MiniReview

The importance of physical isolation to microbial diversification

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Abstract

The importance of physical isolation, defined as the spatial separation of two or more populations, to the evolution of organisms has been well studied in plants and animals yet its significance regarding microbial evolution has not been fully appreciated. Here we review the theoretical paradigm of physical isolation for the diversification of organisms in general and then provide a variety of evidence indicating that microbial populations also fit into a similar evolutionary framework.
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1. Introduction

With respect to the diversification or speciation of prokaryotes, nearly all studies focus on the importance of variation generated by mutation and/or lateral gene transfer (LGT) and subsequent Darwinian selection through competition for resources (i.e. adaptation [1–12]). However, evolution as we understand it, involves additional mechanisms such as physical isolation, which can lead to non-selective or neutral means that advance and promote population divergences. Curiously, neutral mechanisms of divergence associated with physical isolation, like genetic drift, have not been generally considered in prokaryotic evolution, as evidence for prokaryotic population bottlenecks and/or population isolation events in nature have rarely been observed. Furthermore, two general assumptions regarding free-living bacterial populations in nature have suggested a priori that genetic drift might be an uncommon feature of prokaryotic evolution: (i) populations are extremely large, and (ii) prokaryotes freely disperse to all parts of the globe. In recent years, with the advent of molecular

techniques for the detection of prokaryotic diversity, some studies have begun to reveal the existence of populations occurring in isolation in many different types of habitats (ranging from insect hosts to marine and soil environments). Our objective is to review some examples of studies that demonstrate microbial population isolation, and consider their findings relative to well-established general evolutionary theory. We also wish to promote the concept of population isolation as an important co-occurring component of microbial evolution, but one that has been largely overlooked as microbial ecologists have focused more on adaptive evolution through natural selection to explain patterning of diversity detected by molecular methods. We first review evolutionary theory, proceed to the evidence for physical isolation in microorganisms, and then consider the ramifications of isolation for microbial evolution.

2. Physical isolation in the evolution of sexual species

In modeling the processes involved in the speciation of plants and animals, evolutionary theorists proposed that the physical isolation of organisms away from the parental population generates a barrier to gene flow between these two populations and that physical isolation events are considered "extrinsic impediments...envisioned as prerequisite for genetic divergence..." [13].

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For sexual organisms, gene flow within a population tends to act as a cohesive force preventing genetic divergences by homogenizing the gene pool. When organisms become isolated from their parental population (for whatever reason) this cohesive force is escaped. Subsequent to an isolation event, the restriction of gene flow between the two resulting populations allows a nascent population to independently accumulate mutations and/or lose allelic diversity through genetic drift. This might culminate in the formation of a new species if divergence leads to populations that meet criteria for defining species (e.g., if diverged populations are sexually isolated, they satisfy criteria established in the Biological Species Concept). An example would be different species of flightless birds on different continents. Indeed, it is well established that "the evolution of new species is equivalent to the evolution of genetic barriers to gene flow between populations" [14]. Divergence due to physical isolation is real and detectable even if species criteria are not met, so long as sensitive enough measures are applied. For instance, on the island of Borneo, orangutans (*Pongo pygmaeus*) that are physically separated by river systems form genetically distinct and diverging populations even though members of the different populations can still interbreed if artificially mixed (i.e., distinct subpopulations of a single biological species) [15]. Hence, with adequate resolution, the impact of physical isolation on divergence of populations (i.e., the process of speciation) can be observed independently of whether or not the populations meet any particular species criteria. Clearly, there is much argument about the criteria that should be used to demarcate when divergence is sufficient to warrant classification of distinct populations into distinct species [16] and microbiologists have entered the species debate [1–3,7–10,12,17–20] that still rages in plant and animal biology [21,22]. Fortunately, we do not need to enter the debate in order to witness the effect of physical isolation on the evolution of diverging populations.

Allopatric speciation (a process that potentially generates new species through physical separation of populations) is the best documented and is perhaps the easiest isolation process to illustrate. As a result of discrete geographical ranges, interpopulation reproduction is prevented because individuals from the different populations rarely or never co-occur. Isolation can also be viewed as a pre-mating mechanism for the prevention of gene flow. Other pre-mating mechanisms include evolved barriers to genetic exchange, such as mate recognition or behavioral displays. Additionally, post-mating mechanisms can also isolate populations (i.e. chromosomal inversions or different numbers of chromosomes), but these mechanisms tend to reduce reproductive success, not completely eliminate it [14]. While pre- and post-mating mechanisms are not usually thought to apply to microbial evolution, they certainly can in the case of restrictions to the acquisition of laterally transferred DNA/genes.

