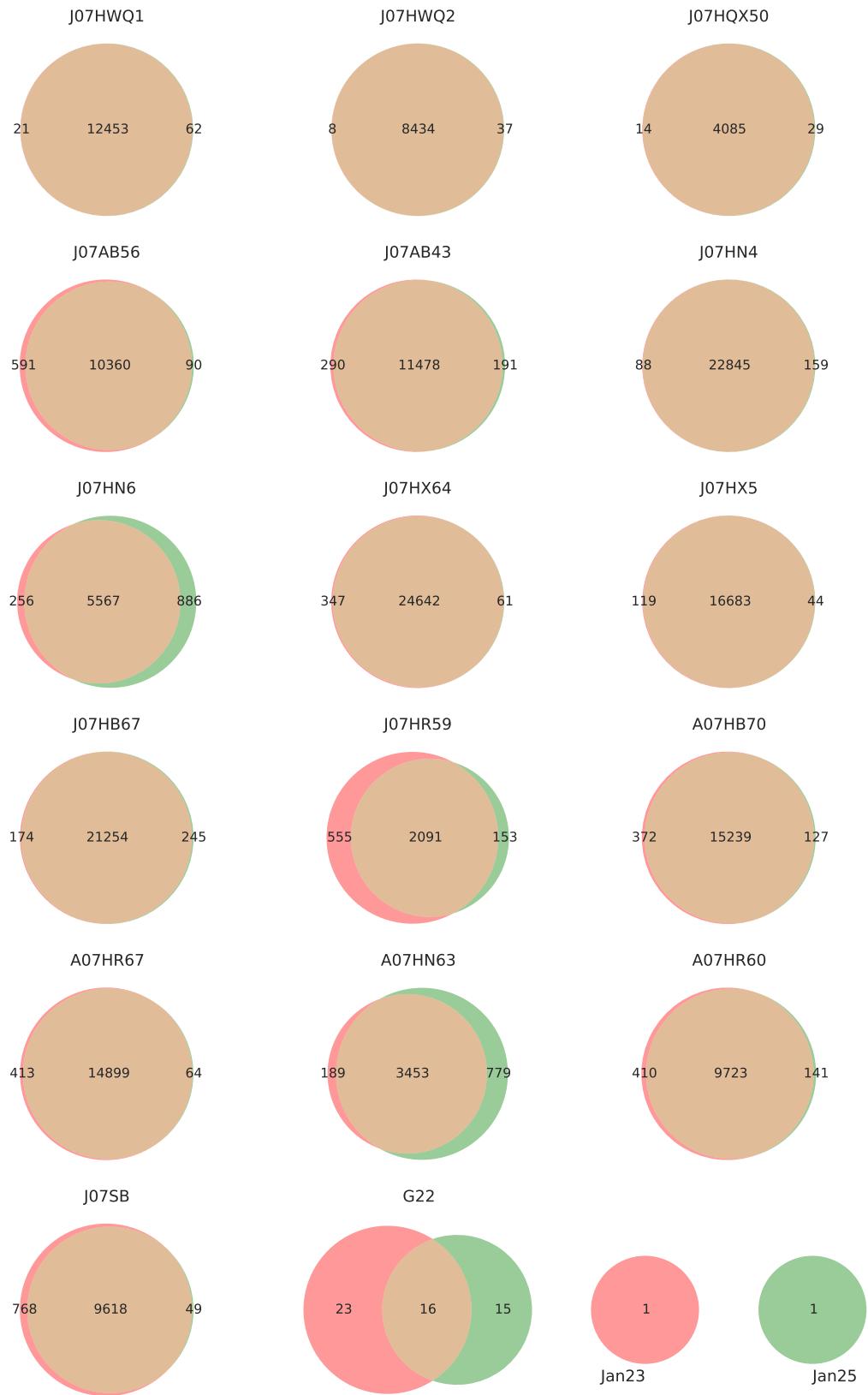


Figure 5.7: JanuarySNPs

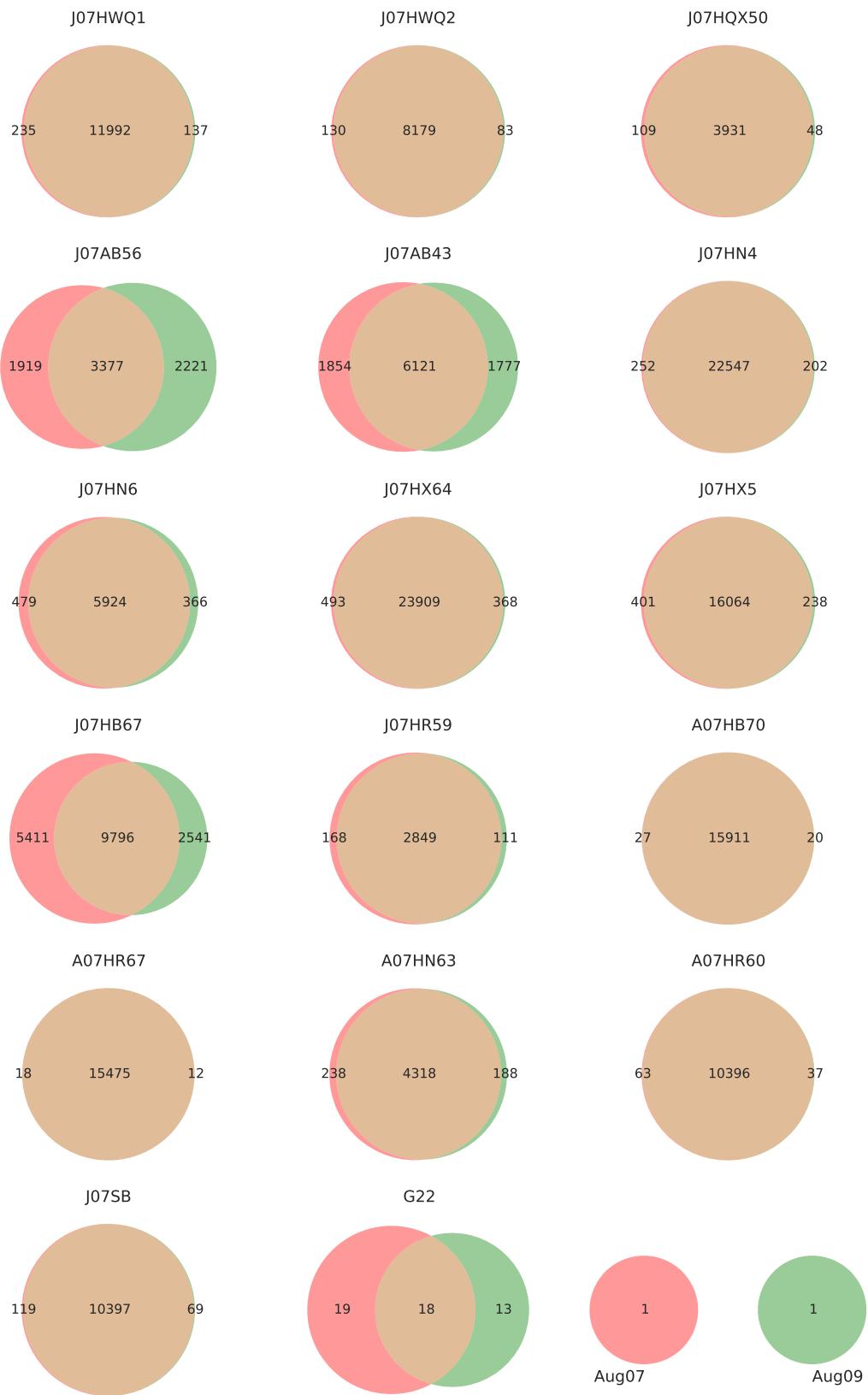
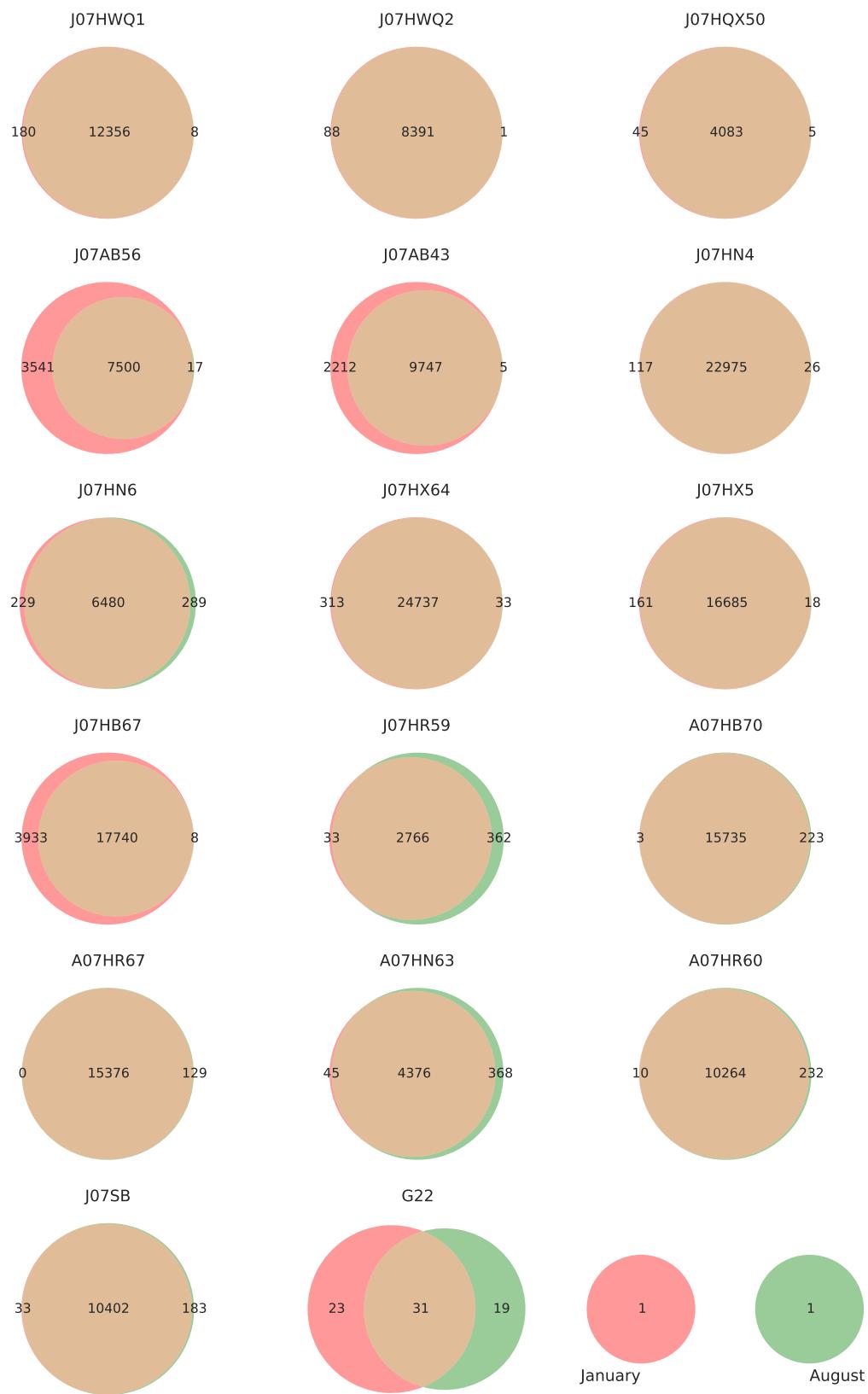


Figure 5.8: AugustSNPs

**Figure 5.9:** BothSNPs

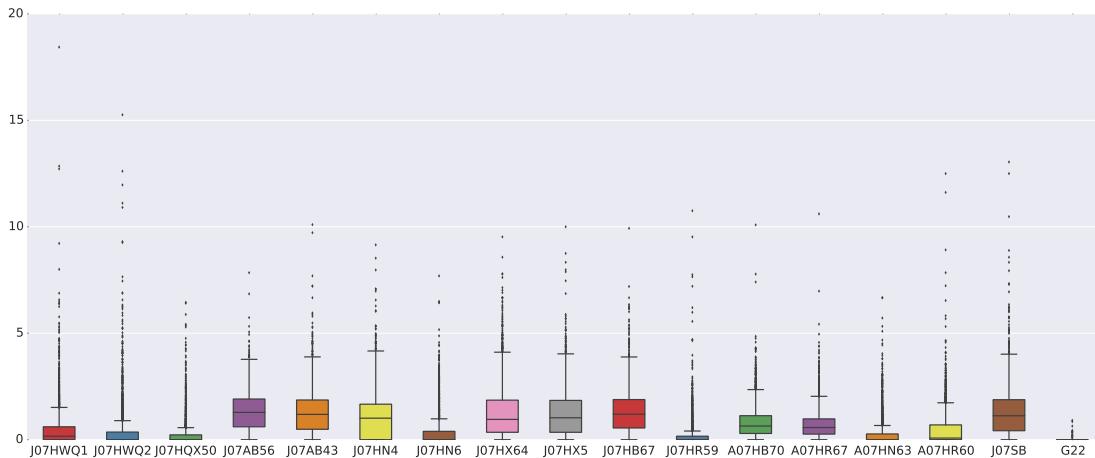


Figure 5.10: SNpp frequency

5.5 Conclusions

5.6 Acknowledgments

List of genes, product

Functional categories, differences from the annotation?.

