EXPERIMENTAL STUDIES OF THE CAUSES AND CONSEQUENCES OF BIODIVERSITY OVER ECOLOGICAL AND EVOLUTIONARY TIMESCALES

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EXPERIMENTAL STUDIES OF THE CAUSES AND CONSEQUENCES OF BIODIVERSITY OVER ECOLOGICAL AND EVOLUTIONARY TIMESCALES

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To the fuchsia and chartreuse

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SUMMARY

This dissertation presents four microbial microcosm-based experimental studies addressing questions related to the causes and consequences of biodiversity. All four studies adopted an approach that integrates ecology and evolutionary biology. Two studies explored the utility of knowledge on species phylogenetic relationships for understanding community assembly (chapter 1) and invasibility (chapter 3). The other two studies investigated the impacts of important ecological factors, including competition (chapter 2) and temporal niches (chapter 4), on adaptive radiation, using the rapidly diversifying bacterium *Pseudomonas fluorescens* SBW25 as the model organism.

The first study, described in Chapter 1, examined how phylogenetic relatedness between competing species affected the strength of priority effects and ecosystem functioning during community assembly. Strong priority effects emerged only when competing bacterial species were phylogenetically most closely related, resulting in multiple community states associated with different assembly histories. In addition, the phylogenetic diversity of bacterial communities effectively predicted bacterial production and decomposition.

The second study, described in Chapter 2, explored the role of competition in the adaptive radiation of *P. fluorescens*. The adaptive radiation was generally suppressed by competition, but its effect was strongly modulated by the phylogenetic relatedness between the diversifying and competing species and their immigration history. The inhibitive effect of competition on adaptive radiation was strongest when phylogenetic relatedness was high and when competitors were introduced earlier.

The third study, described in Chapter 3, evaluated the relative importance of phylogenetic relatedness between resident and invading species and phylogenetic diversity of resident communities for invasibility. Laboratory bacterial communities

containing a constant number of resident species with varying phylogenetic diversity and relatedness to invaders were challenged by nonresident bacterial species. Whereas invader abundance decreased as phylogenetic relatedness increased as predicted by Darwin's naturalization hypothesis, it was unaffected by phylogenetic diversity.

The final study, described in Chapter 4, presented the first experimental demonstration of the maintenance of biodiversity that emerged from adaptive radiation in the presence of temporal niches. Only when provided with temporal niche opportunities were multiple derived phenotypes of *P. fluorescens* able to coexist as a result of negative frequency-dependent selection. When temporal niche was absent, the specialized phenotypes either did not emerge or were predominated by one superior phenotype.

CHAPTER 1

PHYLOGENETIC RELATEDNESS, PRIORITY EFFECTS, AND ECOSYSTEM FUNCTIONING

Abstract

Species immigration history can structure ecological communities through priority effects, which are often mediated by competition. As competition tends to be stronger between species with more similar niches, we hypothesize that species phylogenetic relatedness, under niche conservatism, may be a reasonable surrogate of niche similarity between species, and thus influence the strength of priority effects. We tested this hypothesis using a laboratory microcosm experiment in which we established bacterial species pools with different levels of phylogenetic relatedness and manipulated the immigration history of species from each pool into microcosms. Our results showed that strong priority effects, and hence multiple community states, only emerged for the species pool with the greatest phylogenetic relatedness. Community assembly also resulted in a significant positive relationship between bacterial phylogenetic diversity and ecosystem functions. Interestingly, these results emerged despite a lack of phylogenetic conservatism for most of the bacterial functional traits considered. Our results highlight the utility of phylogenetic information for understanding the structure and functioning of ecological communities, even when phylogenetically conserved functional traits are not identified or measured.

Introduction

Understanding mechanisms underlying the assembly of ecological communities is one of the central goals of community ecology (Gleason 1927; Diamond 1975). Ecologists now recognize that both niche-based deterministic processes (Chase and Leibold 2003) and neutral stochastic processes (Bell 2001; Hubbell 2001) can operate during the process of community assembly. Niche-based processes involve the interaction between species' niches and the conditions of the environment in which they live, which can jointly regulate the structure of the assembling communities. In habitats with similar environmental conditions and under the same regional species pool, such processes often result in convergent communities with similar species composition and abundance. Stochastic processes, highlighted by the neutral theory (Bell 2001, Hubbell 2001), can also strongly impact ecological communities. In particular, stochasticity in the order and timing of species colonization events, as demonstrated by both theoretical and empirical studies (e.g., Drake 1991; Law and Morton 1993; Jiang and Patel 2008; reviewed by Chase 2003), can result in divergent communities dominated by different species. These multiple community states associated with different species colonization histories frequently arise from priority effects, in which early colonizing species affect the establishment and abundance of later colonizers.

One factor that can potentially influence the relative importance of deterministic and stochastic processes, and hence the strength of priority effects, is ecological similarity of species in the regional species pool. Both theory (e.g., MacArthur and Levins 1967) and experiments (e.g., Gause 1934) have demonstrated the difficulty for species with similar niches to coexist, which prompted Hardin (1960) to coin the competitive exclusion principle. A corollary of this principle, applying to community assembly, is that increasing ecological similarity of species in the regional pool may make it more likely for species already established at a locality to have strong negative

impacts on newly colonizing species, promoting inhibitive priority effects. As species niches are often difficult to quantify and phylogenetically closely related species tend to possess similar niches (i.e., phylogenetic niche conservatism; Harvey and Pagel 1991; Prinzing et al. 2001; Webb et al. 2002; Donoghue 2008), we suggest that species phylogenetic relatedness may be used as a surrogate of niche similarity to predict the strength of competition and priority effects. The positive relationship between species phylogenetic relatedness and competition was in fact first hypothesized by Darwin (1859), and supported by a recent experiment (Violle et al. 2011). However, whether phylogenetic relatedness of the regional species pool influences the strength of priority effects during community assembly remains an open question.

Phylogenetic relatedness of the regional species pool may also have consequences for the functioning of the assembled communities. For example, if phylogenetic relatedness serves as a reasonable surrogate for species ecological similarity, then low phylogenetic relatedness (i.e., high phylogenetic diversity) may translate into increased niche complementarity among species in the assembled communities, potentially resulting in high levels of ecosystem functioning (Cavender-Bares et al. 2009). On the other hand, high phylogenetic relatedness among species within the regional species pool would indicate possible redundancy in species' niches, likely leading to reduced ecosystem functioning. So far only a handful of studies have investigated the relevance of species phylogenetic relatedness for ecosystem functioning (Maherali and Klironomos 2007; Cadotte et al. 2008, 2009; Jiang et al. 2010), but the potential interactive effects of phylogenetic relatedness and assembly history on ecosystem functions have not been explored.

Here we describe an experimental study examining how species phylogenetic relatedness affects priority effects and ecosystem functioning by using a laboratory model of bacterial communities. We established bacterial species pools with different levels of

species phylogenetic relatedness and manipulated the immigration history of bacteria from each species pool into the assembled communities. We showed that significant dissimilarity among communities subjected to different assembly histories emerged only when bacteria in the species pool were phylogenetically closely related. We also found significant effects of phylogenetic relatedness and assembly history on bacterial ecosystem functions (i.e., bacterial production and decomposition).

Methods

Our experiment used eight strains of common environmental bacteria from freshwater ecosystems (Fig. 1.1), all of which can form colonies with unique morphological characteristics on agar plates. To estimate phylogenetic relatedness between these bacteria, we constructed phylogenies based on bacterial 16S rRNA sequences (Fig. 1.1a). We sequenced the 16S rRNA gene of each bacterial strain, aligned the sequences with Clustal X (ver. 2.0; Larkin et al. 2007), selected the best sequence evolution model—GTR+G with MrModeltest (ver. 2.3; Nylander 2004) by using the Akaike Information criterion, and built the phylogenetic tree with Bayesian method in MrBAYES (ver. 3.1.2; Huelsenbeck and Ronquist 2001). Three archaea were used as the out-group. The phylogenetic distance between bacteria was obtained by summing lengths of the intervening branches between the two species on the phylogeny; smaller phylogenetic distance between bacteria indicates greater phylogenetic relatedness. Using these eight strains of bacteria, we established four species pools—*Serratia*, *Staphylococcus*, *Bacillus*, and a mixed-genus pool with one bacterium randomly selected from each of the single-genus pools (Fig. 1.1a). The phylogenetic diversity (hereafter PD) of each species pool was calculated by summing the lengths of all the intervening branches of all the species in each pool (Faith 1992). PD is thus an aggregate measure of

the phylogenetic relatedness of each species pool; higher PD values indicate larger phylogenetic distances and thus weaker phylogenetic relatedness among species.

We estimated functional trait diversity of each species pool based on the bacteria's ability to utilize a variety of carbon substrates that may appear in the bacterial growth medium used in our experiment (see below). We measured 55 bacterial traits with Biolog MicroPlates (Biolog, Hayward, CA). Following the manufacturer's instructions, we prepared and inoculated Gram-positive and negative bacterial cultures into their corresponding type of Biolog MicroPlates. Gram-positive and -negative Microplates, each containing 96 wells, share 55 carbon substrates in common, so we only recorded the results of these 55 traits. We scored positive results—indicating that the species was able to use carbon sources in the wells—as 1 and negative results as 0. In addition, we tested the ability of these bacteria to utilize two common carbon substrates—cellulose and starch. We spread diluted cultures of each bacterial strain on carboxymethylcellulose (Wohl et al. 2004) and starch agar plates, incubated them at room temperature (~22 ℃) for 5 days, and flooded plates with 1% Gango Red and Lugol's iodine solutions, respectively. Colorless zones around bacterial colonies on agar plates were observed if bacteria utilize cellulose or starch. Based on the total 57 traits, we calculated functional trait diversity of each species pool in two ways. First, we calculated functional richness (hereafter FR) by counting the total number of carbon substrates that bacteria from a species pool could utilize. Second, we calculated functional diversity (hereafter FD) of each species pool. We performed a UPGMA (unweighted pair group method with arithmetic mean)-based cluster analysis with the Euclidean distance between bacteria in the 57-dimensional trait space, produced the functional dendrogram (Fig. 1.1b), and calculated FD as the total intervening branch lengths of the dendrogram of all the species in each pool (Petchey and Gaston 2002). To test for phylogenetic conservatism of the measured traits, we conducted a Mantel test based on 10,000

permutations that evaluated the correlation between bacterial phylogenetic distance and trait Euclidean distance. We also tested the phylogenetic signal of each trait by using Blomberg's K (Blomberg et al. 2003), with the multiPhylosignal function in the Picante package (Kimbel et al. 2010).

Our experiment used 25 mL capped test tubes as microcosms, each of which contained 10 mL of medium. The medium contained 0.55 g of crushed protozoan pellets (Carolina Biological Supply, Burlington, NC) per liter of deionized water. Protozoan pellets are made from plant extracts and include a variety of common carbon resources for bacterial growth. Medium was autoclaved in large flasks and filtered to remove insoluble particles, then transferred into experimental microcosms and autoclaved again before the experiment started. The microcosms were incubated on a shaker at 200 rpm under room temperature (~22℃).

The experiment included all the possible combinations of assembly sequences for each bacterial pool. Thus, we had two sequential assembly treatments for the *Serratia* pool that contained two species, and six for the *Staphylococcus*, *Bacillus* and mixed pools that each contained three species (Fig. 1.1). Each treatment was replicated three times. Prior to the experiment, we prepared stock cultures of each bacterial strain in 8% nutrient broth. At the beginning of the experiment (day 0), we introduced the first species into microcosms by transferring a small volume (<5 μL) of stock culture with an aseptic loop. In the same way, on day 7 and 14, we introduced the second and third immigrants (no third immigrant for the *Serratia* communities), respectively. The weekly interval between species introduction allows the assembled communities to equilibrate before next introduction event. Our pilot experiment, albeit using only half of the eight bacterial strains used in this study, indicated that bacterial populations of individual species, initiated at small size in isolation from other species, require 2-3 days to reach carrying capacity and persist at the stationary phase for at least our experimental duration;