3. Physical isolation in microbial evolution

Despite the clonal reproductive mode of prokaryotes, they do have a "sexual" component to their survival strategy, but unlike truly sexual organisms, genetic exchange is not required for their life cycle or reproductive success. Prokaryotes have the ability to integrate laterally transferred genes or gene segments into their genomes from virtually any donor using homologous recombination (acquisition of a different allele(s) for an existing gene(s)), non-homologous recombination (acquisition of a novel gene(s)) or through the acquisition of mobile genetic elements (i.e. plasmids). The creation of metabolic pathways [23] and operons [24] are prime examples of the importance of LGT in the generation of vast physiologic and genetic diversity both within a single population and among all of the prokaryotes. Like sexual organisms, prokaryotes have mechanisms that can restrict or exclude extraneous or "non-self" DNA and which act as barriers for isolating their genomes. For a successful "sexual" event (i.e. recombination) to take place, close physical proximity of the recipient to the donor cells or their DNA is required. The absence of proximity (i.e. physical isolation) acts as a pre-mating barrier, as does the inability of cells to take up DNA. Furthermore, a series of post-mating barriers must be overcome that include: escape from restriction enzymes that destroy inappropriately modified donor DNA inside the cell, DNA sequence divergence, recombination joint formation, mismatch repair (MMR) and SOS systems, and functional incompatibility of the gene in its new host (for review see [25,26]). The frequency of homologous recombination can be predicted by a mathematical log-linear relationship for *Bacillus* [27], enterobacteria [28] and yeast [29] and is determined by the DNA sequence divergence between the two strands; the more differences that exist between the two strands, the less likely they are to recombine in a transformation assay. However, the frequency of homologous recombination can be skewed away from that predicted by the mathematical models through the regulation of MMR and SOS systems, thus permitting recombination between more distantly related organisms or prohibiting recombination between even the closest relatives [30]. The former is especially true for microorganisms experiencing stress in their environment [31].

In some ways, the role of recombination as a cohesive force for homogenizing prokaryotic population diversity is thought to be fundamentally different from that observed in sexually reproducing organisms. One major difference in the role of recombination is that in a prokaryotic population, acquisition of a new allele via LGT

may allow an individual cell that has gained such variation to out-compete its conspecific relatives for resources, then clonally reproduce and systematically replace the entire genetic structure of the population with a genome descending from that single organism [2,32,33]. In evolutionary terms, the selection coefficient of this variant is greater than that of other members of the population (i.e., it is more fit). Mutations that increase the selection coefficient can have the same effect. This purging effectually homogenizes the diversity within the entire population by replacing the previous variants within the population with the new variant and acts as a cohesive force that prevents the population from diverging. Through successive periodic selection events the population does, however, evolve. When organisms within a population are able to escape such cohesive forces that place boundaries on the population diversity, individuals can then begin to diverge along separate evolutionary paths and subsequently to generate distinct yet diverse populations. One way an individual can escape genetic diversity purges would be to avoid direct competition for nutrients via previously acquired ecological novelty (i.e. adaptation to a new niche not exploited by members of a parental population) [3,6]; in this regard, LGT, especially non-homologous gene flow, may be a key mechanism for acquiring variation and of speciation by introducing genes that allow an organism to utilize a new niche. However, in the light of studies from plant and animal evolution, another way of enabling microorganisms to escape the cohesive forces restraining their population diversity would be physical isolation from the parental population [3,6], a concept not well addressed in the evolution of microorganisms. This could enable populations to diverge through neutral processes (e.g., genetic drift) rather than or in addition to ecological adaptations.

4. Physical isolation of bacterial populations

4.1. Host–symbiont population isolation

If a symbiotic association (mutualistic or parasitic) between a microbe and a host is permanent, and if the period of interaction has extended back in time through a series of host speciation events, it is possible for the symbiont to evolve in parallel with its host. When this occurs, bacterial symbionts associated with each host species have formed distinct allopatric populations. As such, each population is physically removed from potential periodic purges by members of sister populations in other hosts and is thus free to diverge. Furthermore, lack of physical proximity may prevent populations from acquiring DNA from each other via LGT events (i.e. pseudosexual isolation). Host speciation events would then effectively break the cohesive forces that

would ordinarily keep closely related bacterial populations from diverging, thus resulting in the formation of allopatric bacterial populations, that could culminate in the formation of a new species depending on the preferred species criteria. Probably the simplest method for detecting co-evolution events between a host and its symbiont is when their two evolutionary histories are congruent (i.e. phylogenetic trees have identical or nearly identical topologies for hosts and symbionts) (Fig. 1). We do not exclude the possibility that selection has also played a part in the association. For instance, selection has "fine-tuned" flightless birds to their local environments. However, we emphasize that once the initial symbiosis is formed, subsequent allopatric speciation of the symbionts in concert with host speciation need not be the result of different symbiont populations having acquired adaptations and having been differentially selected in the Darwinian sense. We will discuss this in more detail, below.