Fisher test

Plots of depth, dn/DS along genomes



Figure 5.11: Mapping strategy

Table 5.5: Count of Genes under positive selection

Genome	CDS	January	August	Unique (Jan/Aug)
<i>J07HWQ1</i>	3,584	568 (15.9)	570 (15.9)	4/6
<i>J07HWQ2</i>	3,856	565 (14.7)	561 (14.6)	6/2
<i>J07HQX50</i>	2,872	406 (14.1)	405 (14.1)	3/2
<i>J07AB56</i>	1,411	244 (17.3)	236 (16.7)	72/64
<i>J07AB43</i>	1,678	274 (16.3)	281 (16.8)	35/43
<i>J07HN4</i>	3,230	428 (13.3)	427 (13.2)	5/4
<i>J07HN6</i>	2,914	220 (7.6)	218 (7.5)	23/21
<i>J07HX64</i>	3049	603 (19.8)	603 (19.8)	3/3
<i>J07HX5</i>	2,139	374 (17.5)	374 (17.5)	2/2
<i>J07HB67</i>	2,847	494 (17.4)	493 (17.3)	60/59
<i>J07HR59</i>	1,841	145 (7.9)	155 (8.4)	3/13
<i>A07HB70</i>	2,514	479 (19.1)	479 (19.1)	6/6
<i>A07HR67</i>	2,891	507 (17.4)	511 (17.7)	0/4
<i>A07HN63</i>	2,507	212 (8.5)	240 (9.6)	9/37
<i>A07HR60</i>	2,861	375 (13.1)	381 (13.3)	10/16
<i>G22</i>	3,525	5 (0.14)	5 (0.14)	1/1
<i>J07SB</i>	1,641	290 (17.7)	287 (17.5)	6/3

Chapter 6

Comparative genomics and selective pressure analysis; analysis of the *Haloquadratum walsbyi* genome

6.1 Abstract

6.2 Introduction

With the development of novel sequencing technologies, the price to sequence a single microbial genome has decreased considerably over the last years [52], increasing the number of microbial genomes in databases, helping investigation of comparative genomics, etc, etc. (REFs).

More genomes for related strains, and related species.

Whole genome analysis allows to increase phylogenetic resolution among multiple groups, and also to start asking questions about the particular genomic features that affect the adaptation of each organism to the environment.

Now is possible to move from comparing a single genome to several (REFs)
Multiple databases exist where genomes have been compared and the data

is already available in pre-calculated databases (REFs), but in some cases it is difficult to add or analyze recent genomes. A recent software ITEP, allows these comparasions. PSP allows also this, but is a web server

Selection analysis are important, not only gene content (absecnce presence)

In this chapter, I describe a pipeline developed for the comparative analysis of microbial genomes, with an emphasis on pan-genome anlaysis and selection analysis. All of the code is freely available in Github ([link](#)), and is currently being updated to be applied to large scale comparisons (100 genomes).

6.3 Overview of the analysis workflow

The analysis strategy consists on several Python scripts that carry out the different steps of data analysis (Figure 6.1). All of the scripts are available in a public repository on Github (<http://github.com/juanu/CompMicroGenom>). The analysis steps can be summarized as:

- Data preprocessing
- Ortholog search
- Analysis of ortholog data
- Selection analysis
- Result summary

In the following sections, each of these steps will be explained in more detail.

6.3.1 Data preprocessing

Several public resources are available for genome annotation and as repositories. Among some of the most used ones are the Integrated Microbial Genomes database[53], NCBI and RAST [7]. Although some of these repositories overlap in their content (in particular IMG and NCBI), the increase in the rates of submission of newer genomes, makes IMG slightly outdated in comparison to other resources.

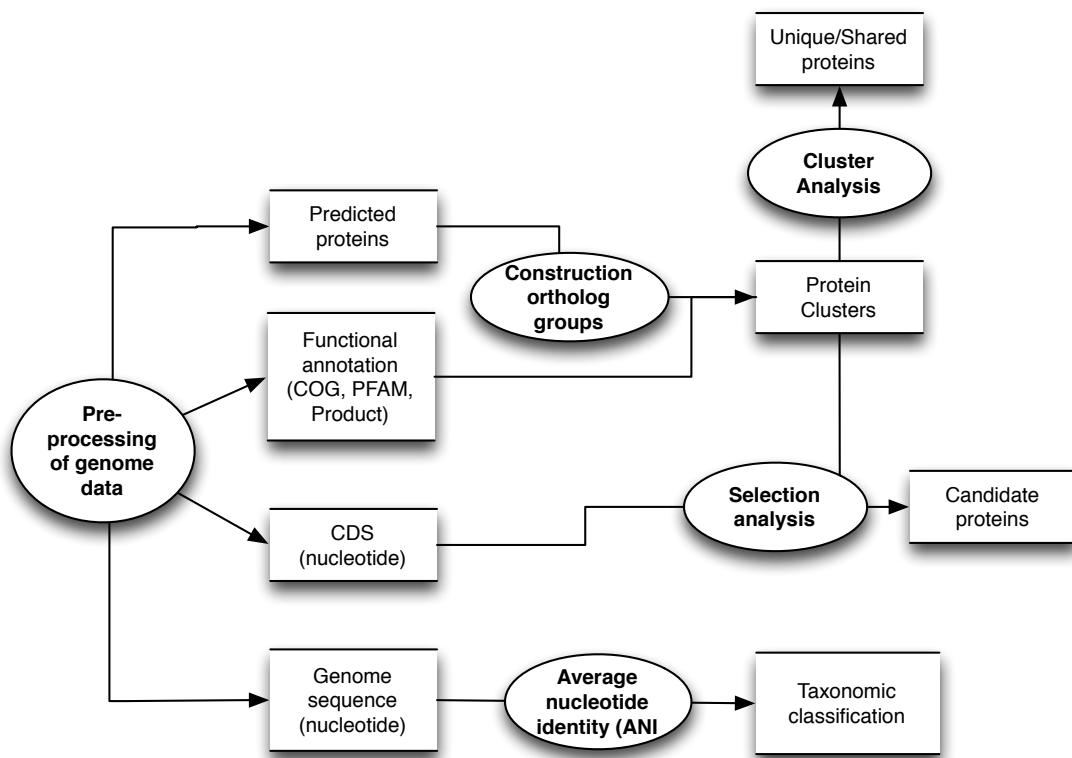


Figure 6.1: Overview of the pipeline developed for the comparative analysis of microbial genomes.

On the other hand, some of the genomes that are present on IMG are not available on NCBI and/or RAST. To be able to exploit all these available information, a pre-processing step is incorporated in the analysis to parse genomes obtained from these different sources, and put them into a common format that can be easily used through all the workflow (Figure 6.2). Using this strategy, also allows to incorporate genomes from newer sources, by just adding a new parsing script. For example, it could be relatively easy to incorporate an in-house sequenced genome into this workflow, by using a genome annotation pipeline like Prokka [78], and connecting into this workflow.

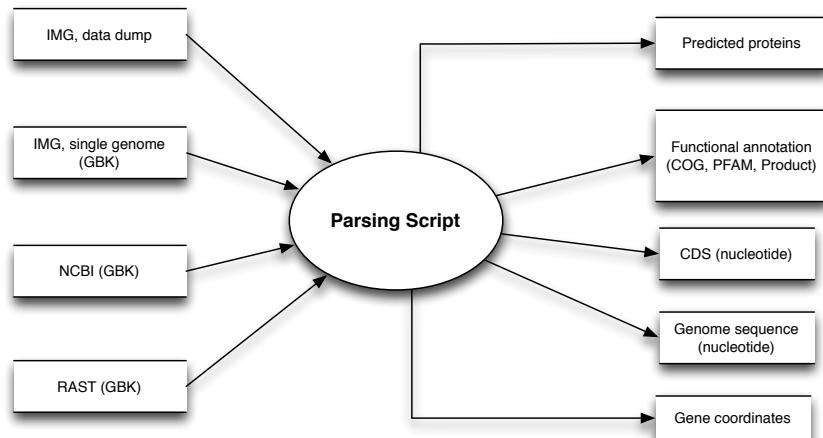


Figure 6.2: Preprocessing step

6.3.2 Construction of orthologous groups

The basis of this comparative genomic approach is the construction of orthologous groups of genes, a collection of all the descendants of an ancestral gene

that diverged after a given speciation event [42]. These groups provide us with a picture of the evolutionary history of the gene family (and its host organisms), and can be used for further studies on the basis for molecular adaptation of its host organisms. Several approaches exists for the construction of ortholog groups [42], which can be broadly classified in two categories: tree-based and heuristic based approaches. Tree based methods are computationally expensive and require a known phylogenetic relationship for the studied organisms, something that sometimes is difficult to generate in light of the presence of horizontal gene transfer events in microbial genomes [41]. On the other hand, heuristic based approaches are easy to automatize and several implementations of these methods are already available [42]. Among the main drawbacks, the generated group of sequences may not reflect the true evolutionary history of the gene, specially in large and complicated protein families [16]. In consideration of this evidence, we choose an heuristic-based approach to generate orthologous groups, because it can be easily scalable to hundreds of genome, and its easiness to incorporate into an automatic pipeline. OrthoMCL [46], performs a bidirectional best hit search in the aminoacid space, using a "all-vs-all" blastp (although, other search methods could be implemented), followed by a subsequent clustering of the results using a Markov Clustering procedure [27].

Within the current workflow, the construction of ortholog groups is based on the OrthoMCL protocol [46], consisting of the following steps:

- Protein blast of "all-vs-all" the proteins
 - Concatenate all the protein sequences for the genomes of interest
 - Create a Blast database
 - Protein blast using these parameters: `-F 'm S' -v 1000000 -b 1000000 -m 8`
- OrthoMCL analysis
 - Create a MySQL database to store the OrthoMCL results

- Run OrthoMCL to generate a list containing pairs of sequences and their similarity score
- Use MCL to create clusters based on the previous results. Different inflation values can be tested, where smaller values give larger clusters.