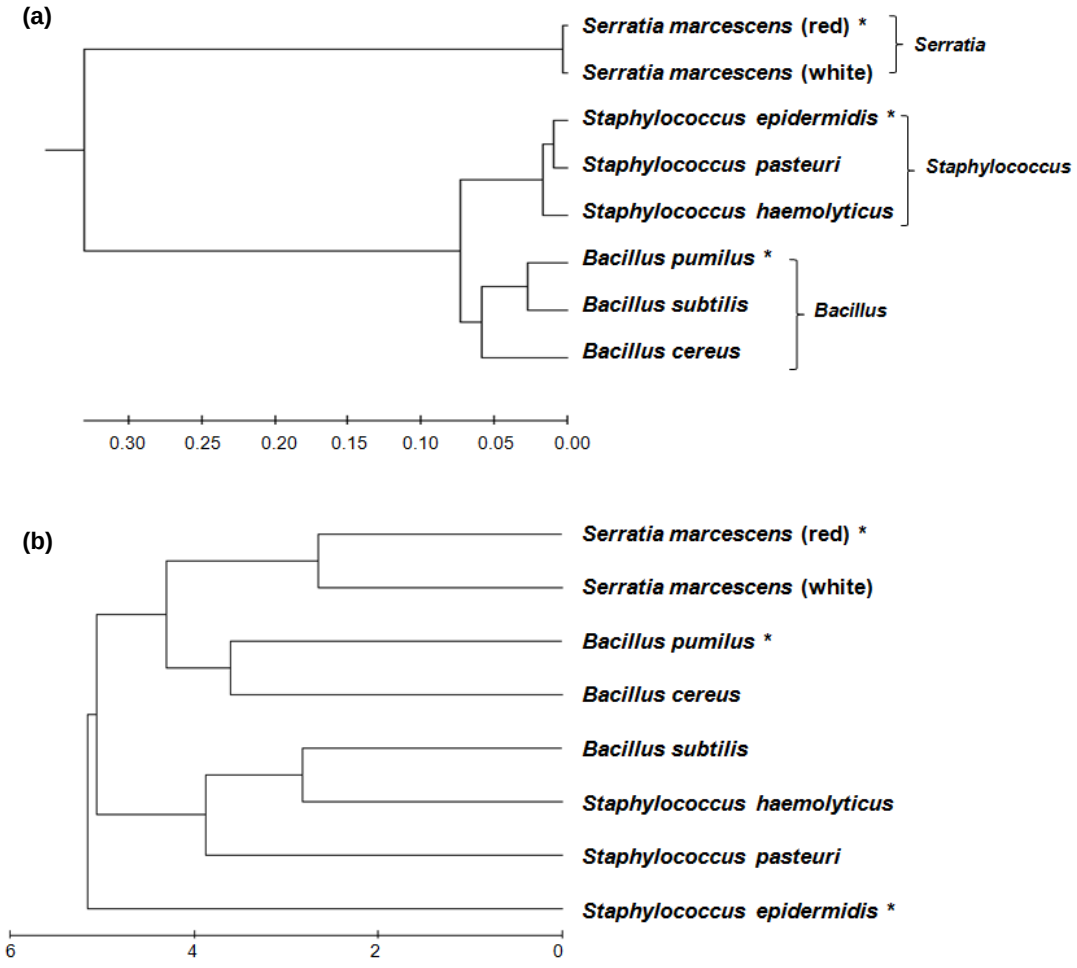

Fig. 1.1 Phylogeny (a) based on Bayesian methods and functional dendrogram (b) based on cluster analysis of 57 traits by UPGMA for the study bacteria. Four species pools, *Serratia* (initial PD: 0.0065; initial FR: 36; initial FD: 2.645), *Staphylococcus* (initial PD: 0.0274; initial FR: 42; initial FD: 7.224), *Bacillus* (initial PD: 0.0959; initial FR: 35; initial FD: 6.959) and the mixed (initial PD: 0.4854; initial FR: 50; initial FD: 7.550) species pool were formed by these bacteria. Asterisk indicates the bacteria constituting the mixed species pool.

bacterial communities containing multiple species generally reach equilibrium in one week and can persist for similarly long periods of time (J. Tan, unpublished data). On day 21, we added a dried, weighed and autoclaved wheat seed to each microcosm. On day 49, we terminated the experiment and destructively sampled the microcosms. The samples from microcosms were serially diluted and spread on nutrient agar plates. After seven-day incubation, we counted the number of bacterial colonies on plates to determine population density (colony formation units per milliliter [CFU/mL]) of each bacterial strain. Wheat seeds were retrieved from microcosms, oven dried and weighed. Two ecosystem functions were recorded. Total bacterial production in each microcosm was obtained by summing the densities of each bacterial strain. Decomposition was measured as the fraction of wheat seed mass lost during the experiment.

We calculated realized community PD, FR and FD, based on the realized species composition measured at the end of the experiment. We calculated β-diversity between communities sharing the same species pool but subjected to different assembly histories, by first calculating the modified Morisita similarity index (Horn 1966), then subtracting it from 1. Calculation of Morisita indices was based on untransformed bacterial density data. For subsequent statistical analyses all the bacteria density data were $\log_{10}$ transformed ($\log_{10}$ [x+1]) to improve normality. We used one-way ANOVA with β-diversity as the dependent variable and different species pools as the class variables to assess the effect of varying phylogenetic relatedness among species pools on history-induced differences in community structure, as represented by β-diversity. Tukey's HSD was further conducted as the post-hoc test. To test the effect of assembly history on the density of bacteria in communities sharing the same species pool, we used MANOVA with bacteria densities for each species pool as the dependent variables and history sequences as the class variables. To test the effect of assembly history on bacterial production and decomposition in different species pools, we used nested ANOVA with

production and decomposition as the dependent variables and history sequences as a factor nested within species pools. To further test the effect of assembly history, we used one-way ANOVA within each species pool, with production and decomposition as the dependent variables and assembly history sequences as the independent variables. To test the effect of phylogenetic and functional diversity on bacterial production and decomposition, we used simple and backward-selection multiple linear regressions to model the ecosystem functions (i.e., bacterial production and decomposition) as functions of realized PD, FR and FD. In all the regressions, explanatory variables were deemed significant if $P < 0.05$.

Results

Our study bacteria did not exhibit significant phylogenetic conservatism when all the 57 traits were considered together (Mantel test, $P = 0.152$). When examined individually, 9 of 57 traits (15%), including D-fructose, L-fucose, α-D-glucose, α-D-lactose, D-melibiose, D-alanine, D, L, α-glycerol phosphate, α-D-glucose-1-phosphate and D-glucose-6-phosphate, showed significant phylogenetic signals (multiPhylosignal function, $P < 0.05$).

β-diversity among communities subjected to different histories varied significantly among the four species pools (ANOVA, $F_{3, 411} = 443.081$, $P < 0.001$). This significant variation mainly resulted from the larger values of β-diversity observed in the *Serratia* pool (see Fig. 1.2; Tukey's HSD). The dominant species in communities of the *Serratia* pool differed depending on history treatments (Fig. 1.3a). In contrast, in the *Staphylococcus*, *Bacillus* and mixed species pools, the dominant species remained the same in different history treatments (Fig. 1.3b-d). Nevertheless, MANOVA still revealed a significant effect of assembly history on species densities in those species pools (*Staphylococcus*: Wilk's $\lambda = 0.010$, $F_{15,28} = 7.882$, $P < 0.001$; *Bacillus*: Wilk's $\lambda = 0.029$,

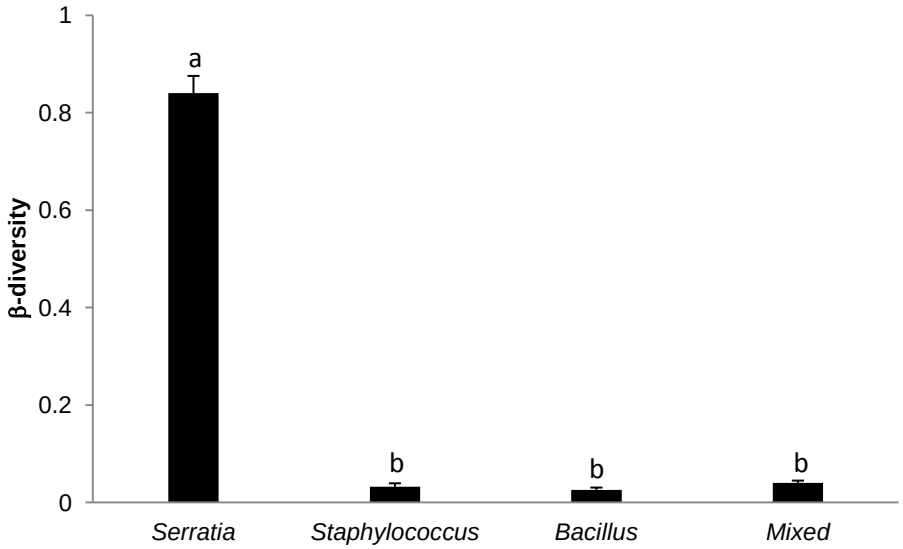

Fig. 1.2 β-diversity among communities assembled from the four species pools with varying phylogenetic relatedness. β-diversity is calculated as 1 - Morisita similarity index. Values are means + SE. Treatments sharing the same letters do not differ in the Tukey's HSD test.

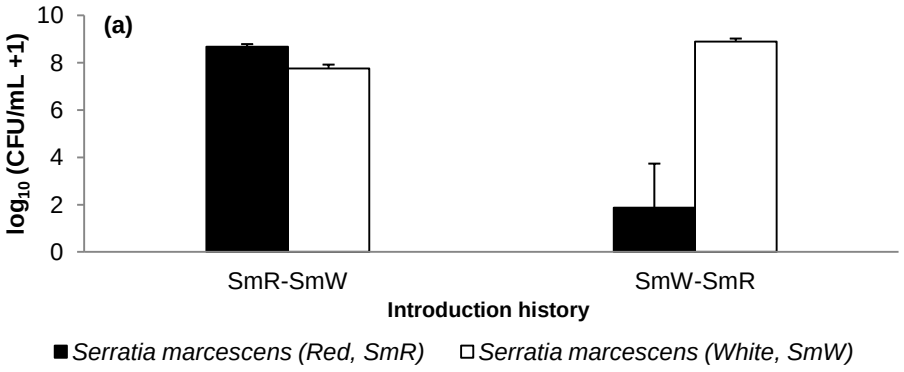

Fig. 1.3 Population density of each bacterium from the four species pools [(a) the *Serratia*, (b) *Staphylococcus*, (c) *Bacillus* and (d) mixed species pool] at the end of the experiment. Values are means + SE with density data measured as colony formation units (CFU) per mL and $\log_{10}$-transformed prior to analysis.

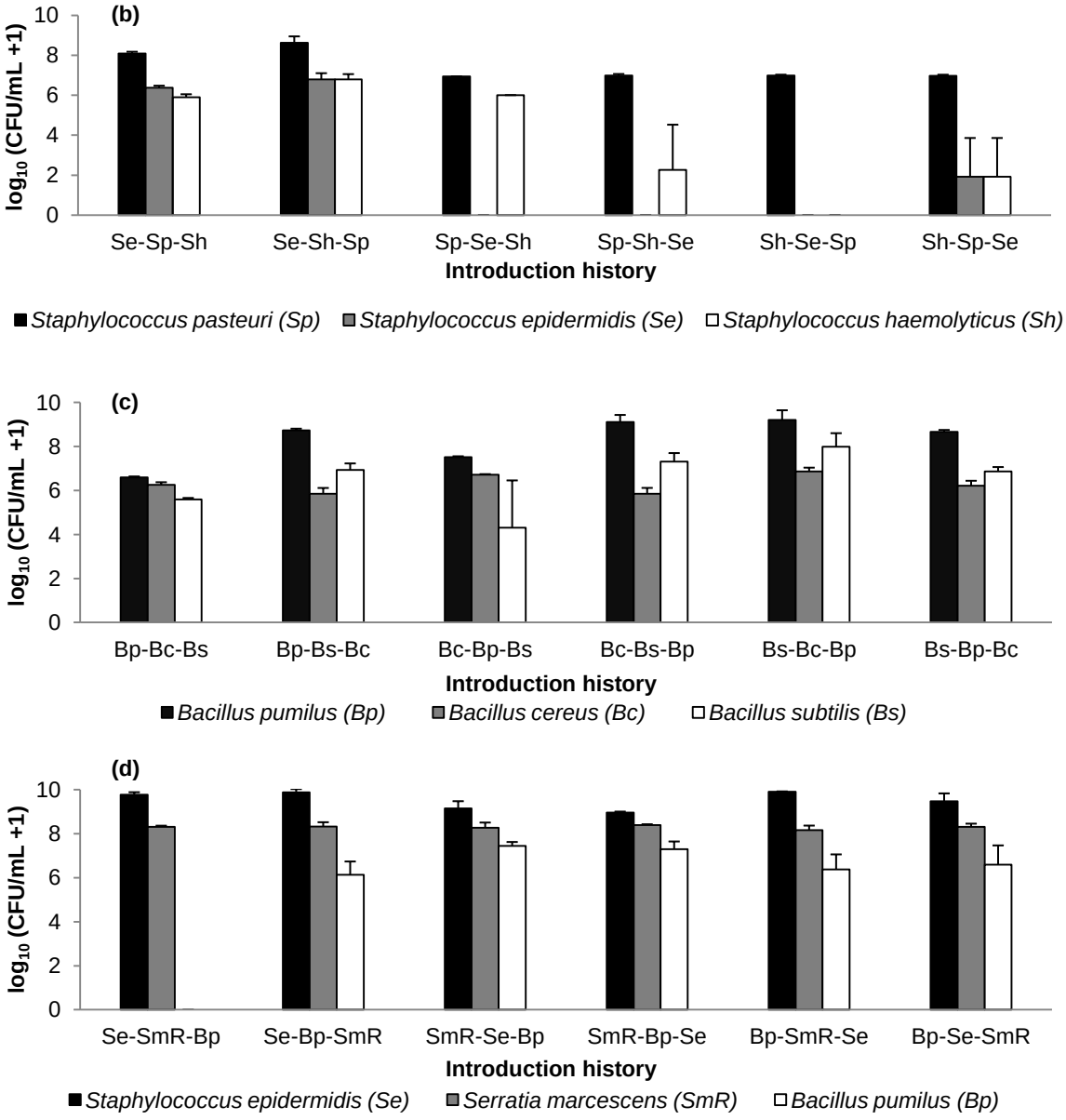

Fig. 1.3 (Continued)

$F_{15,28} = 4.867$, $P < 0.001$; mixed: Wilk's $\lambda = 0.018$, $F_{15,28} = 6.097$, $P < 0.001$), in addition to the significant effect of history for the *Serratia* pool (Wilk's $\lambda = 0.017$, $F_{1,4} = 234.1$, $P < 0.001$).