In the past decade, several microbe–host associations have been discovered to be diverging in concert with one another. Some of the best-studied examples of this type of relationship are host–endosymbiotic relationships that occur with symbionts living in specialized cells of the host (e.g. bacteriocytes). An example of this type of relationship is the deep-sea hydrothermal vent vesicomyid clams and their proteobacterial sulfur-oxidizing endosymbionts. To test the hypothesis that vesicomyid clams have co-evolving endosymbionts, Peek et al. [34] compared the phylogeny of the host with that of its symbiont (Fig. 1). After rigorous statistical analysis of the phylogenetic trees produced from the sequenced genes, congruence between the hosts' and endosymbionts' evolutionary histories was strongly established. This, in combination with other factors, such as the vertical transmission of the symbiont via host eggs, the dependence of the host on the symbiont for nutrients, and lack of evidence for a free-living version of the symbiont in surrounding waters, strongly suggested that these organisms have maintained a long term relationship and have co-evolved as a result of their dependence upon each other. Other well-studied co-evolving animal–microbial host–endosymbiotic relationships have been observed in the following hosts: aphids [35–37], whiteflies [38,39], carpenter ants [40,41], cockroaches and termites [42], tsetse flies [43] and gutless worms [44].

Since co-evolution and population isolation have been observed with respect to endosymbionts, one might expect that genetic drift has played a significant role in the evolution of these prokaryotic organisms. In the case of aphids and their proteobacterial *Buchnera* endosymbiont, such a role for genetic drift has been observed [45]. *Buchnera* populations are small, population bottlenecks occur at each host generation and no evidence for LGT events has been found [46,47]. When selection is a major force in sculpting diversity, slightly deleteri-
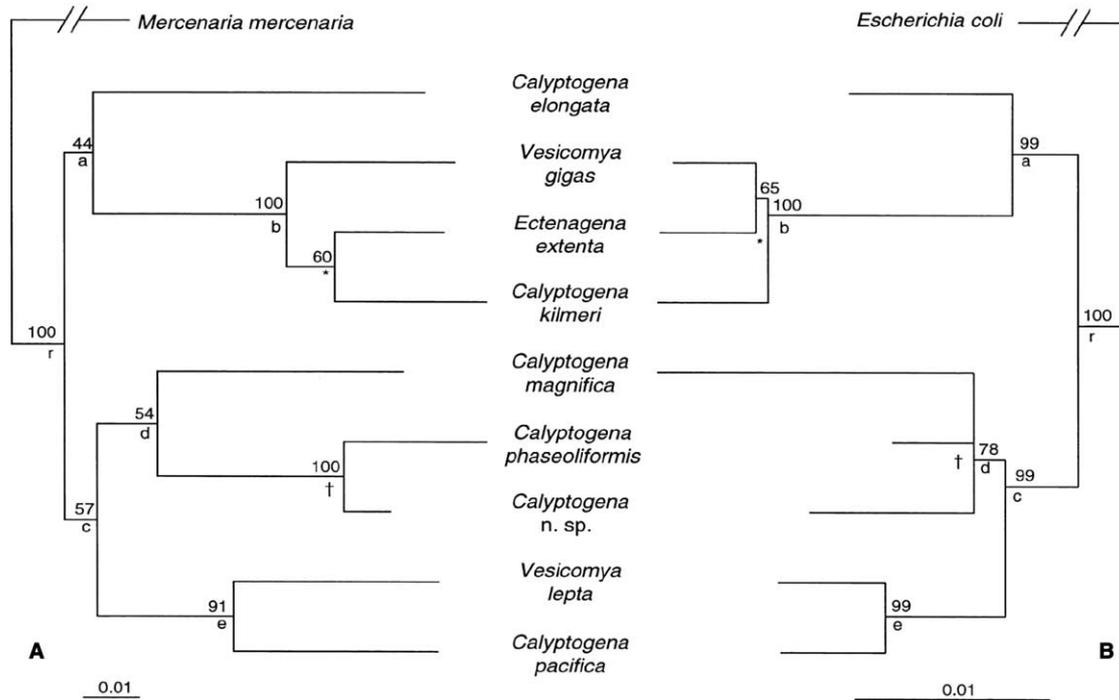
Fig. 1. Maximum likelihood phylogenies for nine species of vesicomyid clams and their associated endosymbionts. (A) The vesicomyid tree was based on combined data from portions of the mitochondrial cytochrome oxidase subunit I and 16S rDNA genes. (B) The bacterial tree was based on a portion of small subunit 16S rDNA. Host and symbiont trees were not drawn to the same rate scale (scale below each tree) After Peek et al. [34].