6.3.3 Analysis of ortholog groups

The clusters of sequences obtained from the previous step are processed using a set of Python scripts to summarize the results. Some of the information obtained here includes:

- List and count of unique sequences in each analyzed genomes
- A list and count of single copy clusters; sequences that are present in single copy in all the analyzed genomes.
- A list and count of shared multiple copy clusters; sequences present in all the analyzed genomes, but that in some of them are present in more than one copy.

Levering on these cluster results, several analysis can be performed using this information:

Functional annotation

The resulting protein clusters can be annotated, based on the information obtained from the annotation files. Currently the annotation incorporates information such as Cluster of Orthologous Groups (COGs), KEGG (REF) and PFAM domains (REF). The current approach for the annotation is to use a majority rule for the cluster, where each cluster is annotated with the function that half plus one of the proteins in the cluster have. This also serves as a validation step, because in classifications such as COGs, is expected that all of the members from the cluster should have the same COG number. Conflicts are recorded for validation as well.

Alignment and tree construction

As part of the pipeline, it is possible to generate alignments and trees for all (or a subset) of the clusters generated. The approach used is to generate an alignment of each cluster using MAFFT on the amino acid sequences, followed by a construction of a phylogenetic tree using FastTree. DNA alignments are generated by replacing each amino acid in the aligned sequences, with its corresponding codon from the DNA sequences, allowing for better DNA alignments. All of the resulting sequences (aligned and unaligned) are saved for future reference.

Phylogenomic analysis

One of the most important goals of the workflow is to generate information useful for phylogenomic analysis. Phylogenetic classification based on 16S rRNA sequences, although highly used, are limited by the sequence space of the 16S rRNA gene. Even species with highly similar 16S rRNA sequences, show differences both in their gene content and the functional capabilities. The use of marker genes, multi-locus sequence typing (MLST) and whole genome analysis, allow to increase the information and therefore the resolution of phylogenetic classifications.

Within the scope of the workflow, the idea is to be able to identify clusters that are present in single copy and are shared across all the genomes in the analysis. This approach is similar to what has been done for taxonomic and metagenomic classification of novel organisms (REF), but in this particular case allows to establish the relationships among the organisms under study. The approach used is very similar to previously proposed analysis (REF, acinetobacter), summarized on Figure ???. All the single copy clusters are identified and the alignments of each one is generated. Each aligned cluster is tested for recombination using the Phipack software, with three different analysis PHI, MaxChi and Neighbour similarity scores (REF), with 1000 permutations, window size of 50 nt and a p-value ≤ 0.05 . The goal of this step is to select recombination free clusters that can be used for phylogenetic analysis, with the bonus by-product of identifying clusters that have evidence of recombination and could be interesting candidates for further analysis (REFs). The alignments of this recombination-free clusters are then

trimmed using Gblocks with default parameters. The trimmed clusters are then concatenated to generate a newer alignment that can be used for phylogenetic analysis.

Complementary to this, another script allows to perform genomic comparisons among the studied organisms using the average nucleotide identity (ANI) between them. The approach used is the method proposed by Goris et al (REF). For each query, two genomes are compared, where the query genome is divided into 500 bp. fragments and then compared using blastn against the target genome. For each fragment, the best hit (highest bit score) is selected if it had at least 70% identity and covers over 70% of the query fragment length. The pairwise comparisons are then collected into a matrix that can be used to evaluate pair of genomes and also for dendrogram construction.

Population and selection

-Why the search of this is important One of the most important features of the analysis is the search for clusters with evidence of selective pressure. For this PAML was used - Statistic models - Comparisons branch -sites

-FDR correction for multiple testing

6.4 Comparative analysis of *Haloquadratum*

6.4.1 *Haloquadratum* core and accessory genome

6.4.2 Evidence of habitat-selection in *H. walsbyi*

6.5 Conclusions and Future Directions

General goals of the pipeline. Goals to complete, including integrations with other tools, documentation. Use of a database for the analysis. Integration of faster tools for selection analysis. Comparison: concatenation of proteins versus consensus of gene trees.

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Figure 6.3: HQPanGenome

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Figure 6.4: Circular plot

6.6 Acknowledgments

Table 6.1: Genes under selection in HQ strains

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Appendix A

Draft Genome Sequence of *Candidatus Halobonum* tyrrellensis Strain G22, Isolated from the Hypersaline Waters of Lake Tyrrell, Australia



Draft Genome Sequence of “*Candidatus Halobonum tyrellensis*” Strain G22, Isolated from the Hypersaline Waters of Lake Tyrrell, Australia

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We report the draft 3.675-Mbp genome sequence of “*Candidatus Halobonum tyrellensis*” strain G22, a novel halophilic archaeon isolated from the surface hypersaline waters of Lake Tyrrell, Australia. The availability of the first genome from the “*Candidatus Halobonum*” genus provides a new genomic resource for the comparative genomic analysis of halophilic Archaea.

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Halophilic Archaea of the class *Halobacteria* (phylum *Euryarchaeota*) are dominant members of extreme hypersaline environments worldwide (1). Numerous genera have been isolated from diverse hypersaline habitats, and many representative genome sequences are available (1, 2). However, recent metagenomic analyses of hypersaline ecosystems have revealed that these reference halophiles are not adequately representative of the dominant microbial populations present in many natural hypersaline habitats (3–5). Here, we report the genome sequence of a novel member of the class *Halobacteria* isolated from the hypersaline surface waters of Lake Tyrrell, Victoria, Australia.

Surface water samples were plated onto minimal medium containing a 23% salt solution amended with various carbon substrates, including glycerol, acetate, or glucose, and incubated at room temperature under aerobic conditions. After 3 to 4 weeks of incubation, the colonies were restreaked for purity and characterized via 16S rRNA gene amplification and sequencing to screen for novel species/stains. “*Candidatus Halobonum tyrellensis*” strain G22 was isolated from minimal medium containing glycerol as the sole carbon source, incubated aerobically at room temperature. Genomic DNA was sequenced using 454 Titanium chemistry at the J. Craig Venter Institute (Rockville, MD). The total number of reads generated was 568,949, with an average length of 428 bp. The sequences were assembled using Newbler (version 2.7), resulting in a total of 72 contigs (N_{50} , 119,067 bp; mean contig length, 45,962 bp; maximum contig length, 303,316 bp), with an estimated genome size of 3,675,087 bp and a G+C content of 70.1%. Functional annotation of predicted gene sequences was performed using the IMG-ER platform (6). A total of 3,525 predicted coding sequences were identified, including 47 tRNAs and a single copy of the rRNA operon.

A phylogenetic tree based on 16S rRNA genes (<http://dx.doi.org/10.6084/m9.figshare.830514>) suggests that “*Ca. Halobonum tyrellensis*” is a member of the *Halobacteriaceae* family and a sister group of the *Halobaculum* genus, sharing 92% 16S rRNA gene sequence identity with *Halobaculum gomorrense* (7). A com-

parison of the “*Ca. Halobonum tyrellensis*” genome with the partial genome sequence available for *H. gomorrense* (632,433 bp) (8) revealed an average nucleotide identity (ANI) of $81.56 \pm 1.18\%$. A phylogenomic approach using multiple amino acid markers (9) supports the placement of “*Ca. Halobonum tyrellensis*” as a new genus (<http://dx.doi.org/10.6084/m9.figshare.830514>). A detailed characterization of the physiology and metabolism of “*Ca. Halobonum tyrellensis*” and a formal description of this strain are currently in progress.

The features found in the genome include the presence of a putative sensory rhodopsin, a high number of ABC transporters and carbon metabolism genes, including trehalose utilization genes, and the absence of conserved haloarchaeal genes encoding a flagellar system or gas vesicle synthesis proteins.

The “*Ca. Halobonum tyrellensis*” genome represents the first high-quality draft sequence for a member of the new candidate genus *Halobonum*. These data expand the breadth of the reference genome sequence information for halophilic Archaea, providing a new resource for comparative genomic analyses and the phylogenetic binning of metagenomic sequence data recovered from hypersaline environments.

Nucleotide sequence accession number. The draft genome sequence of “*Ca. Halobonum tyrellensis*” strain G22 is deposited at DDBJ/EMBL/Genbank databases under the accession no. **ASGZ00000000**.

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Appendix A is a full reprint of the publication XXXX, with permissions from all coauthors.