Nested ANOVA revealed a significant effect of assembly history on bacterial production ($F_{5,40} = 14.449$, $P < 0.001$), but no effect of assembly history on decomposition ($F_{5,40} = 0.886$, $P = 0.499$). One-way ANOVA indicated that assembly history had a significant effect on bacterial production in communities of the *Staphylococcus* ($F_{5,12} = 30.086$, $P < 0.001$), *Bacillus* ($F_{5,12} = 16.888$, $P < 0.001$) and mixed ($F_{5,12} = 3.601$, $P = 0.032$) pools, but had no effects in communities of the *Serratia* pool ($F_{1,4} = 1.136$, $P = 0.346$). In contrast, assembly history significantly affected decomposition in the *Staphylococcus* communities only (*Staphylococcus*: $F_{5,12} = 37.615$, $P < 0.001$; *Serratia*: $F_{1,4} = 1.136$, $P = 0.346$; *Bacillus*: $F_{5,12} = 0.670$, $P = 0.654$; mixed: $F_{5,12} = 2.348$, $P = 0.105$). Nested ANOVA also revealed that ecosystem function levels differed significantly in communities of different species pools (production: $F_{14,40} = 41.161$, $P < 0.001$; decomposition: $F_{14,40} = 6.288$, $P < 0.001$).

Simple linear regressions showed that both bacterial production and decomposition increased with realized PD (Fig. 1.4a; $R^2 = 0.461$, $P < 0.001$; Fig. 1.4b; $R^2 = 0.212$, $P < 0.001$), FR (Fig. 1.4c; $R^2 = 0.586$, $P < 0.001$; Fig. 1.4d; $R^2 = 0.410$, $P < 0.001$) and FD (Fig. 1.4e; $R^2 = 0.268$, $P < 0.001$; Fig. 1.4f; $R^2 = 0.415$, $P < 0.001$), respectively. Multiple regression models retained realized FR and FD as best predictors of both bacterial production and decomposition.

Discussion

The results of our experiment demonstrated the importance of understanding species phylogenetic relatedness when predicting the strength of priority effects. We observed the highest β-diversity among communities in the *Serratia* pool (Fig. 1.2),

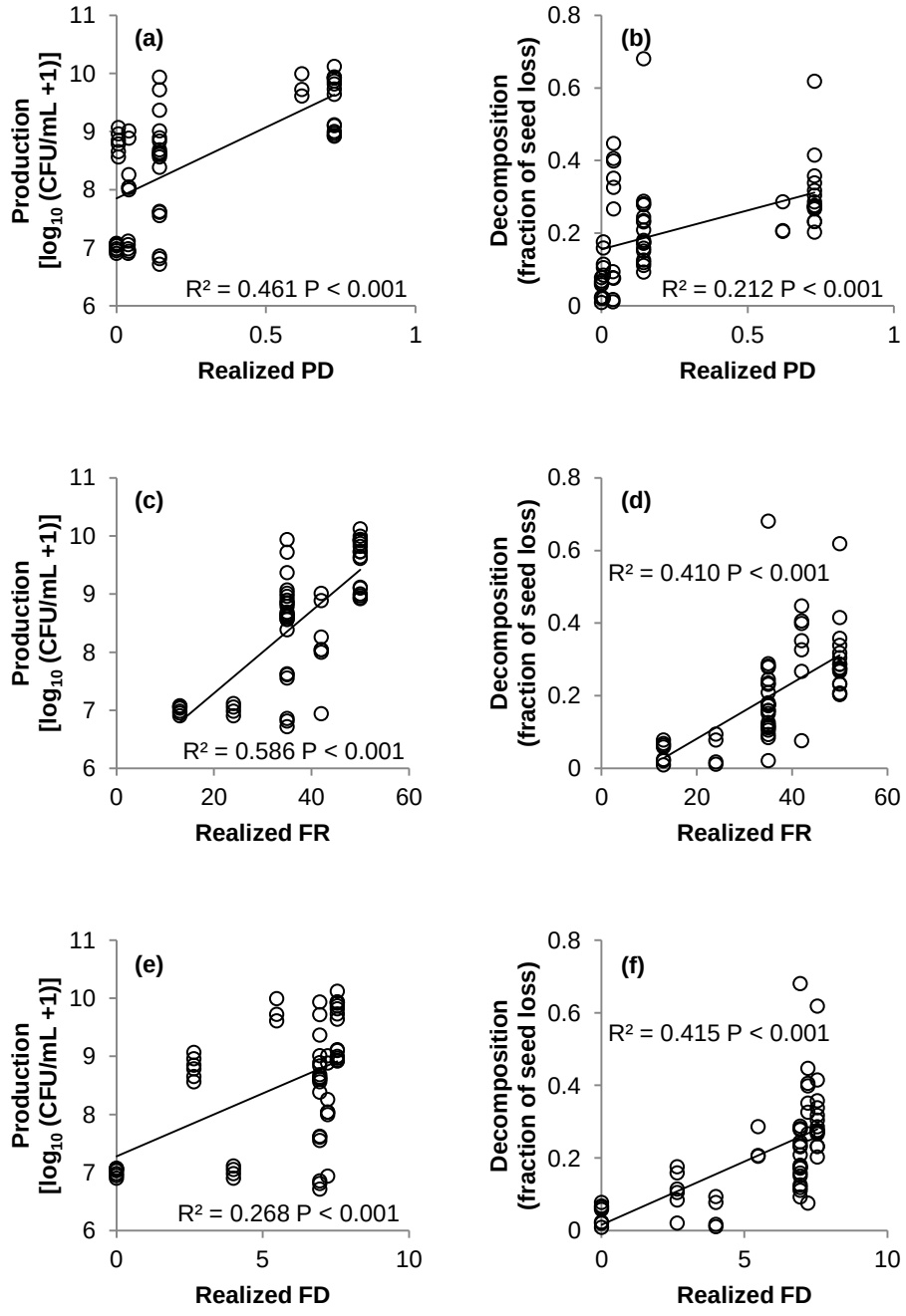

Fig. 1.4 Relationships between realized phylogenetic diversity (PD; a, b), functional richness (FR; c, d), functional diversity (FD; e, f), and production (left column)/decomposition (right column). PD and FD attained zero values in communities with one species. Data are plotted with linear regression lines.

which contained phylogenetically the most closely related bacterial strains (Fig. 1.1a). Different *Serratia marcescens* strains were dominant in these communities when subjected to different assembly histories (Fig. 1.3a). In contrast, communities from each of the other pools with lower phylogenetic relatedness were structurally similar (Fig. 1.2), containing the same dominant species regardless of history (Fig. 1.3b-d). This difference emerged despite the fact that history had a significant effect on the structure of the assembled communities for all species pools, as revealed by MANOVA. These results appear consistent with our hypothesis that stronger competition may occur between species that are more closely related phylogenetically (Maherali and Klironomos 2007; Violle et al. 2011), leading to stronger priority effects that generate multiple community states (Chase and Leibold 2003; Fukami and Lee 2006). However, phylogenetic conservatism was not detected when all bacterial traits were considered together, and non-significant phylogenetic signals were detected for the majority of measured traits. At least three mutually nonexclusive explanations can account for these results. One possibility is that at least some of the phylogenetically conserved traits that we measured are important in defining the ecological niches of our study bacteria. This is supported by the fact that phylogenetic diversity and functional diversity based on measured traits (including FR and FD) were both positively related to bacterial production and decomposition in our experiment. Another possibility is that some unmeasured traits that are important in defining species niches may be phylogenetically conserved, making phylogenetic relatedness a reasonable proxy of functional similarity with regards to these traits. A third explanation is that phylogenetic relationships based on the 16S rRNA gene, which is known to be highly conserved between different species of bacteria (Coenye and Vandamme 2003), may not adequately capture the potentially large variation in traits coded by less conserved genes (see Dahle et al. 2001 for a counterexample). Note that this issue can be circumvented in the future by constructing phylogeny based on whole genomes, which are currently unavailable for most organisms. Regardless, our results

highlight the utility of phylogenetic information for understanding the structure and functioning of ecological communities, even when phylogenetically conserved functional traits are not identified or measured.

Our results indicated that phylogenetic diversity positively affected ecosystem functions (i.e., bacterial production and decomposition), but that ecosystem functioning was better predicted by functional diversity. Using data from plant experiments, Cadotte et al. (2008, 2009) also showed that primary productivity was positively correlated with both plant phylogenetic and functional diversity. However, their results indicated that phylogenetic diversity explained more variation in plant productivity than several measurements of functional diversity. This discrepancy between the results of the two studies may be due to the fact that horizontal gene transfer, which may increase trait similarity among distantly related species and weaken the correlation between phylogenetic relatedness and trait similarity, is much more common for bacteria than plants (Andersson 2005; Richardson and Palmer 2007). Note that phylogenetic diversity nevertheless remained significant in explaining the functioning of bacterial communities in our experiment.

Our results also showed that community assembly history had significant effects on bacterial production in the *Staphylococcus*, *Bacillus* and mixed communities, and on decomposition in the *Staphylococcus* communities. Likewise, Fukami et al. (2010) manipulated the assembly history of wood-decay fungal communities and found a significant effect of assembly history on fungal decomposition. They showed that community divergence in species richness and composition, resulting from different assembly histories, led to the differentiation of ecosystem functioning. However, this mechanism cannot explain the divergence/convergence of ecosystem functioning in communities subjected to different assembly histories in our study. Two distinct alternative states were formed in communities of the *Serratia* pool (Fig. 1.3a), but

ecosystem functions of these two community states were similar. In contrast, a single community state was observed in the *Staphylococcus* pool, but ecosystem functions differed among the assembled communities (Fig. 1.3b). One explanation for the lack of historical effects on ecosystem functioning in the *Serratia* communities is that the two strains of *Serratia marcescens* may play similar ecological roles since they are phylogenetically closely related (99% similarity based on phylogeny) and functionally similar (sharing 50 of 57 traits). The two *Serratia* strains may thus be largely functionally substitutable, resulting in the same levels of ecosystem functions in communities dominated by different *Serratia* strains. In other species pools, although the historical effect was not strong enough to generate multiple community states, the abundance of subdominant species differed under different assembly histories (hence the significant effect of assembly history on species densities in MANOVA), especially in the *Staphylococcus* pool (Fig. 1.3b), which may have caused the differentiation of ecosystem functioning in those species pools. All together, our results showed that assembly history affected ecosystem functioning in some communities, but not in others. Understanding the conditions that promote the relationship between assembly history and ecosystem functioning remains an important topic of future research.

One concern is that each phylogenetic relatedness level in our experiment included only one species combination, so one could argue that the effect of phylogenetic relatedness may have been confounded with the effect of species identity. An ideal solution to this problem would be to use as many species combinations in each phylogenetic level as possible, but this may not be logistically possible. In particular, finding many combinations of phylogenetically closely related bacteria with different colony morphologies (e.g., the red and white *Serratia marcescens*) is difficult. In this experiment, although we cannot exclude the possibility that the effects of species phylogenetic relatedness and identity are confounded, results from a related experiment suggests that this is not the case. That experiment produced results similar to the current

experiment. In particular, strong priority effects were also observed in bacterial communities containing closely related species, specifically those with three strains of *Bacillus pumilus*; weaker priority effects were detected in communities with less related species (J. Tan, unpublished data). In the present experiment, weak priority effects also emerged in all communities of the three species pools with relatively low levels of phylogenetic relatedness, resulting in single community states. Together, these results strongly suggest a linkage between species phylogenetic relatedness and the strength of priority effects. Nevertheless, future studies that manipulate phylogenetic relatedness or diversity should aim to establish multiple species combinations within each treatment in order to eliminate the potential confounding effects from species identity.

In this study, different bacterial species pools exhibited different levels of phylogenetic relatedness permitting an evaluation of how phylogenetic relatedness might govern the relative contributions of niche-based deterministic processes (Chase and Leibold 2003) and neutral stochastic processes (Bell 2001; Hubbell 2001) to community assembly. In the experiment we conducted to accomplish this evaluation, multiple community states resulting from strong stochastic assembly processes (i.e., priority effects) were only observed in the species pool with the highest phylogenetic relatedness and highest functional similarity. Alternatively, single-community states resulting from strong deterministic assembly processes were observed in communities assembled from less phylogenetically related species pools. As such, these observations support our hypothesis that priority effects are stronger between species that are more closely related phylogenetically, although some caution must be exercised when generalizing these results given the limitation of our experimental design (see last paragraph). Further, our study demonstrates a positive relationship between phylogenetic diversity and ecosystem functions in an experiment that directly manipulated phylogenetic diversity. Importantly, we obtained these results despite the fact that many functional traits measured in our experiment exhibited non-significant phylogenetic signals. Our results thus highlight the

difficulty of identifying species functional traits relevant for community assembly and ecosystem functioning, and at the same time, the utility of basic phylogenetic information in predicting the structure and functioning of ecological communities.

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Chapter 2

PHYLOGENETIC RELATEDNESS OF RESIDENT AND INVADING SPECIES, NOT PHYLOGENETIC DIVERSITY OF RESIDENT COMMUNITIES, CONTROLS INVASIBILITY

Abstract

A central goal of invasion biology is to elucidate mechanisms regulating community invasibility. Darwin's naturalization hypothesis, one of the oldest hypotheses in invasion biology, emphasizes the importance of phylogenetic relatedness (PR) between resident and invader species for predicting invasibility. Alternatively, a recent extension of the diversity-invasibility hypothesis predicts that phylogenetic diversity (PD) of resident communities influences invasibility. Neither of these hypotheses has undergone rigorous experimental testing, and relative contributions of PR and PD to community invasibility are unknown. In the reported experiment, we consider both perspectives together by independently manipulating PD and PR in laboratory bacterial assemblages. This experiment demonstrates, for the first time, that PR is more important than PD in regulating invasibility. This novel result illustrates how understanding species evolutionary relationships can guide the prediction, prevention and management of biological invasions.