ous mutations are usually removed from the population, due to the lowered fitness of the variant. However, the observed overabundant accrual of neutral and especially slightly deleterious mutations in *Buchnera* suggests that random genetic drift, not selection is the dominant evolutionary force for these organisms in their specific environment [48]. However, a general absence of selection does not mean that it is non-existent. For example, there is evidence that the highly conserved protein-folding chaperone DnaK is subject to relatively strong selection (i.e. not mutated like the rest of the genome) [47]. Deleterious mutations tend to destabilize proteins (i.e., changes in the amino acid composition make three-dimensional folding difficult) and it is thought that DnaK counteracts those substitutions by properly folding mutated proteins [47].

Another example of host symbiont co-evolution, but one that is less strict in its degree of physical isolation, is the host–pathogen relationship. These are relationships in which the microbial symbiont causes a disease exclusively in its host despite the symbiont's intermediate free-living life stage. The host–pathogen type of symbiotic relationship can be illustrated by the marine red algal genus *Prionitis* (Rhodoyphyta, Halymeniaceae, Gigartinales) and its gall-forming (tumorigenesis) pathogen from the *Roseobacter* group within the *Proteobacteria*. In research presented by Ashen and Goff [49], the phylogeny of different *Prionitis* species was compared to the phylogeny of the pathogens found in galls of each host

(Fig. 2). The results are complicated by the fact that the host algal species *P. filiformis* is not monophyletic (i.e., this species does not belong to a distinct phylogenetic clade). However, the co-evolution pattern is still "consistent and parsimonious" when we consider only the
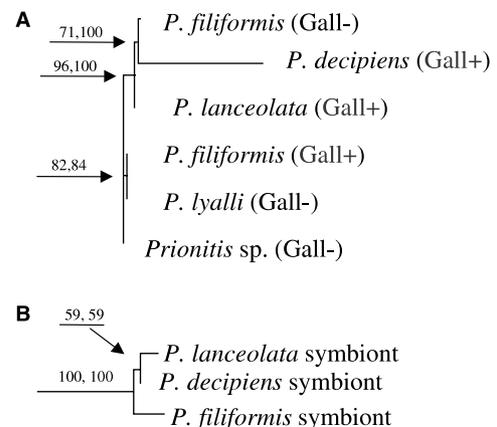

Fig. 2. Red algal species (*Prionitis*) phylogenetically compared to the pathogens found in galls of each host. (A) Phylogeny of different *Prionitis* species using internal transcribed spacer regions. Values at nodes are from bootstrap analysis performed using maximum likelihood. The second number is a bootstrap value after the most diverged sequence was removed from the analysis. (B) Phylogeny of different pathogens using 16S rRNA gene sequences. Bootstrap analysis was performed using parsimony (first number) and maximum likelihood (second number). Modified after Ashen and Goff [49].

gall$^+$ phenotypes: gall$^+$ *P. decipiens* is more closely related to gall$^+$ *P. lanceolata*, with the gall$^+$ *P. filifomis* forming a sister group to the other two species as observed for the phylogeny of the pathogens of these host species. Cross-inoculations of the pathogens to the hosts showed that the host–pathogen relationships were specific, thus increasing confidence that the different gall-forming pathogens were co-evolving with their specific *Prionitis* hosts. Other examples of infectious agents co-evolving with their hosts include *Silene* spp. and its anther smut fungus *Microbotryum violceum* [50], hantaviruses with mice (*Peromyscus leucopus*) in North America [51], and herpesviruses with their mammalian and avian hosts [52]. Similarly, co-evolution patterns have been noted in commensalistic relationships involving microbes and their animal hosts [53].