Appendix B

Genome coverage plots

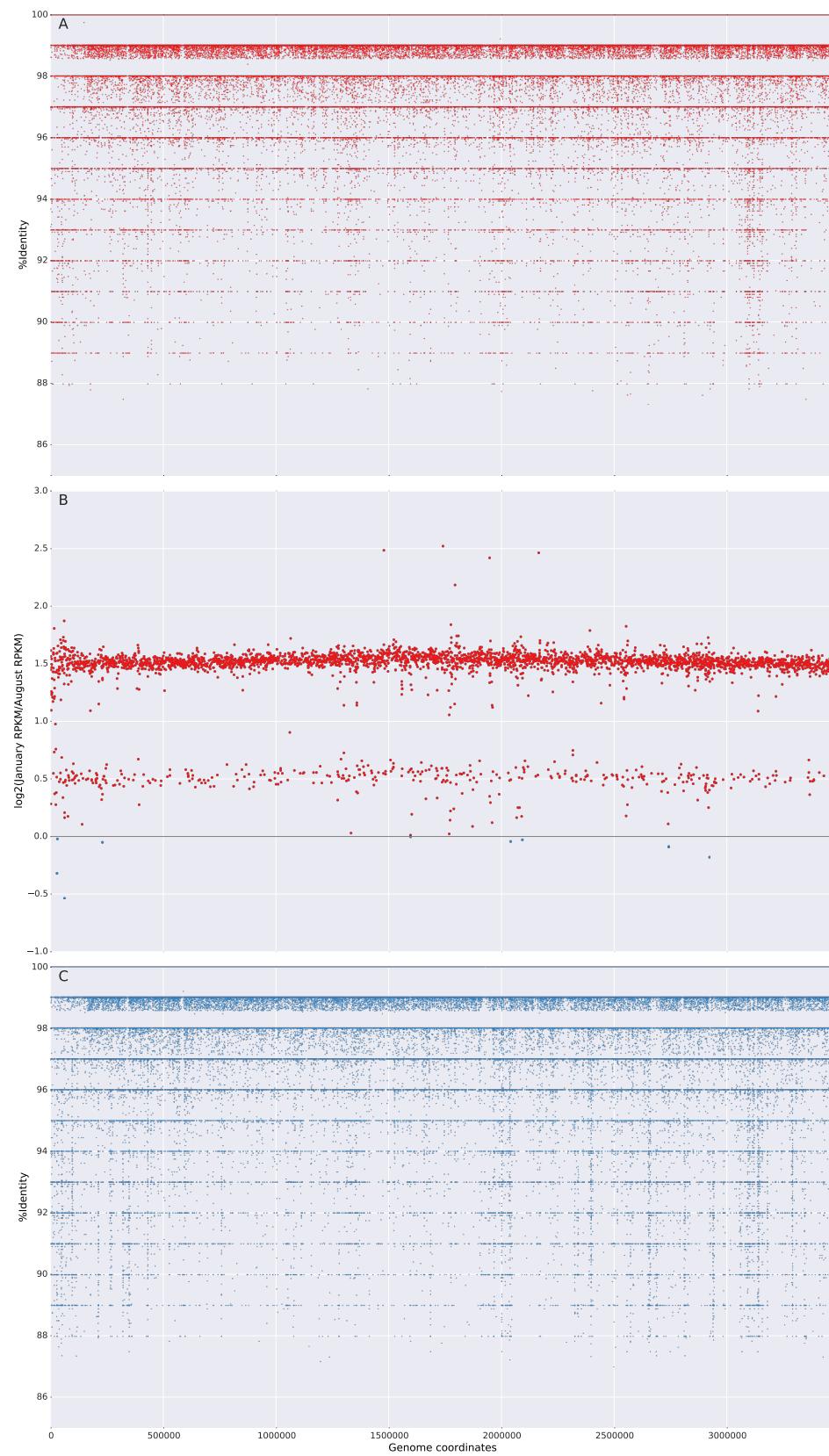


Figure B.1: J07HWQ1coverage

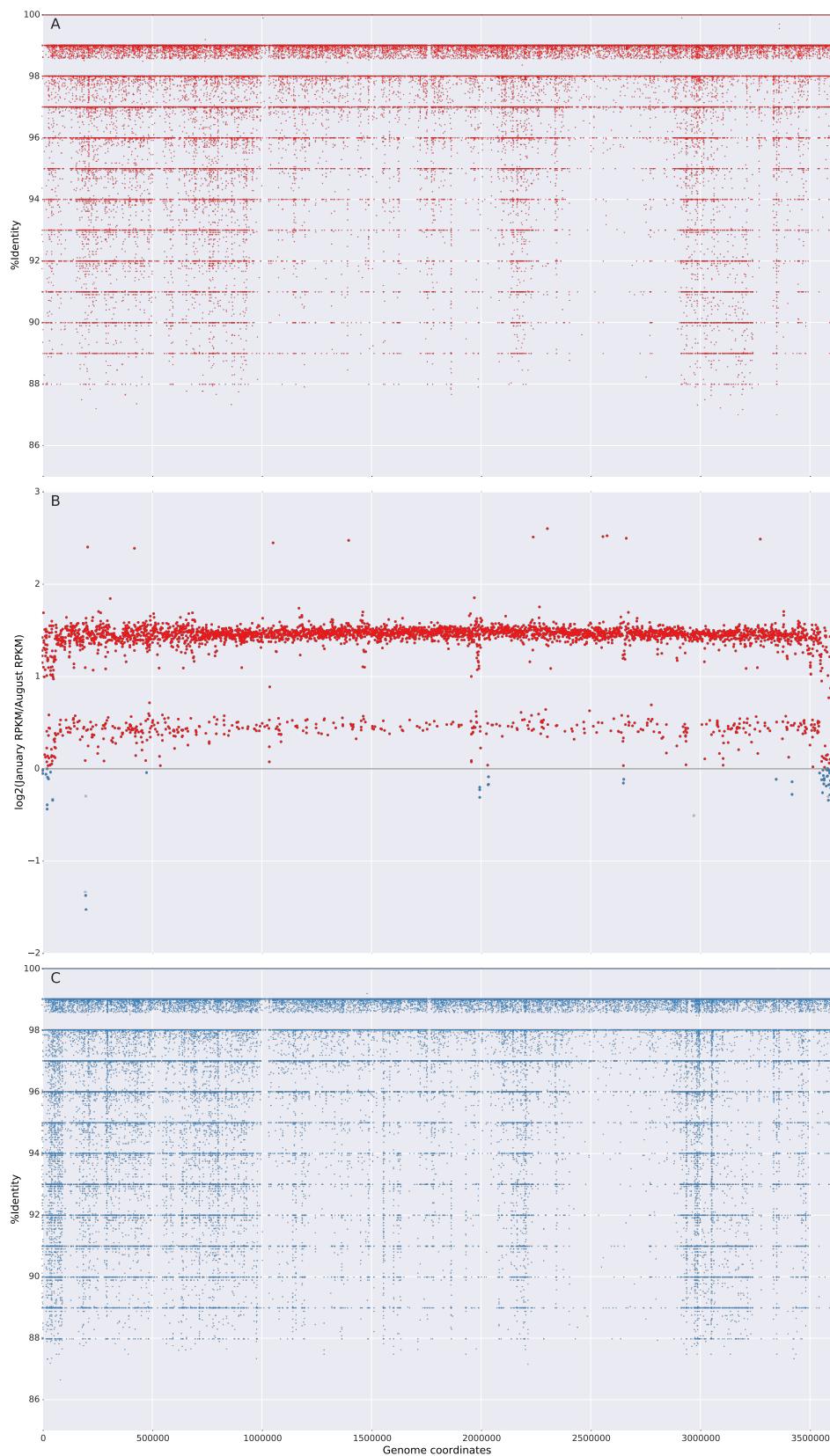


Figure B.2: J07HWQ2coverage

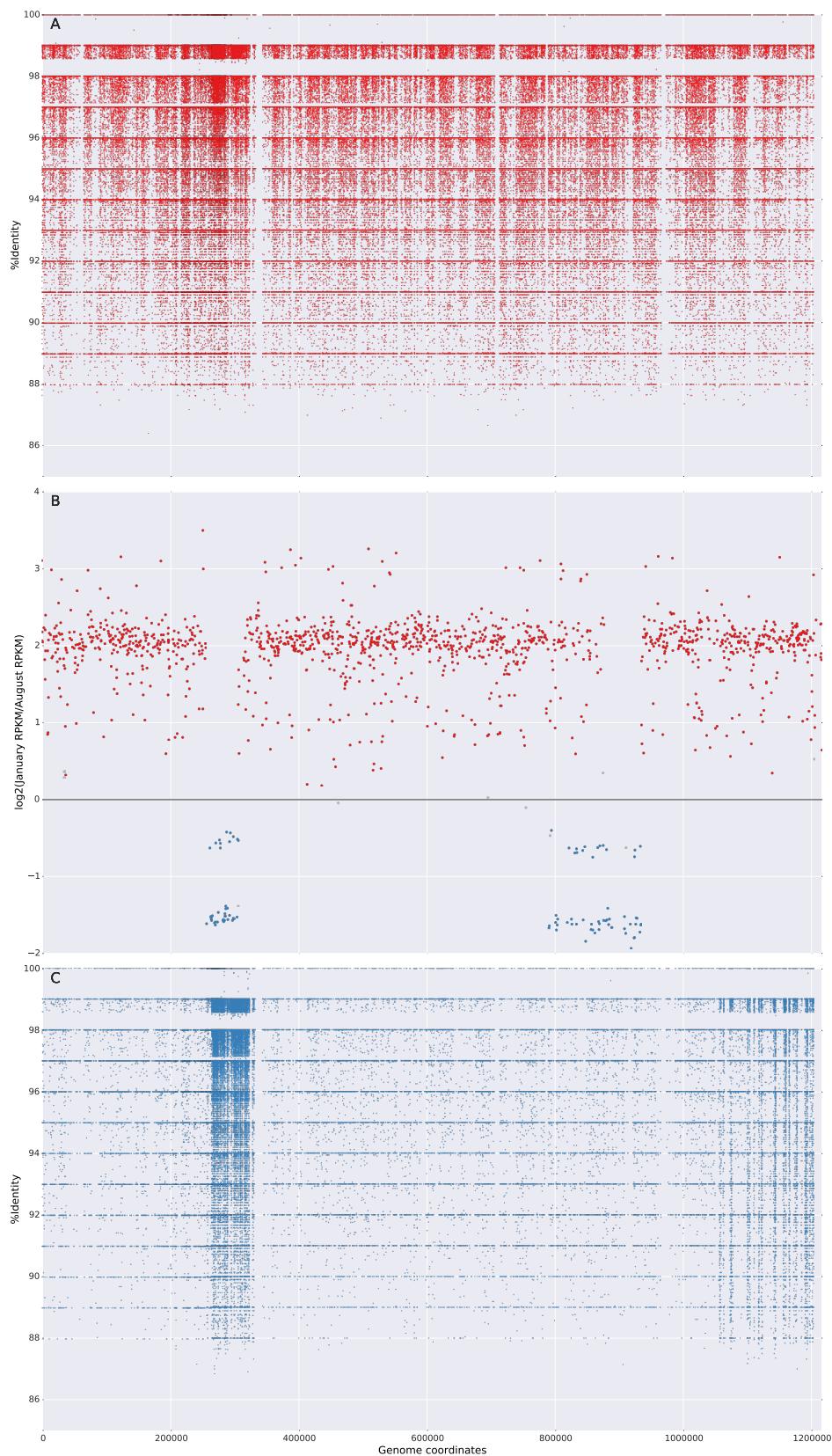


Figure B.3: J07AB56coverage

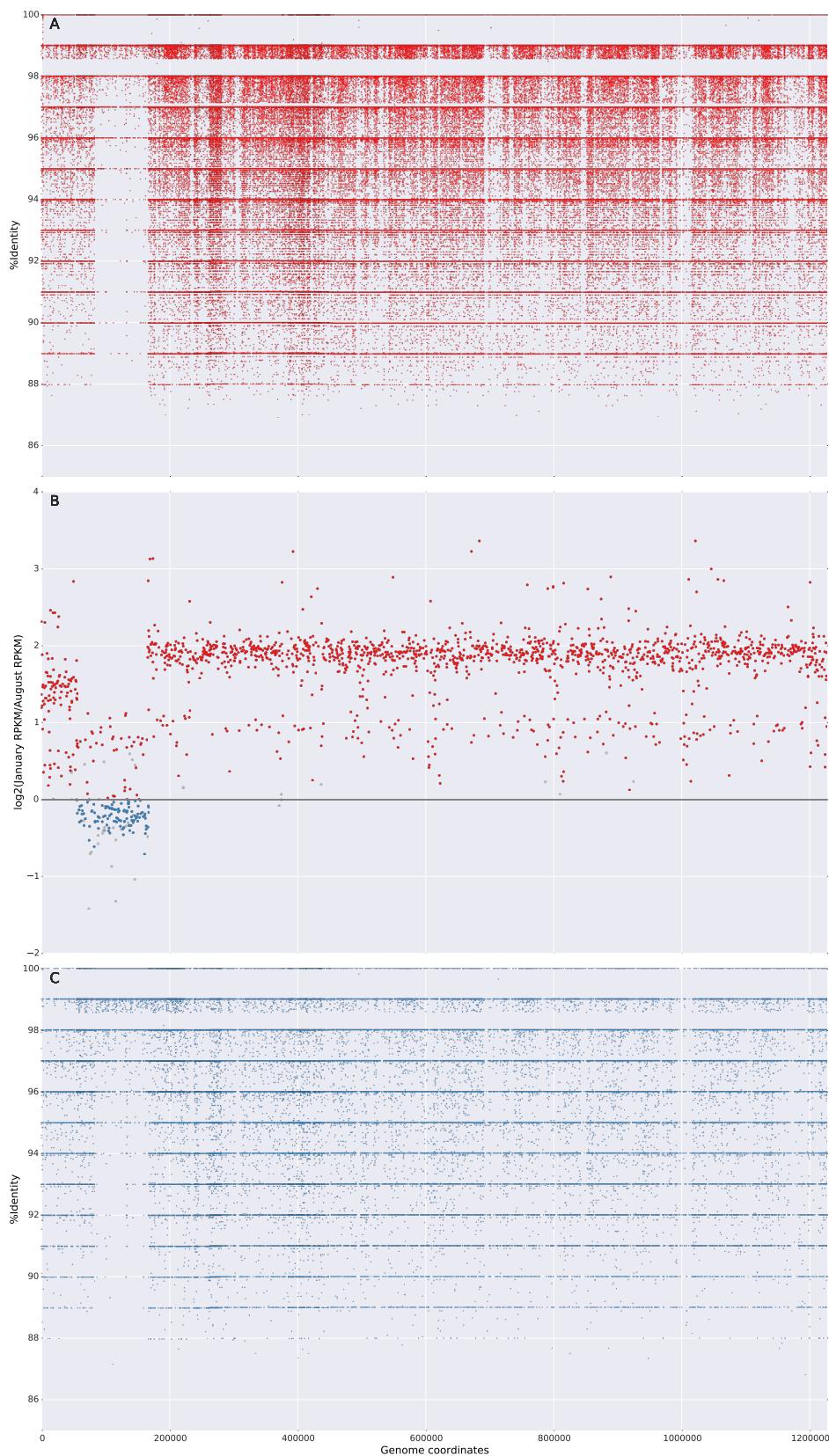