Introduction

The broad ecological and economic consequences of biological invasions have spurred considerable interest among ecologists in exploring mechanisms controlling the invasibility of ecological communities (Vitousek et al. 1996, Tilman 1997, Mack et al. 2000). One aspect of this work has focused on biodiversity, asking the question of whether more diverse communities pose stronger resistance to invaders than their less diverse counterparts. Much of this research has focused on species diversity. Experimental manipulations of species diversity have shown that community invasibility often declines as the number of species in the community increases (Levine et al. 2002, Fridley et al. 2007). This negative diversity-invasibility pattern can be driven by at least one of two mechanisms—the niche complementarity effect (i.e., niche differentiation among resident species leads to fewer unoccupied niches available for invaders in more species-rich communities) and the sampling effect (i.e., there is a greater chance for more species-rich communities to contain species that strongly resist invasion) (Fargione and Tilman 2005).

In light of the large amount of work linking species diversity and invasion, it is notable that species diversity only represents one component of biodiversity and that it treats each species equally. Species, however, are known to differ in their functional traits and evolutionary history. Other, less studied, components of biodiversity may better capture these differences in species characteristics within a community. One such component of biodiversity is phylogenetic diversity (hereafter PD), which has recently drawn much attention from biologists (Forest et al. 2007, Srivastava et al. 2012). This is facilitated by the increased availability of gene sequences for various organisms, and also by our improved knowledge of species functional traits. It has been recognized that species' functional traits, rather than species' identity, determine their contribution to ecosystem functions (Diaz and Cabido 2001, McGill et al. 2006). As a result, functional

diversity (hereafter FD), which better captures functional trait distributions than species diversity, often does a better job predicting ecosystem functions (Diaz and Cabido 2001, Naeem and Wright 2003, Petchey and Gaston 2006). Evolutionary histories, however, place a constraint on the differences in functional traits among taxa (Peterson et al. 1999, Donoghue 2008), such that PD, which accounts for species evolutionary relationships, may often be a reasonable proxy of FD. As it may not always be straightforward to identify or measure functionally important traits, PD has been expected to be of much use as a predictor for ecosystem functions (Cavender-Bares et al. 2009, Srivastava et al. 2012). Here we suggest that increasing PD of resident communities serves to reduce invasibility (Fig. 2.1a), by promoting complementarity and sampling effects. Increasing PD may strengthen the complementarity effect if higher PD communities are characterized by more diverse traits, translating into more niches being occupied by resident species and fewer opportunities available for invaders. Increasing PD may also result in the sampling effect if species with particular traits that confer invasion resistance are more frequently present in higher PD communities.

Ecologists have also long recognized the potential role of phylogenetic relatedness (PR) between resident and invader species in influencing community invasibility. Darwin (1859) proposed that invasive species would be less successful in communities that contain their close relatives (Fig. 2.1b), reasoning that strong competition imposed by resident species on closely related invaders, due to high similarity in their niches, would reduce their success. This naturalization hypothesis is thus related to the sampling effect, where community invasibility is largely determined by certain invasion-resistant species. There have been a number of empirical tests of this hypothesis (Ricciardi and Mottiar 2006, Strauss et al. 2006, Proches et al. 2008, Schaefer et al. 2011, Tingley et al. 2011, Allen et al. 2013, Carboni et al. 2013). These tests, which are based almost entirely on observations of nonnative species in their introduced

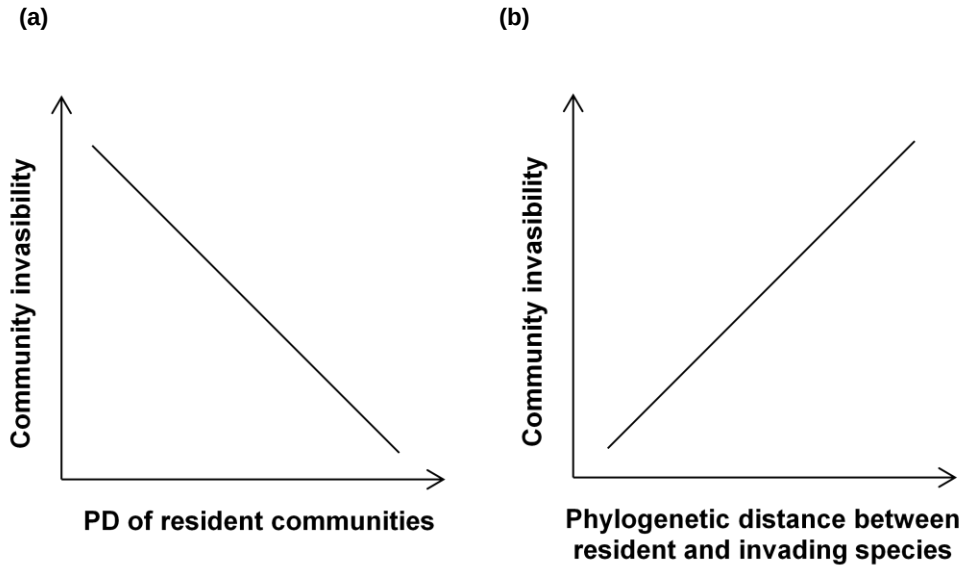

Fig. 2.1 The hypothesized effects of phylogenetic diversity (PD, panel a) of resident communities and phylogenetic distance, the inverse of phylogenetic relatedness (PR, panel b), between resident and invading species on community invasibility. Invasibility is expected to decline as PD increases, but increase as phylogenetic distance increases.

habitats (Jiang et al. 2010), have produced mixed results. These conflicting results are difficult to interpret, given the lack of control for confounding factors in observational studies. Experiments that directly manipulate PR, independent of other factors, are needed to rigorously test the hypothesis.

Recognizing that both PD and PR have the potential to influence community invasibility (Fig. 2.1), we performed the first experiment that independently manipulated PD and PR to evaluate their relative importance for invasibility. We assembled resident communities using bacterial species collected from a single source, and invaded them with a nonresident bacterial species. The PD and PR of each resident community were determined using a phylogenetic tree based on bacterial 16S rRNA sequences (Fig. 2.2). We kept initial resident species richness at a constant level (3 species) to minimize potential confounding effects of PD and PR from species richness. This level of species diversity also permitted the setup of more than one resident community for each PD-PR combination (see Table A.1), reducing the idiosyncratic influences of individual species and communities on experimental results. We hypothesized that both PD and PR are important in regulating community invasibility, much as both the complementarity and sampling effects often contribute to positive relationships between species diversity and community biomass production (Cardinale et al. 2006, Cardinale et al. 2007). In addition, FD of resident communities and functional similarity (FS) between resident and invading species were also determined based on the ability of study bacteria to utilize various organic carbon compounds (see Methods for details and Fig. A.1). We tested for phylogenetic signals in these bacterial functional traits using a Mantel test (all traits considered together; see Methods for details) and Blomberg's *K* statistic (for each individual trait; Blomberg et al. 2003). Similar to PD and PR, we hypothesized that FD and FS combine to affect community invasibility. In addition to the multi-species resident communities, we also established monocultures of each resident species and subjected them to the invasion of the same non-resident species.

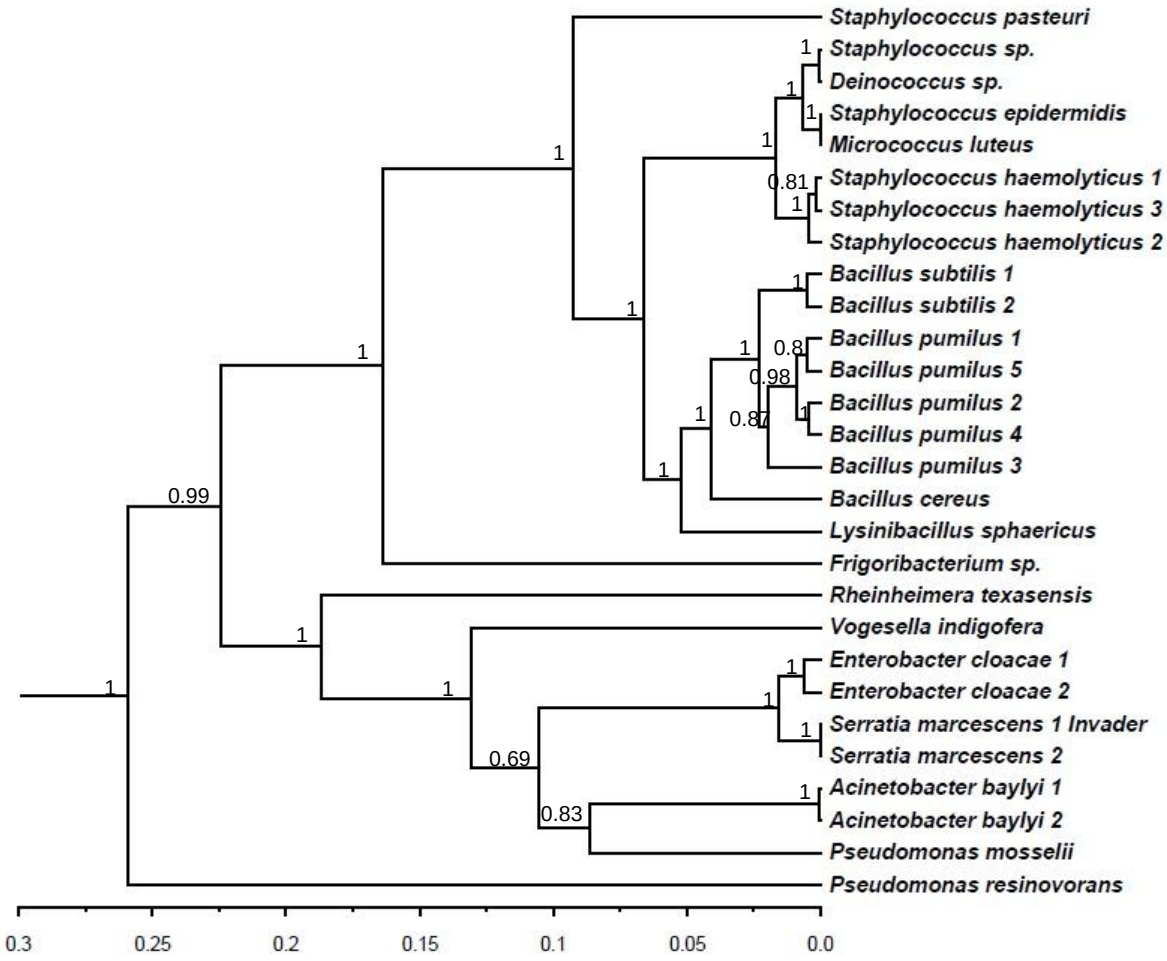

Fig. 2.2 Phylogeny of the bacterial species pool based on the 16S rRNA genes, constructed using the Bayesian methods. Scores on nodes indicate posterior probability.

Methods

Bacteria

Prior to the experiment, we collected bacterial strains from several freshwater ponds, and screened them for suitability as the candidate members of resident communities. Candidate strains must form distinct colonies on agar plates, allowing us to quantify their abundance via simple plate counts. They also must show a wide range of PR, so that resident communities with different degrees of PD can be constructed. The resultant species pool (see Fig. 2.2) consisted of both Gram positive and negative bacteria, the majority of which were collected from a single pond—Lake Clara Meer in Piedmont Park of Atlanta, GA, USA. We obtained a strain of *Serratia marcescens*, whose colony morphology (with a distinct solid red color) differs from that of all resident species, from Carolina Biological Supply (Burlington, NC, USA) as the invader. The stock culture of each bacterial strain was grown in 0.2% LB broth.

Constructing phylogeny

We constructed the bacterial phylogeny based on the 16S rRNA gene of each strain in the species pool (including the invader; Fig. 2.2). After sequencing the 16S rRNA, we aligned sequences with the Nearest Alignment Space Termination Algorithm(DeSantis et al. 2006), selected the best sequence evolution model—TIM3+G with jModelTest (version 0.1; Guindon and Gascuel 2003, Posada 2008) using the Akaike information criterion, and assembled the phylogenetic tree with MrBAYES (version 3.1.2; Huelsenbeck and Ronquist 2001) using the Bayesian method. Three archaeal species were used as the out-group. We also constructed a phylogenetic tree with the maximum likelihood (ML) method, which was similar in structure to the Bayesian tree; results based on the two trees were similar. We thus only reported the Bayesian tree and associated results here.

Experimental design

Based on the phylogenetic tree, we assembled bacterial communities that differed in three levels of PD (low, intermediate, and high) of resident communities and three levels of PR (low, intermediate, and high) between the resident community and invading species, while fixing the number of species in each resident community at three. All the resident species used in our experiment came from Lake Clara Meer. Three-species resident communities varied widely in PD and PR, allowing them to be manipulated independently of each other. This level of species diversity also allowed the setup of more than one resident community for each PD-PR combination (see Table A.1), reducing the idiosyncratic influences of individual species and communities on experimental results. Nevertheless, we were unable to find any suitable resident community for the high PD-low PR treatment, leaving us an incomplete factorial design (Table A.1). Following Faith (1992), we calculated PD of a resident community by summing the lengths of all the intervening branches of the three constituent species with the community. Similarly, we calculated phylogenetic distance (the inverse of PR) between resident and invading species by summing the length of the intervening branches between them on the phylogenetic tree. Both nearest phylogenetic distance (distance between the invader and its closest relative in the resident community) and average phylogenetic distance (average distance between the invader and each species in the resident community) were calculated, and because results based on the two metrics were qualitatively similar, we reported those based on nearest phylogenetic distances only. In addition to the three-species resident communities, we also established monocultures of each resident species used in the experiment and subjected them to the invasion of the same non-resident species. Each of the single- and three-species communities was replicated five and ten times, respectively.