4.2. Geographic population isolation

Much evolutionary thinking regarding free-living bacteria (e.g. non-intracellular symbionts) has been based on the idea that free-living bacteria are incapable of forming isolated populations. The idea appears to date back to the beginning of the 20th century when it was proposed that "everything is everywhere and nature selects" [54,55]. This premise implies that prokaryotic population genetic cohesiveness can never be broken by physical isolation, but by adaptation alone. Support for this position is generally derived from the observations and/or assumptions that microbes (i) are small and therefore easily transported, (ii) can form resistant physiologically inactive stages that allow them to survive transport and hostile environments into which they may fall after transport, and (iii) have extremely large population sizes which indicates a high probability of dispersal [56]. For example, it was reported that an extremely large number of microorganisms (10^{18}–10^{20}) are transported annually through air [57] and that this is an obvious example of how easy it is for microorganisms to be everywhere [56]. However, these values actually represent an extremely small proportion of the estimated number of microbial cells on Earth [58,59]. If the estimates for total cells are correct, it would take approximately 2.2–220 times the age of the Earth for all cells to disperse through the atmosphere. Furthermore, it is unknown whether microorganisms that disperse through the atmosphere are typical of all microorganisms; some microorganisms (e.g., spore formers) would seem better adapted for travel in the atmosphere than those organisms that are sensitive to O_2, UV radiation or desiccation, for example. The assumption that all prokaryotes have extremely large population sizes in nature also deserves scrutiny. For instance, it has been estimated that the rarest prokaryotic species, if distributed evenly in the oceans and in soils, would exist at

densities of 1 cell per 10 km^3 and 1 cell per 27 km^2 respectively [59]. Of course, there are many more rare species than common species.

A further complication is that it is unclear what resolution is needed to detect patterns of prokaryote divergence. For example, some studies that have indicated support for the "everything is everywhere" hypothesis [60–70] are based on evidence obtained from what could be considered as low-resolution analyses. Similarities in morphology and/or physiology, identical restriction patterns of highly conserved genes (e.g. 16S rRNA), or identical and nearly identical 16S rRNA genotypes may be insufficient for detecting important differences in genetic variation among organisms investigated. For instance, genetic comparison between two strains of *Thermotoga maritima* (MSB8 and RQ2), which are 99.7% similar at the 16S rRNA locus, revealed that 20% of the RQ2 genome was unique [71] and strains of *Bacillus globisporus* that are >99.5% at the 16S rRNA gene have less than 70% DNA–DNA hybridization levels [72]. Hence, one of the most commonly used measures of microbial diversity may be inadequate for measuring biodiversity patterning.

Recently, the "everything is everywhere" hypothesis has been invoked to explain why the relationship between body size and species numbers breaks down for organisms smaller than 1 mm [73–76]. Among animals there is a general trend of more species per taxon when the average body size is small, (e.g. 751,000 insect species vs. 281,000 for all other animals [77]). Despite the small size of prokaryotes, there is a paucity of officially recognized prokaryotic species ($\approx$5000) and the low number of prokaryotic species has been hypothesized to be a consequence of ubiquitous dispersal [73–76]. Allopatric speciation, the most common form of speciation among organisms that tend not to disperse over large distances, is inferred to be an extremely rare event in the evolution of free-living microbes [73–76]. It must be appreciated, however, that the number of officially recognized prokaryotic species (i.e., cultivated representatives characterized phenetically and phylogenetically, http://www.bacterio.cict.fr/minimalstandards.html) is considered to be a gross underestimate concerning the total number of prokaryotic species [59,78]. The number of species is, of course, a function of the criterion used to define species. But, even when using a conservative conventional criterion like >70% DNA–DNA hybridization [17,19,20] (a standard that could lump all primates into a single species [79]), it has been calculated that nature contains sufficient genetic diversity among prokaryotes that the number of prokaryotic species may exceed one billion [80]. An increasing number of studies are beginning to challenge the "everything is everywhere" hypothesis, suggesting that physical isolation in free-living microorganisms (prokaryotic and eukaryotic) from rare extreme environments, as well as more common envi-

ronments like the oceans and soils, may be more widespread than previously thought [67,68,81–105].

An excellent example demonstrating the dependence of observing bacterial geographic isolation on the use of suitably sensitive methods comes from work on free-living soil bacteria. From soils collected at 10 sites on four continents (North America, South America, Australia, and Africa), Cho and Tiedje [86] cultivated 248 fluorescent pseudomonads, organisms containing a chromosomally derived selectable trait. Three different levels of resolution were examined: (i) restriction patterns of the conserved 16S rRNA gene, (ii) restriction patterns of the more highly variable 16S–23S rRNA internal transcribed spacer (ITS) region, and (iii) an extremely sensitive method that takes advantage of the entire genome's complexity, repetitive extragenic palindromic-PCR (REP-PCR). Restriction pattern analysis of the 16S rRNA gene revealed very few differences among all of the strains and no endemism. Restriction analysis of the ITS region revealed some endemism. However, with REP-PCR no identical genotypes were found among any of the different regions sampled and geographic clades were observed (Fig. 3), leading the authors to conclude that "geographic isolation plays an important role in bacterial diversification".