Figure B.4: J07AB43coverage

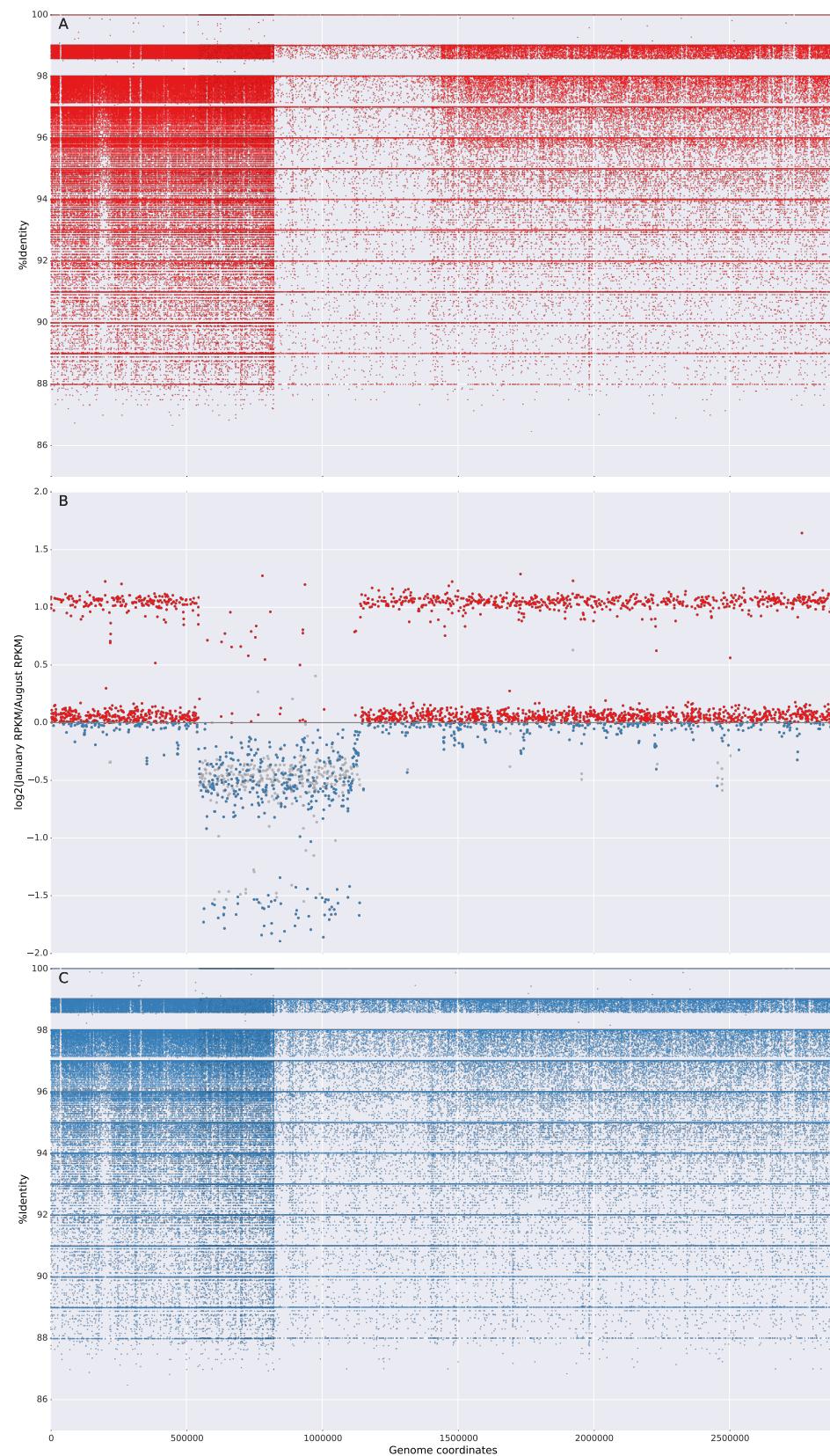


Figure B.5: J07HN4coverage

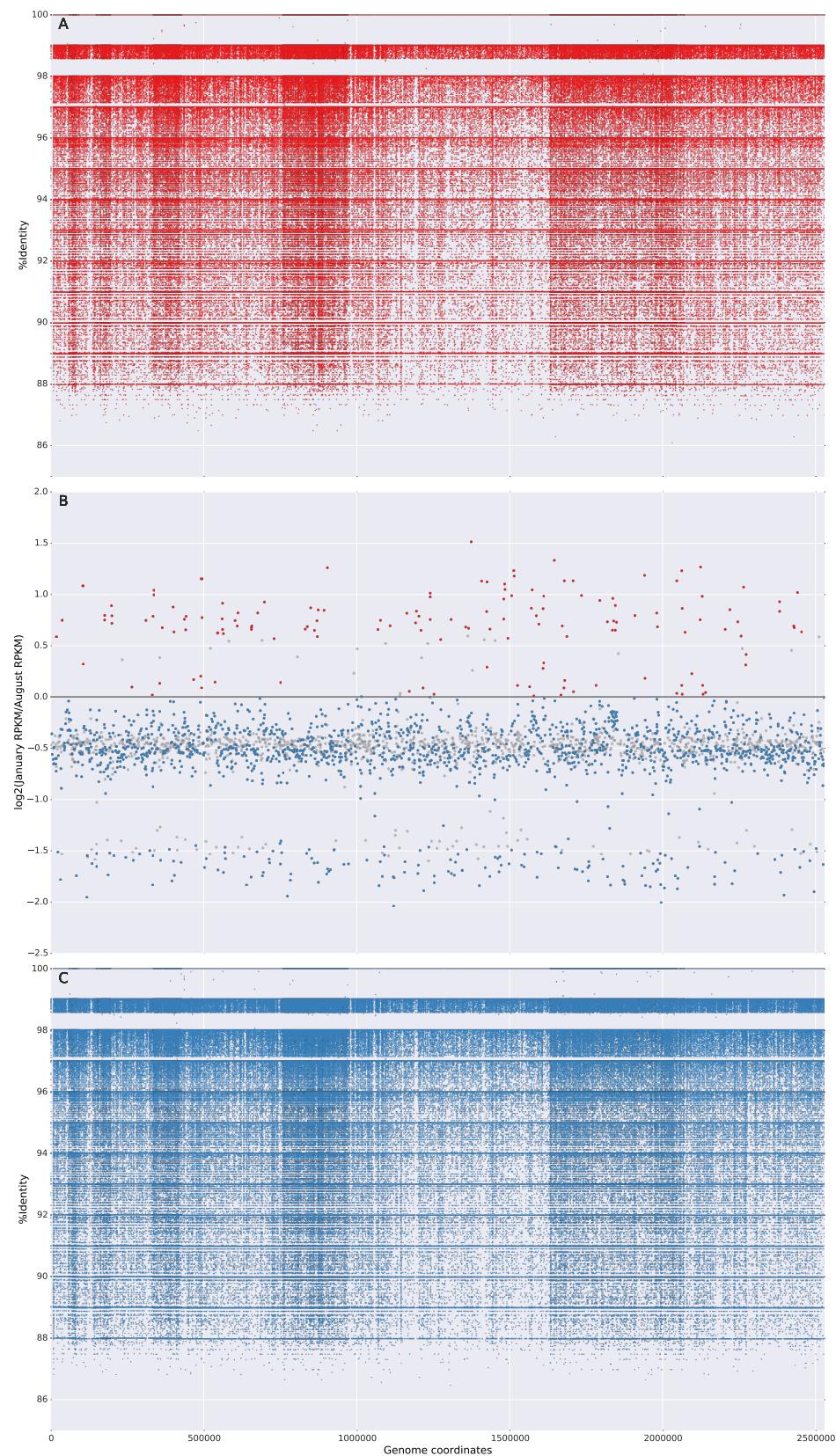


Figure B.6: J07HN6coverage

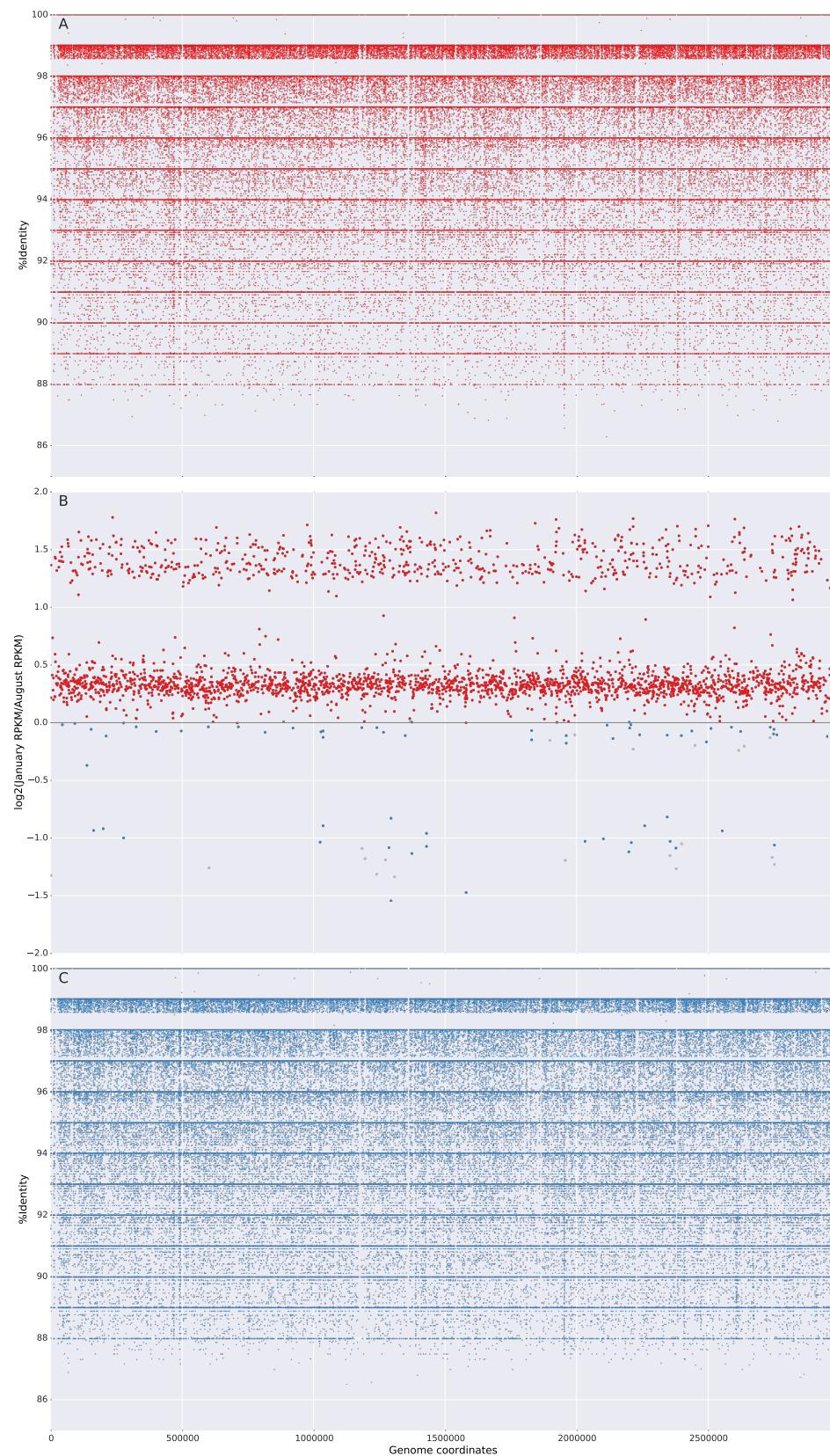


Figure B.7: J07HN64coverage