Each microcosm consisted of one 25-mL loosely capped test tube containing 10 mL of filtered protozoan pellet medium, which, despite its name, supports the growth of bacteria (Jiang 2007, Jiang et al. 2010, Tan et al. 2012). The medium was composed of 0.55 g crushed protozoan pellet (Carolina Biological Supply, Burlington, NC, USA) per liter of deionized water. The pellet is made from plant materials, containing a variety of carbon compounds that can be utilized by bacteria. This medium was autoclaved in large flasks, and filtered afterwards to remove insoluble particles. The filtrate was then transferred into experimental microcosms, and autoclaved again before the experiment started. Each microcosm also received a wheat seed as the additional carbon source. The wheat seeds were dried, weighed, and autoclaved beforehand. During the experiment, all microcosms were incubated on a shaker at 200 rpm under room temperature (~22 °C).

At the beginning of the experiment (Day 0), we introduced resident species into microcosms by transferring a small volume (<5 µL) of their stock cultures with an aseptic loop. We allowed the resident communities to equilibrate for 41 days, before subjecting them to invasion. To determine the realized PD and PR of three-species resident communities at the time of invasion, we destructively sampled half of the microcosms (five replicates of each treatment) on days 40 and 41 to determine resident species composition and abundance. These data were used to calculate realized PD and PR in each sampled microcosm. On day 42, we invaded the remaining microcosms with *S. marcescens*, introduced in the same way as the resident species. The experiment continued for another 21 days to allow the establishment of invader populations. Final sampling was conducted on day 63 to quantify invader abundance as a common metric of invasibility.

Measuring FD and FS

To directly assess the relevance of functional traits for predicting invasibility, we estimated FD of each resident bacterial community and functional similarity (FS) between resident and invading species, based on their ability to utilize a variety of carbon substrates on BIOLOG MicroPlates (BIOLOG, Hayward, CA, USA). Gram-positive and -negative bacterial cultures were inoculated into their corresponding types of MicroPlates, and the results for each of the carbon substrates (positive or negative) were recorded. Our analyses focused on the 55 substrates that Gram-positive and -negative MicroPlates shared in common. As in Tan et al. (2012), we performed a UPGMA-based cluster analysis of these functional trait data and produced a functional dendrogram (Fig. A.1). We then calculated FD of each resident community as the total branch lengths connecting its component species on the functional dendrogram (Petchey and Gaston 2002), and functional distance (the inverse of FS) between the invader and resident species as the branch lengths connecting these species on the functional dendrogram. Both nearest and average functional distances between the invader and resident communities were calculated, and analyses based on the two metrics produce similar results. We thus reported findings based on nearest distances only.

Data analysis

We used a two-way ANOVA to assess the effects of PD and PR on community invasibility, with invader abundance as the dependent variable and initial PD and phylogenetic distance as the class variables. Tukey's HSD post-hoc test was conducted following the detection of significant treatment effects. We performed simple linear regressions modeling invader abundance as a function of realized PD, realized phylogenetic distance, realized FD, and realized functional distance, separately. This was followed by a multiple regression model that considered all four explanatory variables together, with the backward selection procedure used to identify the variables best

explaining invasibility. Bacterial abundance data were log-transformed prior to statistical analyses to improve normality.

We tested for significance of phylogenetic signals of the measured functional traits in two ways. First, we considered all traits together with a Mantel test assessing the association between bacterial phylogenetic and functional distances, with distance matrices permutated 10,000 times. Second, we considered each trait individually with Blomberg's (2003) *K*, using the multiPhylosignal function in the Picante package (Kimbel et al. 2010).

Results

The non-resident invader successfully established its populations in all experimental microcosms. Its abundance, however, differed substantially among the experimental treatments (Fig. 2.3). ANOVA revealed a significant effect of PR ($F_{2,82}$ = 49.958, $P < 0.001$), but not PD ($F_{2,82}$ = 2.291, $P = 0.108$), on invader abundance; changes in PD did not alter the effect of PR on invasibility (PD × PR term in ANOVA: $F_{3,82}$ = 1.117, $P = 0.347$). The significant PR effect was largely driven by lower invader abundance in the high PR (i.e., low phylogenetic distance) treatment than in the low and intermediate PR (i.e., large and intermediate phylogenetic distance) treatments (Tukey's HSD test, $P < 0.001$).

Consistent with the ANOVA results, linear regressions showed that invader abundance in multiple-species resident communities was unaffected by realized PD (R^2 = 0.024, $P = 0.148$, Fig. 2.4a), but declined with increasing realized PR (i.e., decreasing phylogenetic distance; $R^2 = 0.560$, $P < 0.001$, Fig. 2.4b). Neither FD ($R^2 = 0.001$, P = 0.811) nor FS ($R^2 = 0.002$, $P = 0.698$) affected community invasibility (Fig. A.2). The multiple regression model retained realized PR as the best predictor of invasibility. PR ($R^2 = 0.379$, $P < 0.001$, Fig. 2.5a), not FS ($R^2 < 0.001$, $P = 0.931$, Fig. A.3), was a

significant predictor of invader abundance in single-species resident communities. Note that species extinction occurred in some of the resident communities, without causing realized PR and PD to covary ($R^2 = 0.098$, P = 0.357).

When all the 55 measured traits were considered together, our study bacteria exhibited significant phylogenetic signals (Mantel test, P = 0.005). When examined individually, only 8 of the 55 traits (15%), including D-trehalose (Blomberg's *K* = 1.250, P = 0.009), α-D-glucose (*K* = 0.472, P = 0.015), D-melibiose (*K* = 1.032, P = 0.022), D-fructose (*K* = 0.355, P = 0.031), gentiobiose (*K* = 0.305, P = 0.034), β-methyl-D-glucoside (*K* = 0.481, P = 0.035), α-D-glucose-1-phosphate (*K* = 1.016, P = 0.037) and D,L,α-glycerol phosphate (*K* = 0.255, P = 0.046), showed significant phylogenetic signal.

Discussion

Our study demonstrated the important role of PR, relative to PD, for determining community invasibility. We found that invader abundance declined with increasing PR in both multi- and single-species resident communities, providing direct experimental support for Darwin's (1859) naturalization hypothesis. There has been substantial recent interest in evaluating Darwin's naturalization hypothesis in natural assemblages of various organisms. For example, Duncan and Williams (2002) found that exotic seed plant species with their congeneric native species present in New Zealand were more likely to naturalize in the country, a pattern at odds with the hypothesis. By contrast, Strauss et al. (2006) found the invasiveness of exotic grass species was negatively related to their phylogenetic relatedness to native grass species in California, a finding consistent with the hypothesis. These conflicting results, which were based on observations of natural communities, underscored the need for rigorous tests of Darwin's naturalization hypothesis via experimentation (Duncan and Williams 2002, Lambdon and Hulme 2006, Ricciardi and Mottiar 2006, Strauss et al. 2006, Schaefer et al. 2011).

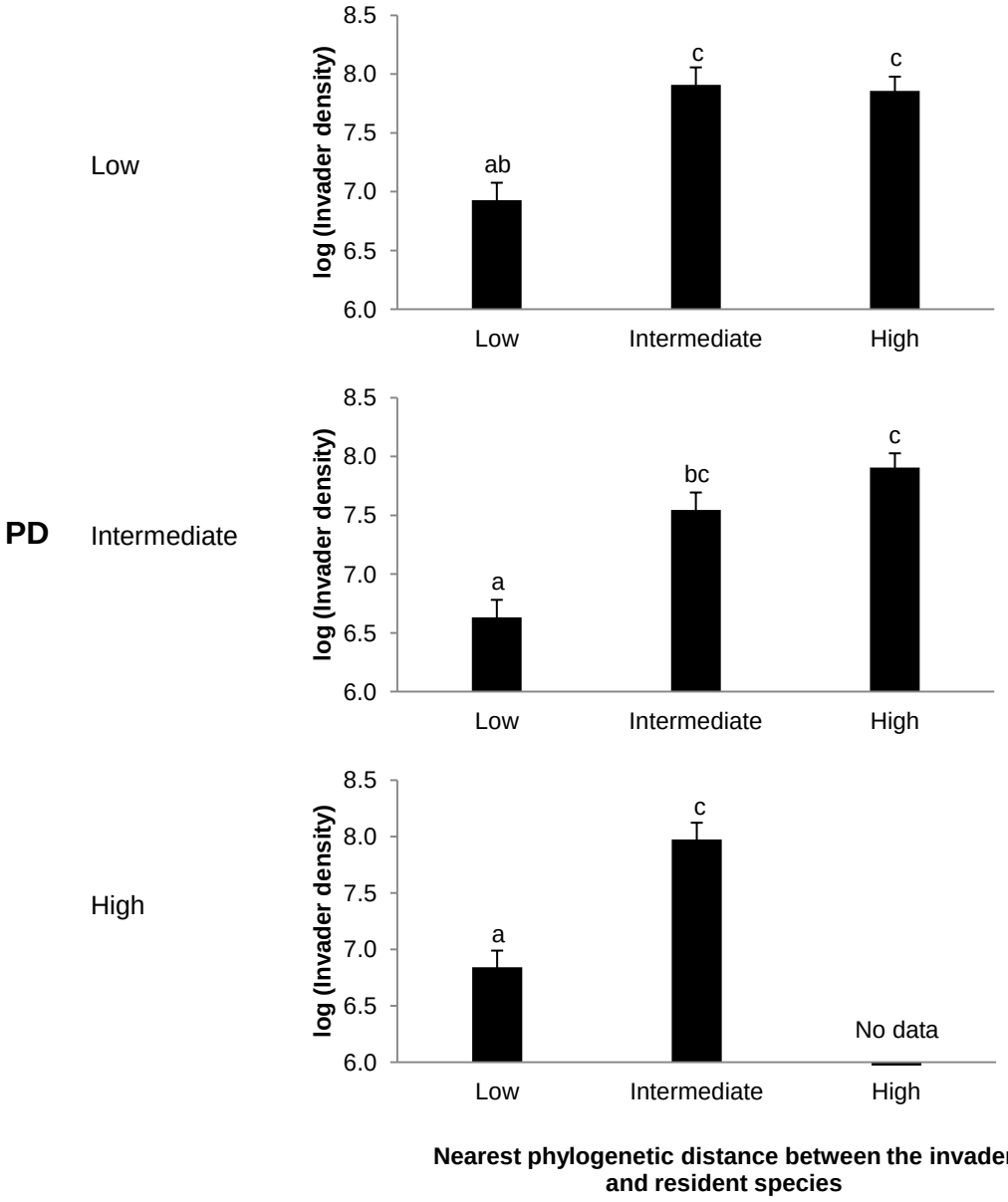

Fig. 2.3 Population densities of the invader (*Serratia marcescens*) in three-species resident communities with different levels of phylogenetic diversity (PD) and nearest phylogenetic distance (the inverse of PR) between resident and invading species. Values are mean + standard error, with population densities measured as colony forming units (CFUs) per mL and $\log_{10}(x+1)$-transformed prior to analysis. Treatments sharing the same letters do not differ from each other according to Tukey's HSD tests. Note the absence of the high PD-high phylogenetic distance treatment.

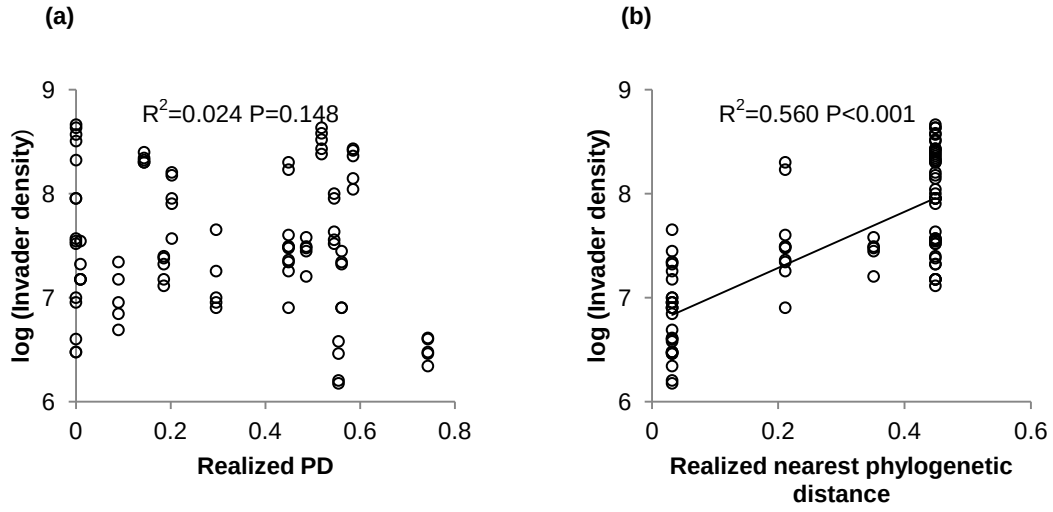

Fig. 2.4 Relationships between invader population density and (a) realized PD of three-species resident communities; and (b) realized nearest phylogenetic distance (the inverse of PR) between the invader and three-species resident communities. Realized PD and phylogenetic distance were calculated based on data collected immediately before invasion. Data are plotted with significant linear regression lines. Invader population densities are in the unit of CFUs per mL and were $\log_{10}(x+1)$-transformed prior to analysis.