A second example of free-living bacteria experiencing geographical isolation comes from our own observations of hot spring cyanobacterial mats [81]. From 42 hot springs located in four countries (United States, Japan, New Zealand and Italy), the biogeographic patterns of indigenous thermophilic cyanobacteria were determined using PCR amplification of the 16S rRNA gene (Fig. 4) and 16S–23S rRNA ITS region (Fig. 5) followed by cloning and sequencing. Lineage-specific probing (16S rRNA hybridization) was used to verify that the cloning approach detected dominant populations and lineage-specific PCR was used to search for rare genotypes. Each of the four main cyanobacterial lineages detected appeared to vary with respect to dispersal capabilities: the A/B *Synechococcus* lineage was detected only in North America, the C1 *Synechococcus* lineage was detected in North America and Japan, the *Oscillatoria amphigranulata* lineage was detected in Japan and New Zealand, and the C9 *Synechococcus* lineage was detected in all of these countries. *Synechococcus* morphotypes and 16S rRNA genes were not detected in Italian hot springs. Each country's hot springs were dominated by a different cyanobacterial lineage. We never found identical cyanobacterial 16S rRNA genotypes in the different countries. Sequences found in different countries and subregions often exhibited distinct clades at the 16S rRNA or ITS loci (Fig. 5), providing evidence that endemism exists within as well as between countries. To test the hypothesis that chemical composition of hot springs might have influenced the distribution patterns of detected cyanobacterial populations,
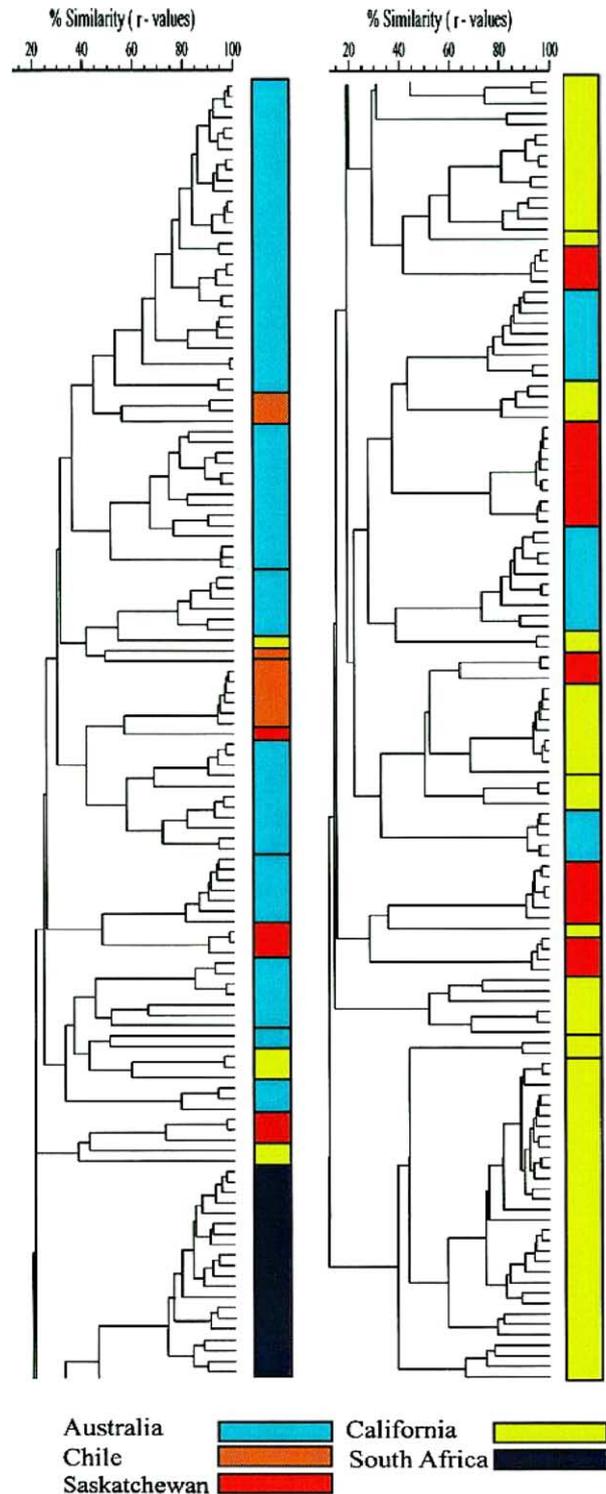

Fig. 3. REP-PCR band pattern similarity dendrogram demonstrating the relationship between the genetic diversity of fluorescent *Pseudomonas* cultivated from soils and the geographical origins of the isolates. Modified after Cho and Tiedje [86].