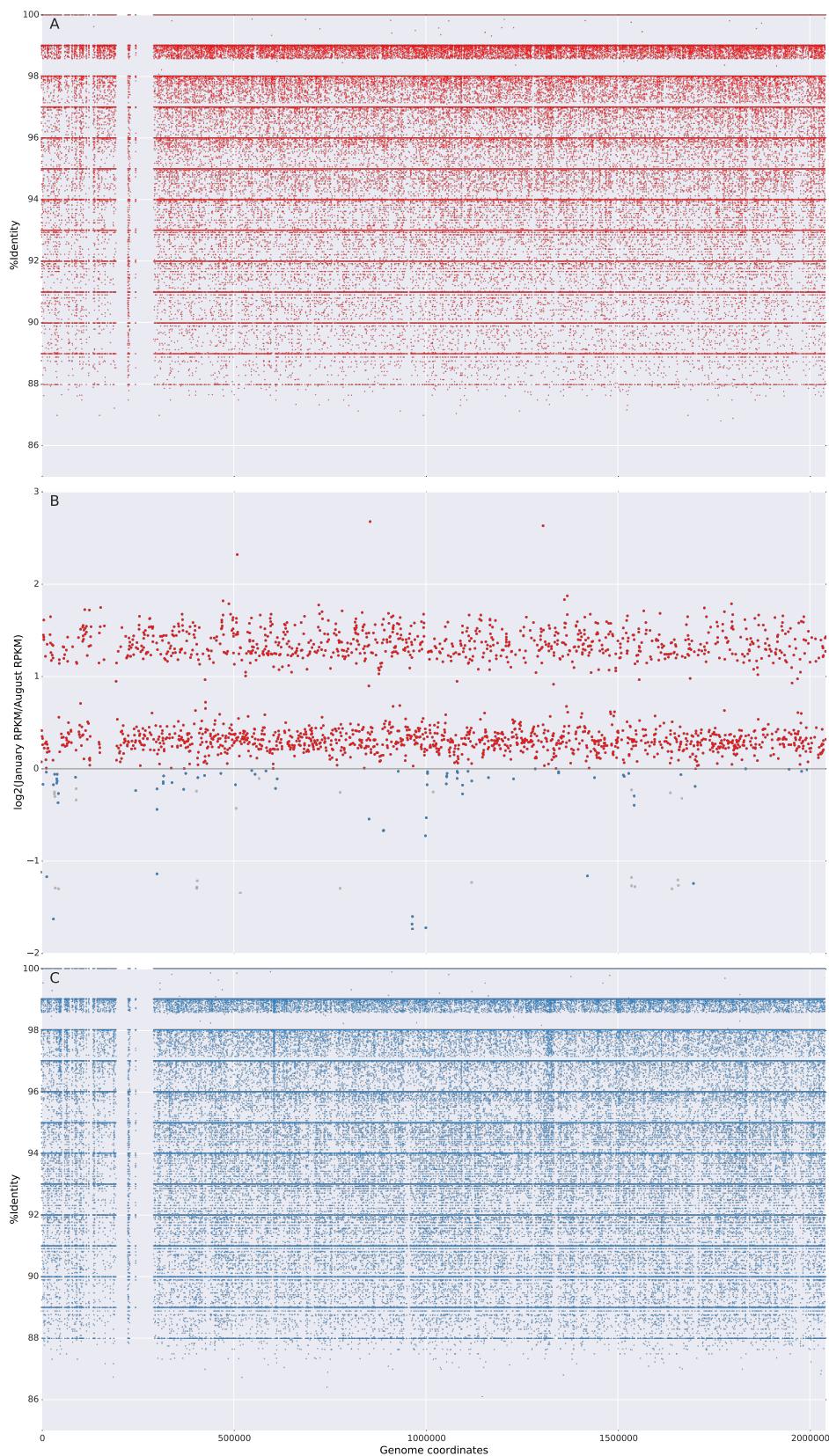


Figure B.8: J07HX5coverage

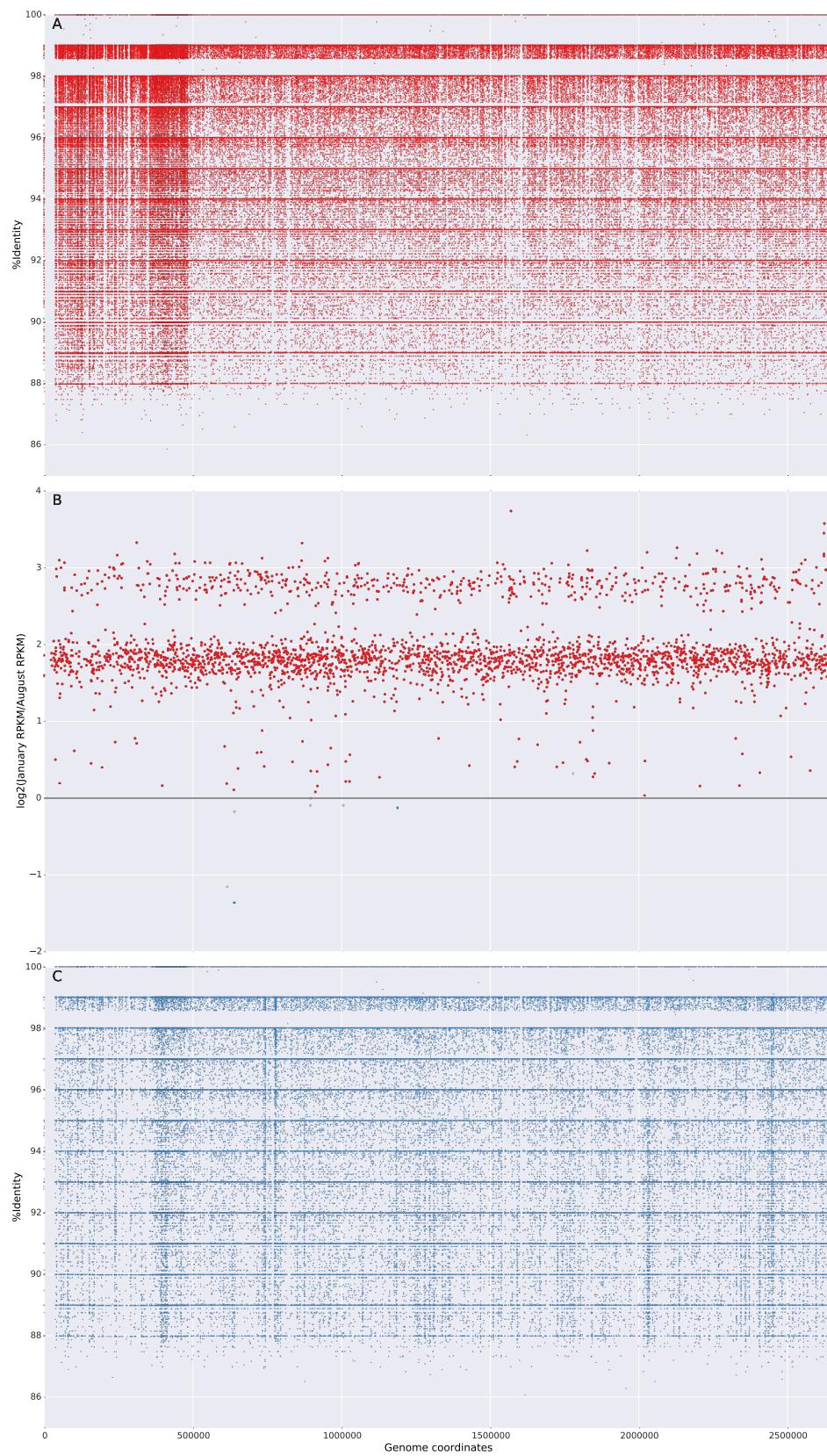


Figure B.9: J07HB67coverage

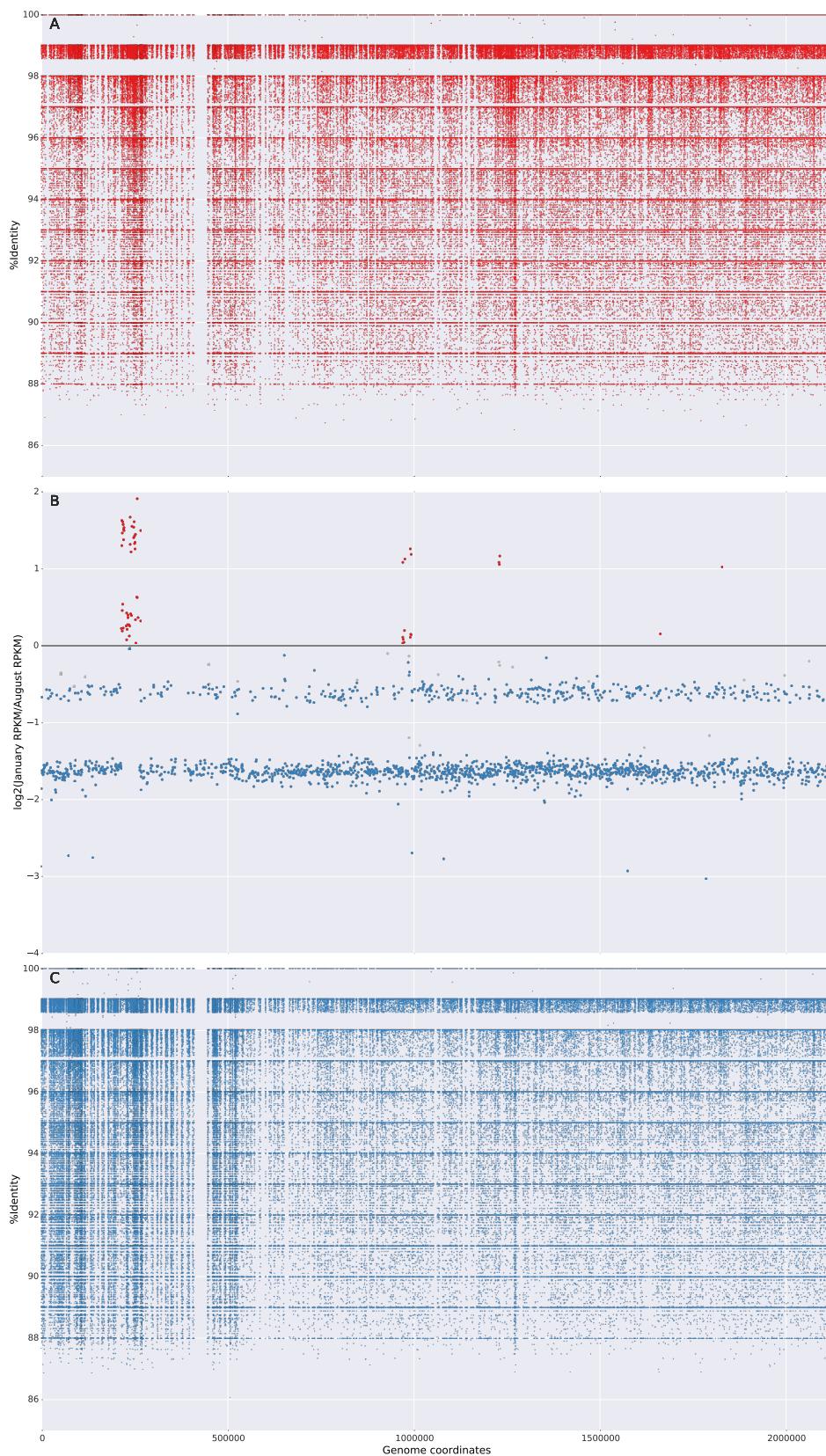


Figure B.10: J07HR59coverage

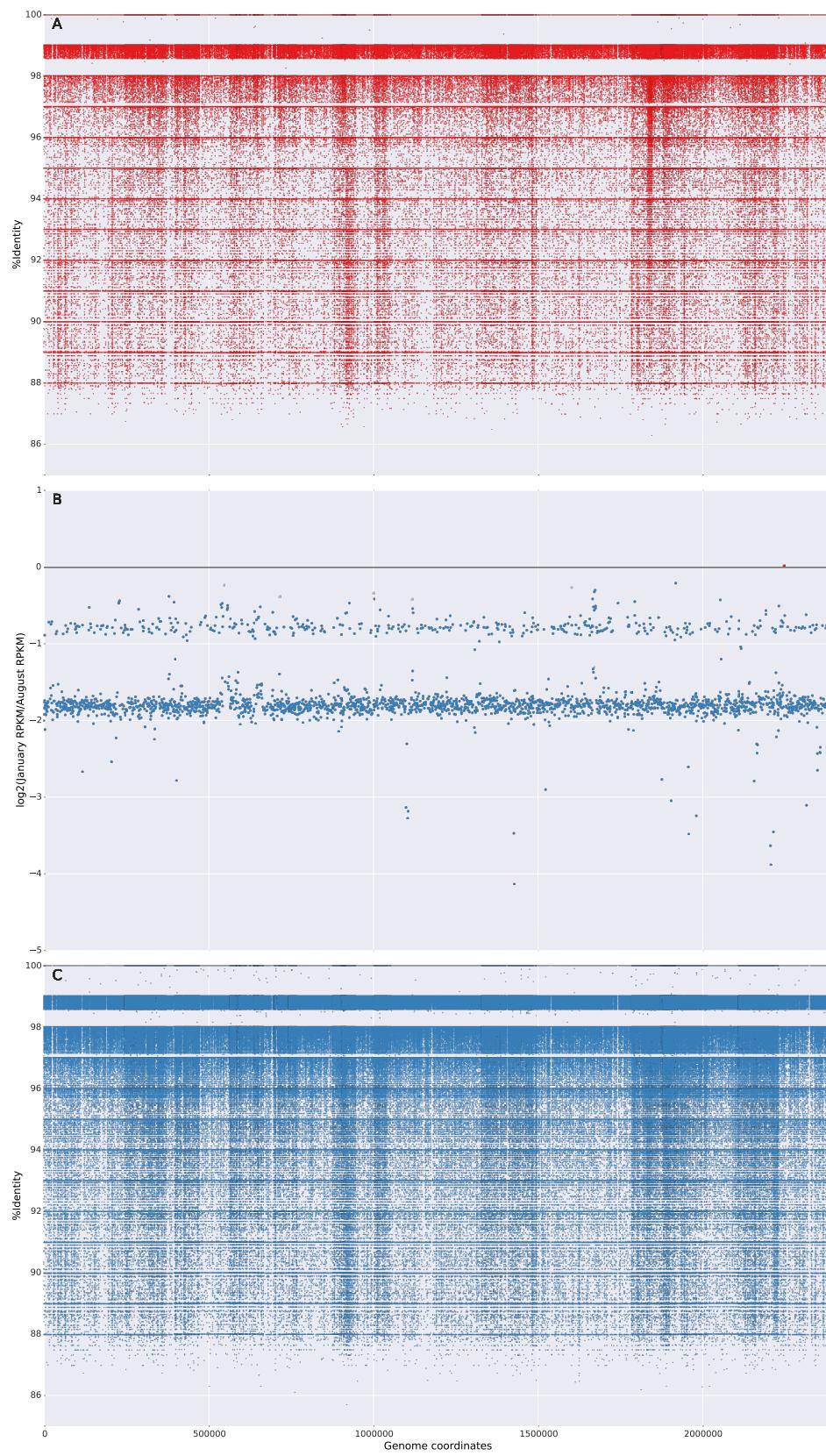


Figure B.11: A07HB70coverage