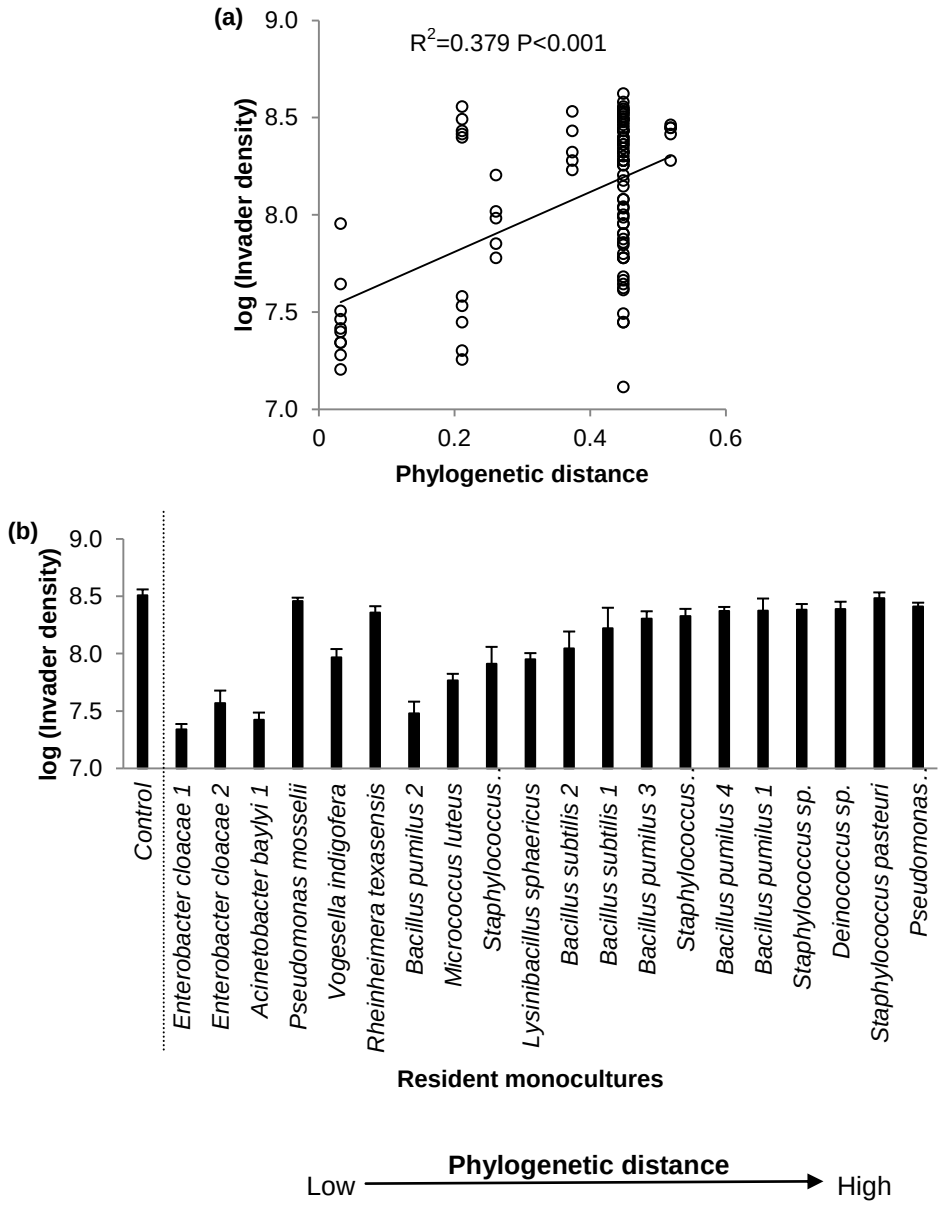

Fig. 2.5 Invader population densities in single-species resident communities. (a) The relationship between invader population density and its phylogenetic distance (the inverse of PR) within the single-species resident communities; and (b) invader population density in each single-species resident community (ordered by phylogenetic distance between the species and invader). Invader population densities are in the unit of CFUs per mL and were $\log_{10}(x+1)$-transformed prior to analysis. Data in panel (a) are plotted with the linear regression line. Values in panel (b) are mean + standard error.

Jiang et al. (2010) conducted such an experiment using bacteria and reported results consistent with Darwin's prediction, but the strength of this study was diminished by the fact that PD was left uncontrolled, and that the invader and its most closely related resident species were in fact two strains of the same species. These issues were not a problem in the experiment reported here, in which PD and PR were manipulated independent of each other with each PD-PR combination represented by multiple resident communities, and where the bacterial invader and residents did not share the same species identity. In particular, the six resident communities characterized by the highest PR, containing different *Enterobacter cloacae* strains, were most closely related to the invader (Fig. 2.2) and least receptive to invasion (Fig. 2.5b). As predicted by Darwin (1859), more closely related bacterial species were more similar in their niches (i.e., phylogenetic conservatism in carbon usage patterns), presumably translating into stronger competition between the invader and its closer resident relatives (Violle et al. 2011). Taken together, our study provided strong experimental evidence for Darwin's naturalization hypothesis, supporting the role of PR between resident and invader species in predicting community invasibility.

In contrast to the strong effect of PR, we found no effect of PD of resident communities on invasibility. This surprising result is at odds with the traditional view that more diverse communities are more resistant to invasion (Elton 1958). It also contrasts with the result of a recent observational study that higher PD plant communities in the Netherlands were less receptive to alien plant species (Gerhold et al. 2011). One possibility that could potentially explain this apparent discrepancy is that the strength of the relationship with PD of resident communities may differ for different invasibility metrics. Invader establishment success, the metric used in Gerhold et al. (2011), may be tightly linked with PD because higher PD communities would have less unoccupied niche space left for invaders, resulting in their higher establishment failure. On the other hand, invader abundance, the metric used in our study, may be closely associated with PR but

not PD because invaders may not be able to attain large abundance if their optimal niches are occupied by their closely related resident species (i.e., those having high PR with invaders). Here we focused on examining invader abundance, given that all our experimental communities were receptive to invasion. Studies considering both metrics together would be needed to test the above hypothesis. A second possibility associated with the lack of PD effect on invasibility is that lateral gene transfer among bacteria may reduce the degree of trait conservatism (Boucher et al. 2003), making PD an ineffective proxy of niche complementarity and sampling effects in bacterial communities. However, current evidence indicates that many bacterial functional traits exhibit phylogenetic signals despite lateral gene transfer (Martiny et al. 2013). Some of the bacterial traits examined in our experiment were also phylogenetically conserved, and the whole set of traits was conserved when considered together. The significant effect of PR on invader abundance is also indicative of the conservatism of important functional traits for resident-invader interactions that determined invader abundance. A third possibility is that the PD metric used in our experiment, which is based on species presence/absence data (Faith 1992), may not necessarily capture the actual phylogenetic diversity of real communities typically characterized by uneven species abundances (Cadotte et al. 2010). To address this issue, we calculated several abundance-based PD metrics for each resident community using the methods described in Cadotte et al. (2010). None of these abundance-adjusted PD metrics, however, resulted in an improved ability to predict invader abundance ($P > 0.05$ in all cases).

Also somewhat surprisingly, metrics based on bacterial functional traits, including FD of resident communities and FS between resident and invading species, failed to predict community invasibility. This contrasts with several plant invasion experiments reporting FD of plant resident communities (Dukes 2001) and FS between plant residents and invaders (Fargione et al. 2003) as significant predictors of invasibility (measured as invader abundance, biomass or cover). Note that these results were obtained despite the

fact that these studies used discrete FD and FS measures based on simple plant functional group classifications. These findings thus indicate that classifying plants into even simple functional groups can effectively capture key differences in functional traits among plant species. Indeed, much interest in trait-based ecology has focused on plants (Violle et al. 2007, Kattge et al. 2011), resulting in a substantially better understanding of important functional traits for plants than for other groups of organisms. In particular, we know relatively little about important microbial traits associated with various ecosystem functions and their distributions across different lineages. This is reflected in our experiment, where the 55 bacterial functional traits were measured without a priori knowledge on their relevance for community invasibility. The non-significant effects of FD and FS on invasibility could thus have been caused by the lack of involvement of these measured traits in modulating the interactions between residents and invaders. The significant PR effect on invasibility, on the other hand, suggests that at least some of the important functional traits for resident-invader interactions, though not measured in our experiment, are phylogenetically conserved. The greater predictive power of PR demonstrated here supports the idea that species phylogenetic knowledge would be particularly useful for predicting ecosystem functions where important species traits cannot be identified or measured (Srivastava et al. 2012).

Two aspects of our experiment warrant clarification. First, only one invader species was used to challenge resident communities in our experiment, raising the concern that our findings may be influenced by idiosyncratic characteristics of the species. This concern, however, is alleviated by the fact that within each PD-PR combination treatment the invader faced multiple, different resident communities, which reduced the impacts of individual species (including both resident and invader species) on invasion success. Nevertheless, we suggest that future studies could include multiple invader species to further evaluate the robustness of our results. Second, bacterial functional traits were measured based on their ability to exploit different carbon sources in our

experiment. While the number of measured traits was relatively large (55 total), it can be argued that they all belong to the same group of traits related to carbon and energy acquisition. It remains to be seen whether considering other bacterial traits, such as cell size, capsule formation, and swimming behavior (Matz et al. 2002, Mulder et al. 2005), would improve the ability of FD and FS to predict invasibility.

In conclusion, our experimental results did not lend support to our original hypothesis that PD of resident communities and PR between resident and invading species combine to regulate community invasibility. Rather, only PR was a significant predictor of invader abundance. This result provides unequivocal support for Darwin's naturalization hypothesis, reinforcing the importance of knowledge of phylogenetic relationships between invading and resident species for predicting invasibility. Mechanistically, this result suggests that trait similarity between resident and invading species is more important than trait diversity of resident communities in modulating invader success. These results demonstrate the utility of species phylogenetic information for improving our understanding of regulatory mechanisms of ecosystem functions, and we encourage future investigations to be conducted in various types of ecosystems to evaluate their robustness. Our study also highlights the need to identify and measure functionally important traits to better our ability to predict ecosystem functions. Until a thorough understanding of species traits relevant for various ecosystem functions is attained, combining species phylogenetic information with knowledge of known functional traits may be our best tool for predicting the ecological consequences of changes in biodiversity.

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Chapter 3

SPECIES PHYLOGENETIC RELATEDNESS AND IMMIGRATION HISTORY MODULATE THE EFFECTS OF COMPETITION ON ADAPTIVE RADIATION

Abstract

Understanding ecological mechanisms regulating the evolution of biodiversity is of much interest for ecologists and evolutionary biologists. Adaptive radiation constitutes an important evolutionary process that generates biodiversity. Competition has long been recognized to influence adaptive radiation, but the direction of its effect and associated mechanisms remain ambiguous. Here we report an experimental test of the role of competition on adaptive radiation using the rapidly evolving bacterium *Pseudomonas fluorescens* interacting with different competing bacteria. Overall, competition suppressed adaptive radiation. This effect, however, was modified by the phylogenetic relatedness of *P. fluorescens* and its competitors and their immigration history, such that only when competitors were phylogenetically closely related to *P. fluorescens* was the extent of diversification affected by history. Immigration history further altered the relative importance of ancestor population size and niche availability for adaptive radiation. Our results highlight the context dependency of competitive effects on adaptive radiation.

Introduction

One important evolutionary process that generates biodiversity is adaptive radiation, in which a lineage diversifies rapidly to occupy available niches within a habitat (Schluter 2000, Gillespie 2004, Gavrilets and Vose 2005, Meyer et al. 2011). Often found in insular environments, such as islands (Losos et al. 1998, Grant and Grant 2002, Gillespie 2004), lakes (Schluter and McPhail 1992, Seehausen et al. 1997), and mountains (Heenan and Mitchell 2003), adaptive radiation has been thought to be influenced by two important factors: the availability of ecological niches, and the size of ancestral populations (Grant 1998, Schluter 2000). Whereas niche availability may affect the fitness of newly formed species and thus selective pressure (MacArthur and Wilson 1967, Grant 1998, Schluter 2000), ancestral population size governs the supply of genetic variation and thus potential for diversification. By affecting these two factors, competition—one of the most ubiquitous species interactions in many ecosystems—may influence adaptive radiation (Roughgarden 1972, Schluter 1994, Dieckmann and Doebeli 1999, Doebeli and Dieckmann 2000, Zhang et al. 2012). Several empirical studies (Schluter 1988, Gillespie and Roderick 2002, Grant and Grant 2006) have assessed the role of competition for adaptive radiation. The majority of these studies, however, are based on comparisons of lineages found on islands (where fewer competitors are present) versus those found on the mainland (where more competitors are present) (Schluter 1988, DeSalle 1995, Grant and Grant 2008), or experiments manipulating the presence/absence of intraspecific competitors (Schluter 1994, Fukami et al. 2007, Bailey et al. 2013). While the findings of observational studies are vulnerable to alternative explanations, experimental studies of intraspecific competition tell little about how species from evolutionarily more distant lineages affect adaptive radiation. The few experimental tests of interspecific competition, which have either considered a single competitor species (Zhang et al. 2012), or competition from complex natural communities (Gomez and Buckling 2013), have produced mixed findings. Here we provide a rigorous

experimental test of how competition influences adaptive radiation by allowing the diversifying species to interact with various known intraspecific and interspecific competitors.