20 chemical parameters were measured for each sampling site. The observed cyanobacterial distribution patterns did not correlate with hot spring chemical parameters. Geographic clades have also recently been
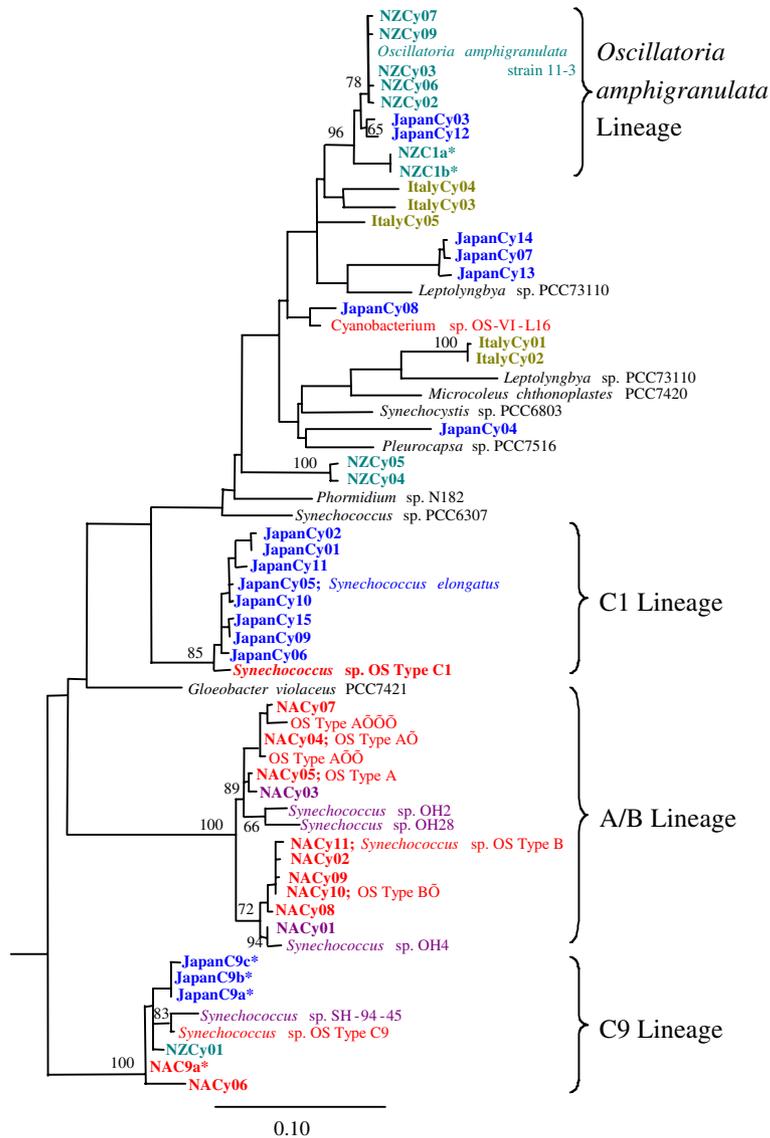
Fig. 4. 16S rRNA gene tree demonstrating the relationships of cyanobacterial clones retrieved from hot spring mats in North America, Japan, New Zealand and Italy relative to other cyanobacterial 16S rRNA gene sequences including hot spring *Synechococcus* spp. and *Oscillatoria amphigranulata* isolates. The tree was rooted with *Escherichia coli* and *Bacillus subtilus* 16S rRNA sequences. Values at nodes indicate bootstrap percentages for 1000 replicates. Values less than 50% are not reported. Scalebar indicates 0.10 substitutions per site. *, genotypes recovered exclusively from linage-specific PCR study. Color highlighting: Green, New Zealand; blue, Japan; yellow Italy; red, Greater Yellowstone Ecosystem; purple, Oregon. Taken from Papke et al. [81].

observed in a study of multiple protein-encoding genes of *Sulfolobus* isolates with nearly identical 16S rRNA sequences, which were cultivated from geographically isolated acid hot springs [102]. Some evidence for geographic isolation has been reported for other hot spring bacteria [85,94,97,98].

5. The ramifications of prokaryote evolution due to physical isolation

The examples above along with numerous other studies demonstrate that physical isolation can be an integral element in the evolution of microbial populations (symbiotic and free-living). Therefore, genetic drift, founder effects and/or neutral evolution should be considered when studying microbial diversity, evolution and ecology. Physical population isolation may be more common than is currently appreciated, as the ability to detect isolation clearly depends upon the resolving power of the genetic marker(s) used and the habitats and organisms examined.