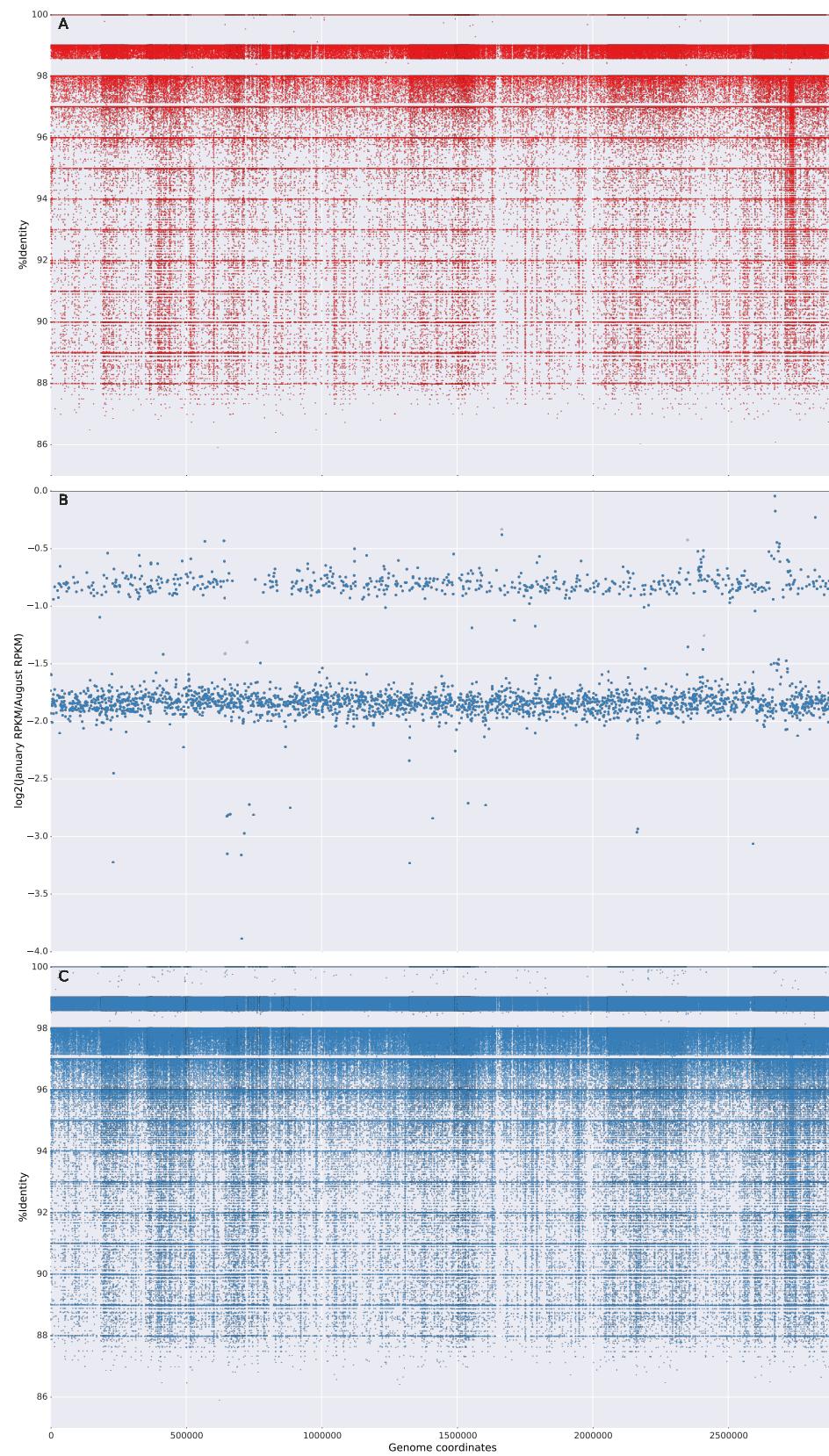


Figure B.12: A07HR67coverage

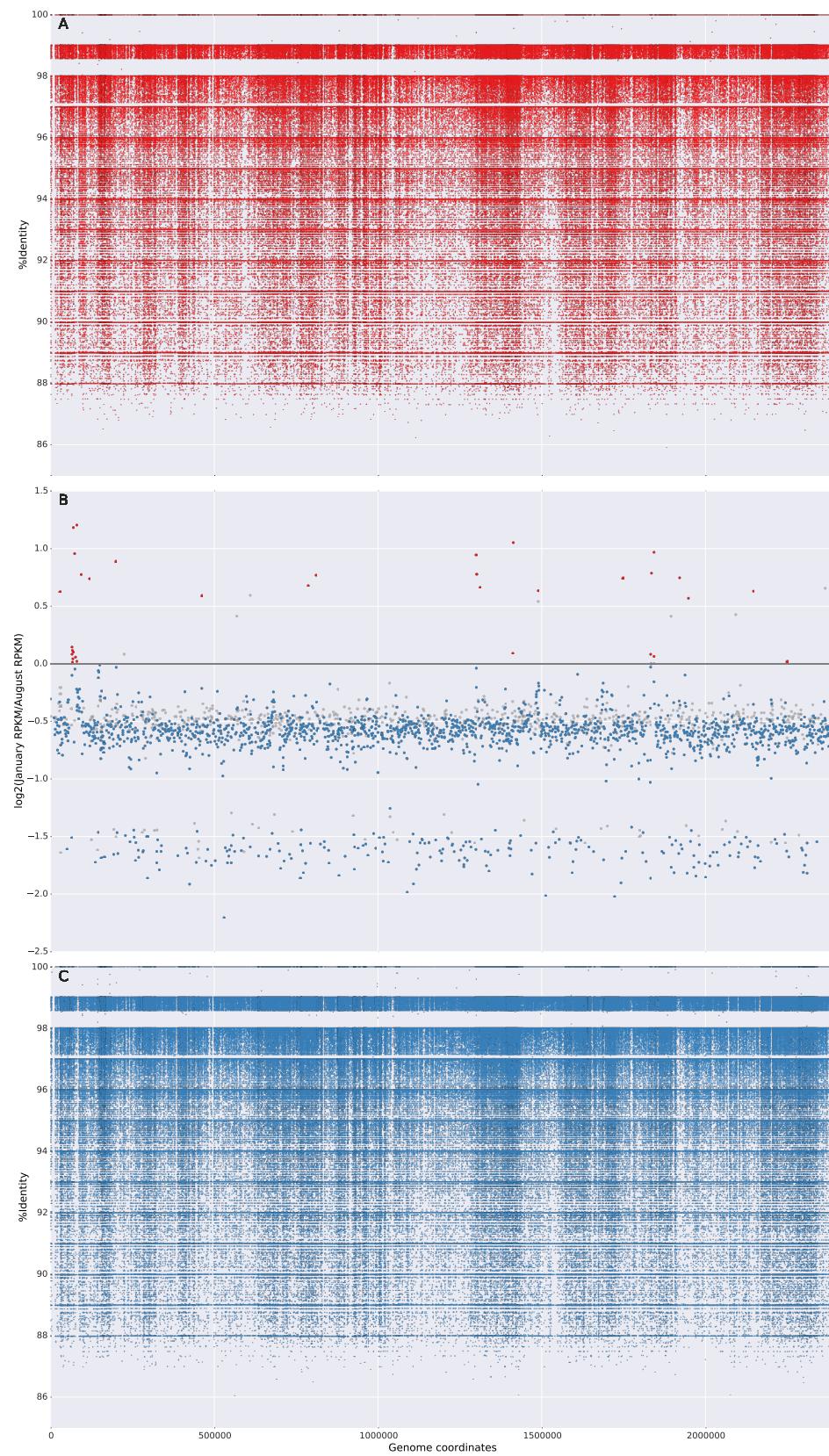


Figure B.13: A07HN63coverage

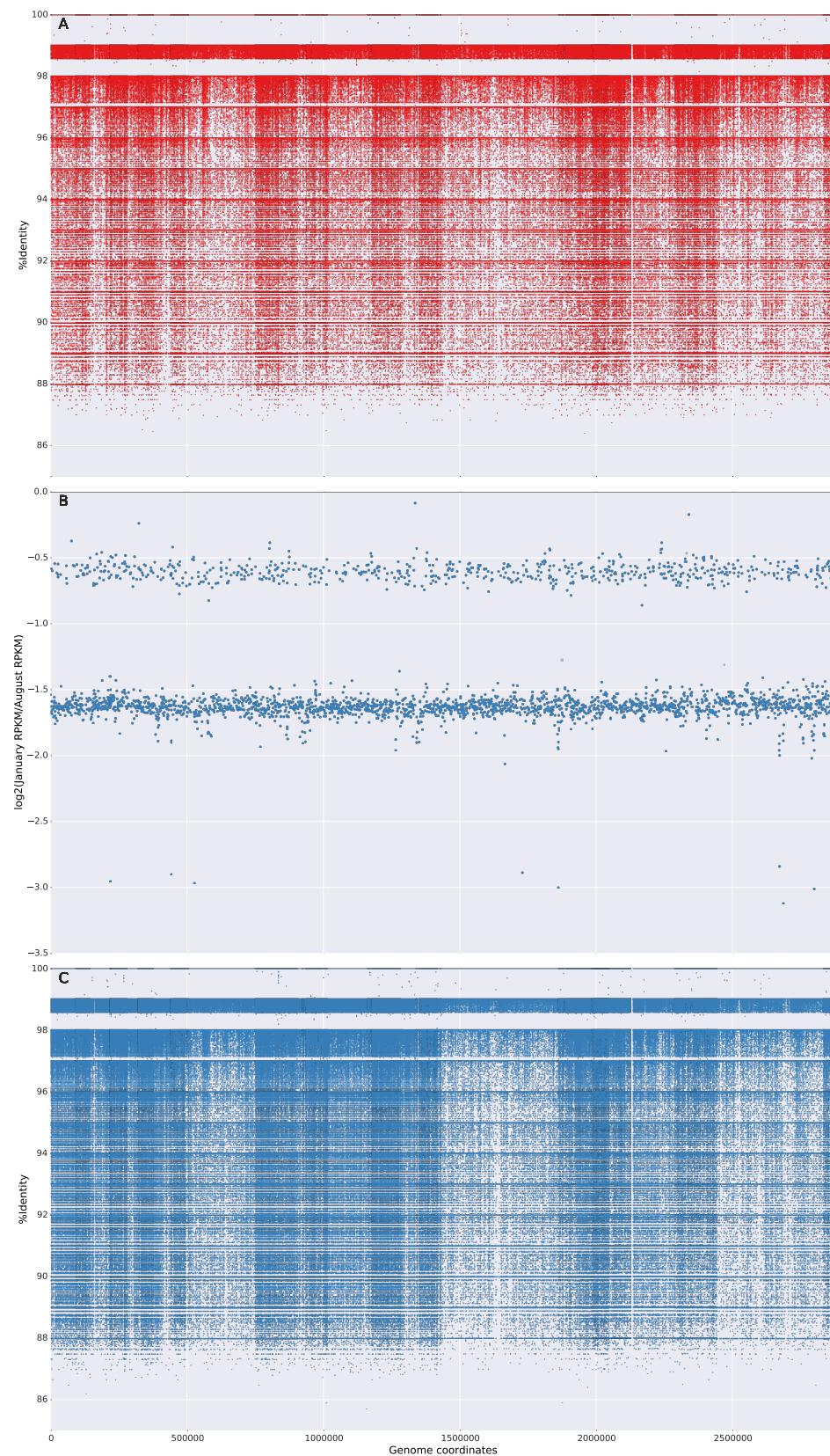


Figure B.14: A07HR60coverage

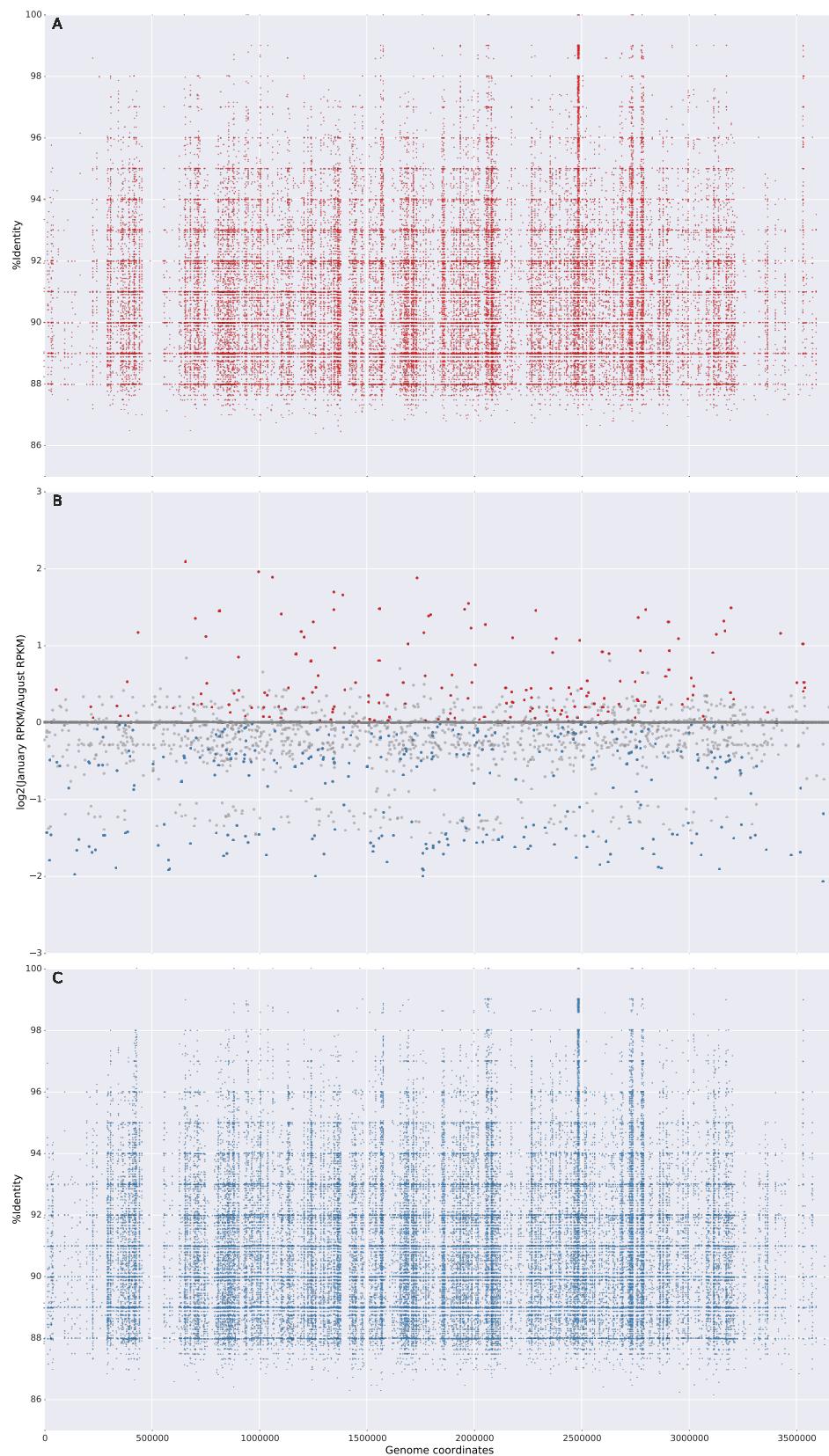