In situations where the diversifying lineages have the opportunity to interact with different competitors, the strength of competition could vary, with potential consequences for adaptive radiation. In particular, the evolutionary relationship between the diversifying and competing species may have considerable influence on competition, as species traits that determine their interactions with other organisms are often constrained by their evolutionary histories (Darwin 1859, Harvey and Pagel 1991, Wiens 2004). Recognizing the general tendency for more closely related species to share more similar niches, Darwin (1859) reasoned that it would translate into stronger competition between more closely related species—a hypothesis supported by several recent experiments (Jiang et al. 2010, Violle et al. 2011, Peay et al. 2012, Tan et al. 2012). Applying this idea to adaptive radiation, one may expect that greater phylogenetic relatedness (PR) between the diversifying species and its competitors would result in more intense competition, and in turn stronger effects on diversification. We note that this hypothesis has not been experimentally explored, but can be readily tested by including multiple competitors with different degrees of PR to the diversifying species.

While PR is associated with different competitors faced by the radiating lineage, species immigration history may affect adaptive radiation by influencing the interaction between the radiating lineage and the same competitor species. The importance of immigration history for adaptive radiation was emphasized in the first-arrival hypothesis of David Lack (1947), who suggested that earlier colonizing species should diversify to a greater extent than later colonizing species. According to this hypothesis, earlier arrival allows a species to undergo adaptive radiation before its competitors that arrive later exert their force, whereas the earlier arrival of competitors suppresses diversification by

occupying available resources and niches. Lack's first arrival hypothesis was initially proposed in 1947 to explain why Darwin's finches, but not other avian taxa, radiated on the Galapagos Islands (Lack 1947, Grant and Grant 2008). Experimental evidence for this 67-year-old hypothesis, however, is extremely lacking (but see Fukami et al. 2007 for an intra-species study).

Here we report an experimental study examining the effect of competition on adaptive radiation, using the rapidly diversifying bacterium *Pseudomonas fluorescens* SBW25 (hereafter SBW25) (Rainey and Bailey 1996, Rainey and Travisano 1998, Fukami et al. 2007) as the model organism of adaptive radiation, and multiple environmental bacterial species, which vary in their phylogenetic distance (the inverse of PR) to SBW25, as the competitors (Fig. 3.1). We found that competition tended to have an overall negative effect on adaptive radiation, with its effect modified by the PR of the diversifying and competing species and their colonization history, and that species immigration history further altered the relative importance of ancestor population size and niche availability for adaptive radiation.

Methods

We used a smooth morph (SM) phenotype colony of *P. fluorescens* SBW25, as the ancestral bacterium (Rainey and Bailey 1996, Rainey and Travisano 1998). This SM phenotype was marked by lacZ, which makes colonies of bacteria derived from SBW25 in our experiment exhibit a distinct blue color on agar with 40 mg/L 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Fukami et al. 2007). In a static microcosm containing nutrient rich aqueous medium, SM, which prefers the broth phase, diversifies to utilize different niches in the microcosm. Specialized phenotypes, including fuzzy spreaders (FS) inhabiting the bottom and wrinkly spreaders (WS) forming the biofilm at the air-broth interface of the microcosm, emerge within days (Rainey and Travisano

1998). Within each phenotype, multiple subtypes can be further identified (e.g., small-, large-, wheel-, and SM-like-WS within WS) (Fukami et al. 2007). We treat each identifiable phenotype/subtype as a biological species, as each of them is genetically identified and reproduces asexually with little recombination (Rainey and Travisano 1998, Spiers et al. 2002, Bantinaki et al. 2007, Ferguson et al. 2013).

We used six environmental bacterial strains, which show different degrees of phylogenetic relatedness to SBW25, as its competitors. We sequenced the 16S rRNA gene of our study species, and constructed the phylogenetic tree based on the gene sequences (Fig. 3.1). We first aligned these sequences with ClustalX2 (Larkin et al. 2007), confirmed the alignment manually, and selected TIM3+G as the best evolution model with jModelTest 2 (Guindon and Gascuel 2003, Darriba et al. 2012), by using the Akaike information criterion. Then we constructed the phylogenetic tree with the Bayesian method in MrBAYES (version 3.1.2) (Huelsenbeck and Ronquist 2001), with one archaeal species as the out-group. Based on the phylogenetic tree, we calculated phylogenetic distance between SBW25 and each competing bacterial strain by summing the length of intervening branches between them; lower values of phylogenetic distance correspond to higher values of PR. We classified the competing bacterial species into three groups according to PR values: *Pseudomonas fluorescens* (PF) and *Pseudomonas putida* (PP) in the high PR group, *Aeromonas hydrophilia* (AH) and *Serratia marcescens* (SER) in the intermediate PR group, and *Bacillus pumilus* (BP) and *Bacillus cereus* (BC) in the low PR group. Each PR level included two competitors to reduce possible idiosyncratic effects of competitor identity. The diversifying SBW25 and one competitor—*P. fluorescens* (PF), which was obtained from the Carolina Biological Supply (Burlington, NC, USA), belong to the same species. However, there is significant genetic difference between the two (0.7% in their 16S rRNA sequence). PF did not carry the lacZ marker, and did not diversify within the duration of our experiment.

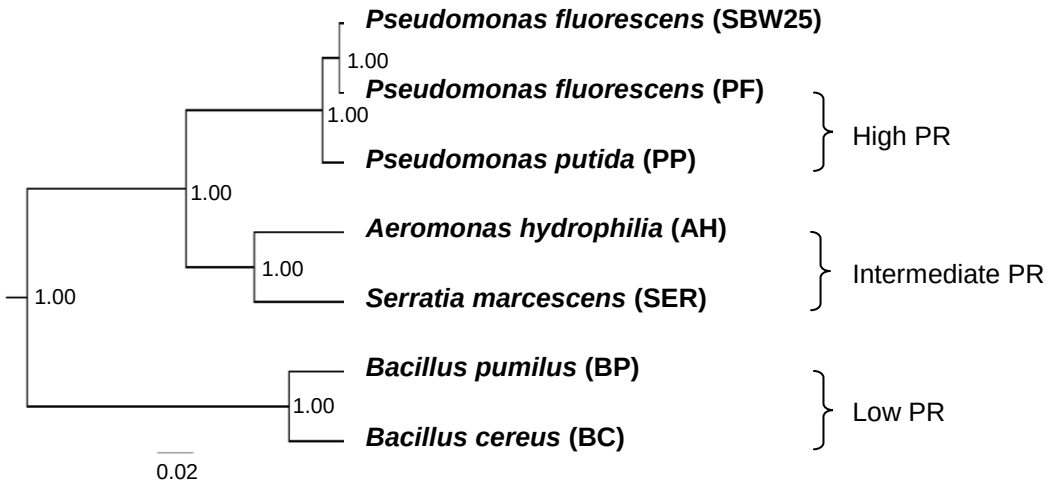

Fig. 3.1 Phylogeny of the study bacteria based on the Bayesian method. The scale indicates the posterior probability.

Our experiment used a two-way factorial design with three immigration histories (competitor introduced first and SBW25 second [competitor-SBW25], SBW25 and competitor introduced simultaneously [competitor & SBW25], and SBW25 introduced first and competitor second [SBW25-competitor]) crossed with three levels of PR (low, intermediate, and high). Each treatment combination was replicated six times. Experimental microcosms comprised 25 ml capped test tubes, each containing 6 ml King's Medium B (KB). Prior to the experiment, we plated each bacterial species on agar, randomly selected one colony for each species (one SM colony for SBW25), transferred the selected colonies into test tubes with 6 ml KB, and incubated the cultures under shaking (250 rpm) at 28℃ overnight. We introduced ~10^4 colony forming units (CFU) of each bacterial species into their designated microcosms on the day of their introduction (day 0 for the first colonizer, and day 1 for the second colonizer). We ended the experiment after the microcosms were incubated statically under 28 ℃ for 12 days, which were sufficiently long for a variety of SBW25 phenotypes to emerge (Tan et al. 2013). We collected the sample from each microcosm, serially diluted and plated it on agar with X-gal, and quantified the abundance of each species/phenotype after another three days of incubation under 28 ℃.

To characterize the niche similarity between SBW25 and its competitors, we measured their ability of utilizing a variety of carbon resources with BIOLOG MicroPlates (BIOLOG, Hayward, CA, USA). Previous work (Bailey et al. 2013, Gomez and Buckling 2013) has shown that different phenotypes of SBW25 differentiated among these carbon resources. First, we prepared monocultures of the SBW25 SM phenotype and the six bacterial competitors, and propagated them separately on KB agar. Then, we incubated each bacterium in their corresponding type of BIOLOG MicroPlates (GN2 for gram-negative bacteria, and GP2 for positive), each of which has 96 wells containing different carbon substrates. The GN2 and GP2 plates share 55 substrates, and we

recorded the results of these 55 traits, as positive if the bacterium was able to utilize the carbon substrate, and negative if not. We counted the number of traits shared by SM and each of the competitors as a proxy of the niche similarity between them. WS phenotypes constituted the majority of SBW25 diversity, and the competition between WS and competing species within the air-broth interface (WS's preferred habitat) may be affected by the similarity of carbon resource utilization of these species. We thus also quantified the ability of the mixture of a total of four WS phenotypes, found in our experiment, to utilize carbon substrates on BIOLOG MicroPlates, and calculated their niche similarity to competitors, using the same protocol as described above.

To assess the overall effect of competition on adaptive radiation, we lumped data from all competition treatments together and compared SBW25 phenotypic richness in these treatments to that in the controls (where competition was absent), using ANOVA. We then conducted a two-way ANOVA with PR and species immigration history as class variables to examine their effects on SBW25 phenotypic richness and abundance, followed by Tukey's HSD tests to identify difference among the competition and history treatments. To explore whether SBW25 shared more similar niches with more closely related competitors, we regressed SBW25-competitor trait similarity against their phylogenetic distance. To explore whether ancestral population size affected adaptive radiation, we regressed SBW25 phenotypic richness against its abundance, using data from all three immigration histories together as well as data from each history treatment; data from microcosms with no SBW25 surviving at the end of the experiment were excluded from the regression analyses. Similar regressions were also performed to assess whether niche availability affected adaptive radiation, using WS phenotypic richness as the dependent variable and niche similarity between WS and competitors as independent variables. In all regressions, data were $\log_{10}$-transformed when necessary to make the relationships linear.

Results

When all interspecific competitors were considered together, competition had a negative effect on the diversification of SBW25 (Fig. 3.2a; ANOVA: $F_{1,113} = 5.057$, $P = 0.026$). The effect of competition, however, varied substantially among the PR and immigration history treatments (Fig. 3.2a; ANOVA: PR, $F_{2,99} = 155.467$, $P < 0.001$, history, $F_{2,99} = 38.094$, $P < 0.001$, and PR × history, $F_{4,99} = 12.370$, $P < 0.001$). In particular, the negative effect of competition on diversification was observed in the intermediate and high PR treatments (i.e., PF, PP, AH and SER), but not in the low PR (i.e., BP and BC) treatments. Moreover, species immigration history affected diversification only in the intermediate and high PR treatments (ANOVA: PR × history, $F_{4,99} = 12.370$, $P < 0.001$). Within these two treatments, SBW25 phenotypic richness tended to be higher when it was introduced before its competitors than when introduced after or simultaneously with competitors (Fig. 3.2a; Tukey's HSD: $P < 0.05$). Similar patterns were observed when examining the response of SBW25 abundance to competition and history treatments (Fig. 3.2b; ANOVA: PR, $F_{2,99} = 12.824$, $P < 0.001$, history, $F_{2,99} = 16.478$, $P < 0.001$, and PR × history, $F_{4,99} = 3.980$, $P = 0.005$).

SBW25 phenotypic richness was positively correlated with SBW25 population abundance when all treatments were considered together ($R^2 = 0.238$, $P < 0.001$). However, when considering different histories separately, we found that SBW25 phenotypic richness was unaffected by SBW25 abundance when competitors were introduced prior to SBW25 (Fig. 3.3a; $R^2 = 0.042$, $P = 0.359$), but was positively correlated with SBW25 abundance when competitors were introduced either simultaneously with (Fig. 3.3c; $R^2 = 0.408$, $P < 0.001$) or after SBW25 (Fig. 3.3e; $R^2 = 0.150$, $P = 0.002$).