Physical isolation of microbial populations is unlikely to fit into a dichotomous either/or model. Realistically, there is likely to be a gradient of isolation or a relative frequency of dispersal among microorganisms, with
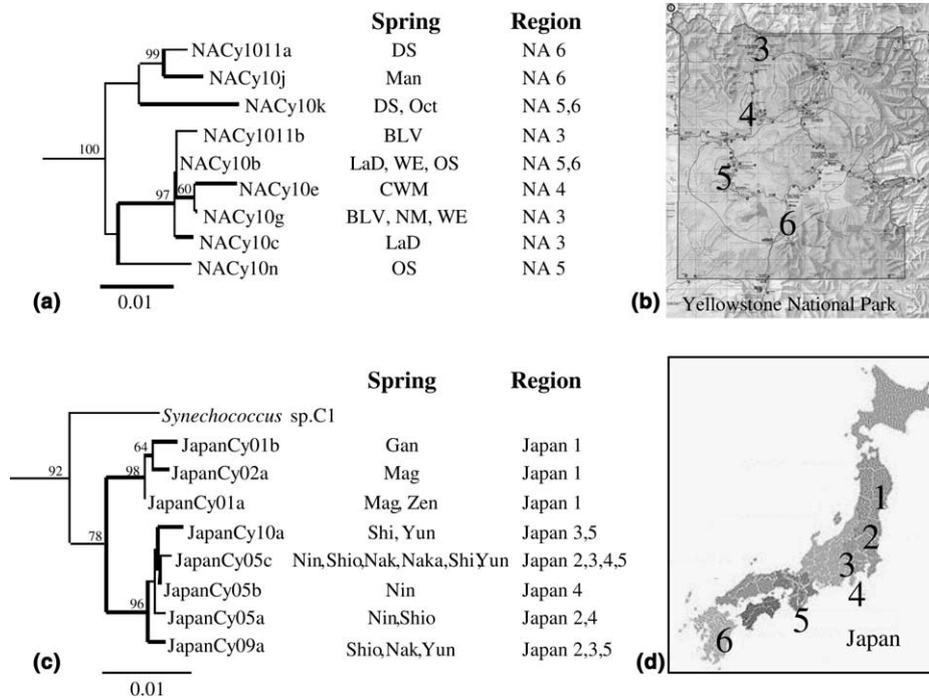
Fig. 5. Phylogenies for 16S–23S rRNA ITS variants detected in Yellowstone or Japan relative to springs and subregions from which they were retrieved. (a) ITS genotypes with an identical 16S rRNA genotype (NACy10 corresponding to the type B' 16S rRNA sequence from previous work [11]). (b) Map of Yellowstone National Park (www.yellowstone_natl_park.com/ywstone.htm) indicating regions sampled (c) Concatenated 16S rRNA and ITS sequence data for members of the C1 lineage. (d) Map of Japan (http://jin.jcic.or.jp/region/index.html) indicating regions sampled. Values at nodes indicate bootstrap percentages for 1000 replicates. Values less than 50% are not reported. Scalebar indicates 0.01 substitutions per site. Abbreviated spring names defined in [81]. Taken from Papke et al. [81].

endosymbionts on one end of the spectrum and cosmopolitan organisms on the other. Microbial evolution is predictably complex, involving both isolation and adaptation. To fully unravel a microbe's natural history we need to measure the relative contribution of all the different forces that can affect its evolution.

Including isolation when contemplating microbial evolution has important general implications. For instance, physical proximity and barriers to gene flow are realities that must affect the LGT process. Isolation should also play a role in how we think about bacterial species. For instance, as of the writing of this manuscript, microbial taxonomists have not officially recognized host-specific diverged *Buchnera* endosymbionts as separate species though insect taxonomists recognize the aphid hosts as being separate genera. If the criterion for species were terminal phylogenetic clades formed by physical isolation, each endosymbiont would be considered a separate species. Since allopatry has been observed in free-living prokaryotic populations, perhaps biogeography could also be a useful criterion in defining prokaryotic species. In a general theory of microbial evolutionary ecology, isolation should be thought of as one, not the only, mechanism influencing population divergence. Adding physical isolation to a developing theoretical framework for microbial evolu-

tion is an enhancement that does not displace other mechanisms, such as adaptation and selection arising from mutation, LGT, or both. As with plants and animals, each of these mechanisms must be tested to gain complete insight into how different microbes evolve in nature.

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