Figure B.15: G22 coverage

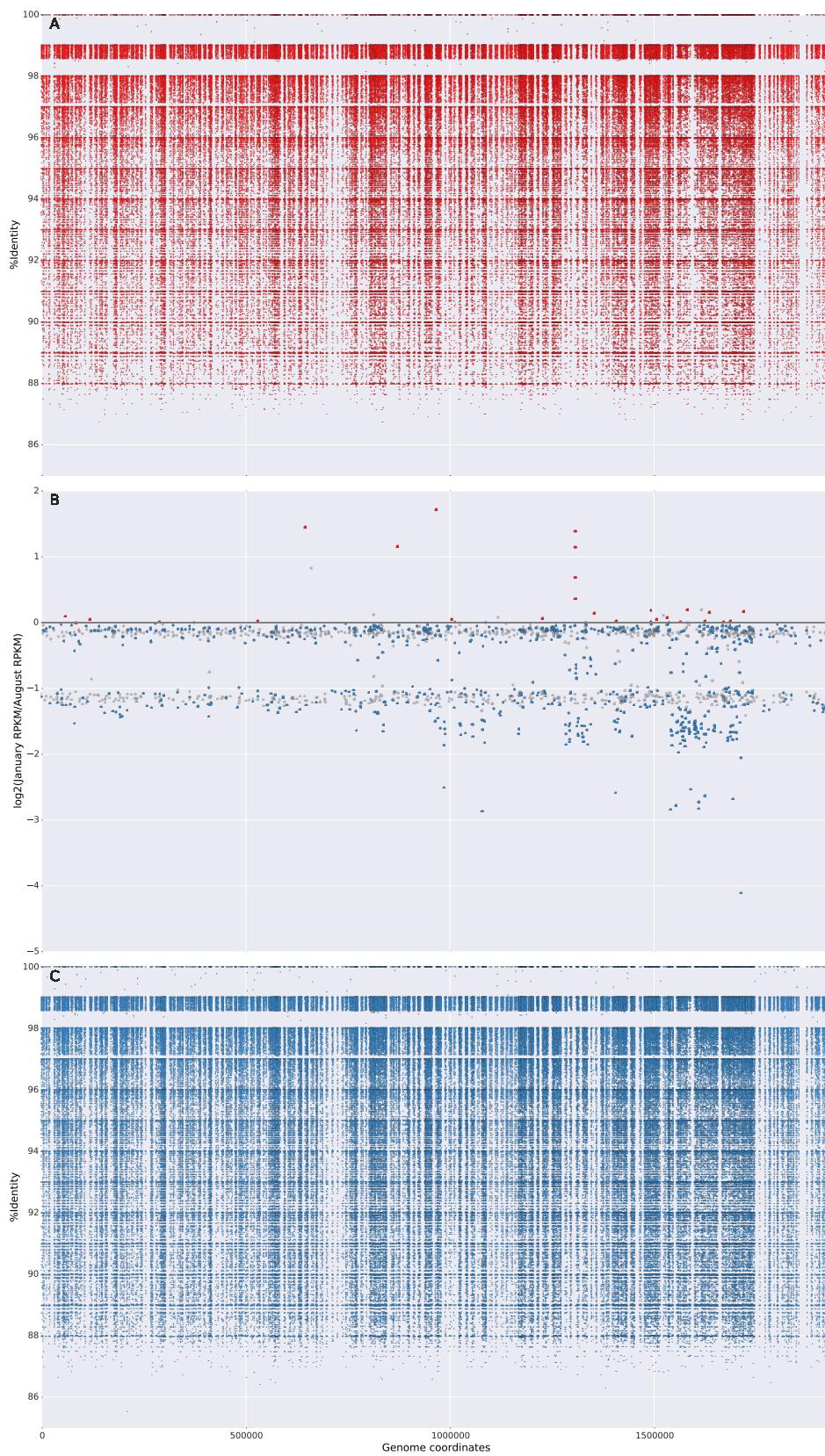


Figure B.16: J07SB coverage

Todo list

Check if it is ok this as bold	i
List of genes, product	76
Functional categories, differences from the annotation?. Fisher test	81
Plots of depth, dn/DS along genomes	81