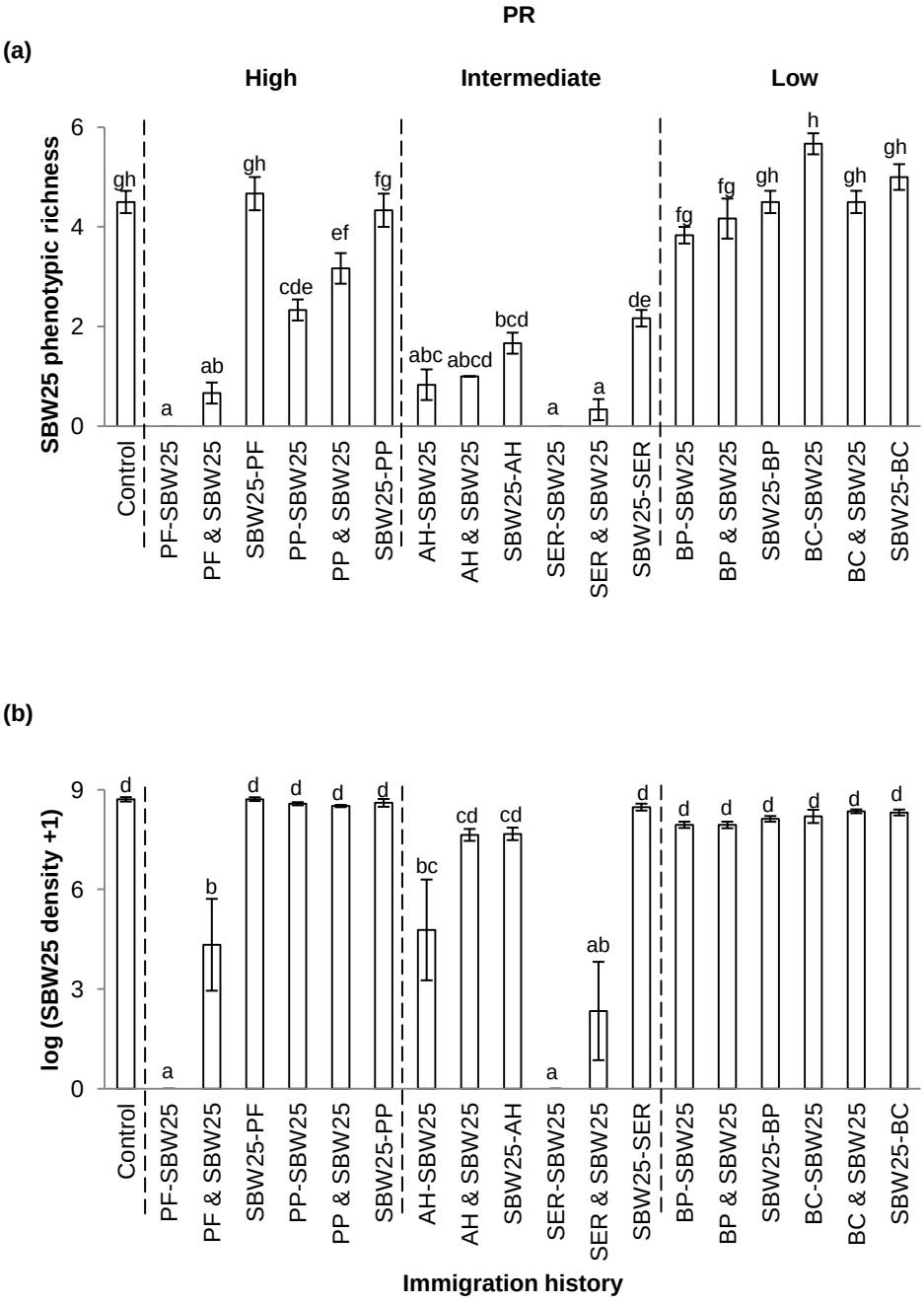

Fig. 3.2 The effect of different competitors and colonization histories on the phenotypic richness (a) and abundance (b) of *Pseudomonas fluorescens* SBW25. Bars indicate the phenotypic richness or abundance of SBW25 in monocultures (control) and polycultures (with competitors present). Values are means ± SE. Treatments sharing the same letter are not different from each other in Tukey's HSD tests.

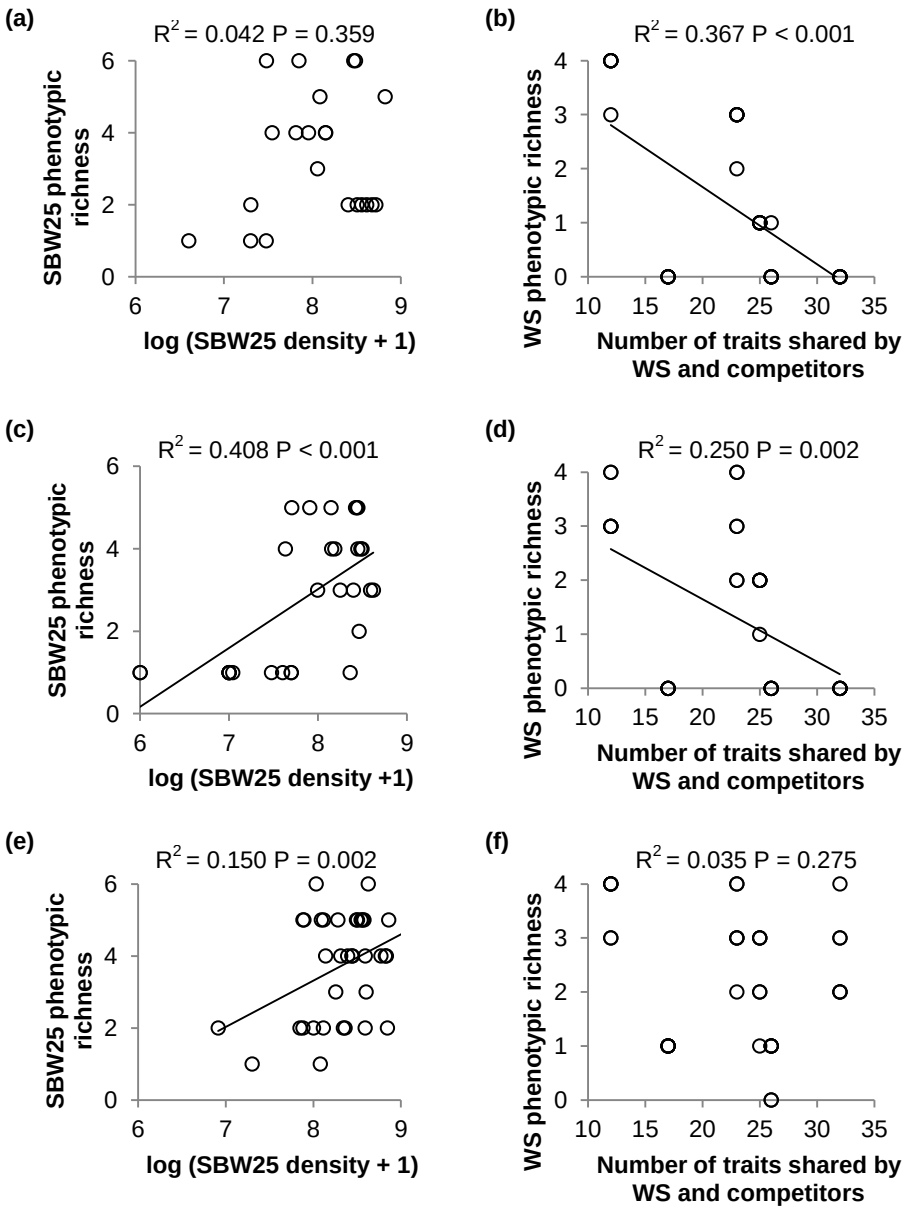

Fig. 3.3 Relationships between SBW25 population density and phenotypic richness (left column), and between WS-competitor trait similarity (the number of traits shared by WS and competitors) and WS phenotypic richness (right column), under different immigration histories (a, b, competitors-SBW25; c, d, SBW25 & competitors; e, f, SBW25-competitors). Data are plotted with linear regression lines. Note that population density data were recorded as colony forming units (CFU) per mL and $\log_{10}(x+1)$-transformed.

When data from all the treatments were considered together, we also found that the richness of WS phenotypes, which constituted most of the SBW25 diversity, was negatively correlated with niche similarity between WS and competitors ($R^2 = 0.182$, $P < 0.001$). However, when considering different histories separately, we found a negative relationship between WS genotypic richness and niche similarity when competitors were introduced before (Fig. 3.3b, $R^2 = 0.367$, $P < 0.001$) or simultaneously with SBW25 (Fig. 3.3d, $R^2 = 0.250$, $P = 0.002$), but not when competitors were introduced after SBW25 (Fig. 3.3f, $R^2 = 0.035$, $P = 0.275$).

There was a significant negative relationship between SM-competitor PR and the similarity in their carbon usage (Fig. 3.4; $R^2 = 0.741$, $P = 0.028$), indicating that competitors that were more closely related to SBW25 also shared more similar resource niches with it.

Discussion

Competition is widely considered as the key driver of adaptive radiation, but the direction of its effect is unclear. A common perception among evolutionary biologists is that intraspecific competition tends to promote divergent selection and, hence, diversification in the presence of ecological niches, and that interspecific competition tends to inhibit diversification by reducing population size of the focal lineage and niche availability (Roughgarden 1972, Doebeli and Dieckmann 2000, Schluter 2000). Experimental tests of the role of competition for adaptive radiation, however, are relatively few. Using the same SBW25 strain as the model organism, Brockhurst et al. (2007) and Bailey et al. (2013) found that the presence of intraspecific competitors reduced, rather than, increased the extent of adaptive radiation. Their results indicate that reduced niche opportunities and/or population size, often thought to be associated with interspecific competition, can also result in reduced diversification under intraspecific

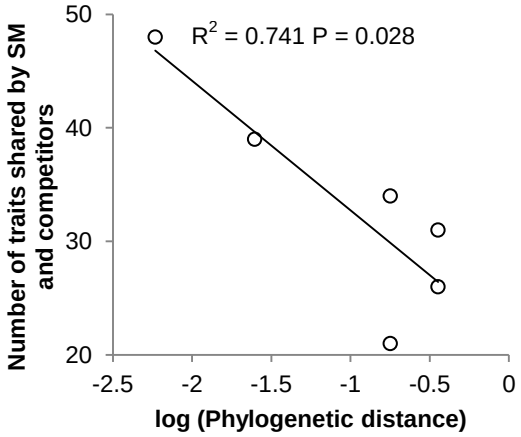

Fig. 3.4 The relationship between SBW25-competitor phylogenetic distance (the inverse of PR) and functional similarity (number of traits shared by SM and competitors). Data are plotted with the linear regression line. Note that phylogenetic distance was $\log_{10}$-transformed.

competition. Indeed, both Brockhurst et al. (2007) and Bailey et al. (2013) have considered different phenotypes of SBW25 as different species in their experiments. Moreover, Gomez and Buckling (2013) reported that competition from natural soil microbial communities impeded adaptive radiation of *Pseudomonas fluorescens* SBW25 via reducing niche availability. Together, these studies suggest that the same mechanisms (i.e., niche occupation and/or population reduction), irrespective of the nature of competition (i.e., intraspecific or interspecific), may operate to influence adaptive radiation.

In our experiment, we have adopted a comprehensive approach by considering a gradient of PR between the competitors and the focal diversifying bacterium, which in fact shared the same species identity with its most closely related competitor. Consistent with the aforementioned work (Brockhurst et al. 2007, Bailey et al. 2013, Gomez and Buckling 2013), our results show that competition tends to work in the direction of reducing the extent of adaptive radiation. Note that in our experiment the presence of a congener competitor, *P. putida*, reduced the diversification of SBW25 when the two colonized microcosms simultaneously. Zhang et al. (2012), however, found no effect of *P. putida* on the extent of SBW25 diversification in the same treatment of their experiment. It is unknown what caused this discrepancy between the two experiments. One possibility is that the two *P.* strains, which came from different sources, may be somewhat different, causing differences in their interactions with other species. Importantly, our study produced two notable findings that have not been previously reported. First, the effect of competition on adaptive radiation strongly depends on the evolutionary relationship between the competitor and the diversifying species and their colonization history, such that competition from a particular species may not necessarily reduce diversification. Second, the mechanisms governing the extent of adaptive radiation strongly depend upon the history of species colonization, such that either niche

occupation or population reduction or both may be important in different community assembly scenarios. We discuss these findings in more detail below.

We had hypothesized that both the PR of the radiating lineage and its competitors and their colonization history would influence adaptive radiation. While supporting this hypothesis, our results also indicated that the effects of PR and colonization history were not independent. Competitors that were closely related to the radiating lineage tended to reduce diversification as hypothesized, but not when they were late colonizers. On the other hand, the earlier arrival of the competitors relative to the radiating lineage tended to have a negative effect on the extent of diversification, but not when they were distantly related, providing only partial support for the first arrival hypothesis. One explanation for this pattern is that more closely related species may share more similar niches and thus compete more strongly (Darwin 1859), translating into stronger inhibitive priority effects on population abundance during community assembly (Tan et al. 2012). Consistent with this idea, we found that the diversifying species in our experiment shared more similar carbon usage patterns with its more closely related competitors (Fig. 3.4). Correspondingly, we also found that the earlier arrival of competitors strongly reduced the abundance of the diversifying species in the high and intermediate treatments. In particular, the priority effect on abundance was strongest when the competitor exhibited the highest PR with the diversifying species (the two were the same species), with the early colonizer completely preventing the establishment of later species (Fig. 3.2). This contrasted with the complete lack of priority effects in the two low PR communities, where the presence of competitor had little effect on SBW25 abundance (Fig. 3.2).

The rough correspondence between SBW25 abundance and diversification patterns suggests that competition influenced adaptive radiation through reducing population size of the diversifying species. Small population size tends to discourage adaptive radiation for several reasons. First, small population size means reduced

intraspecific competition, translating into weak disruptive selection. Second, even given constant per capita mutation rates, small ancestral populations would produce fewer mutants than large ones. Third, cooperation that may create novel niches is less likely to occur in smaller populations. In our experiment, biofilm formation that requires cooperation may be reduced in small *P. fluorescens* populations, with a negative effect on the emergence of new WS phenotypes (Bantinaki et al. 2007, Brockhurst et al. 2007). It should be noted, however, that population size alone only explained 23.8% of the variation in SBW25 diversification. The lack of population size effect is most dramatic in communities with *P. putida* as the competitor, where its earlier arrival resulted in the reduction in SBW25 phenotypic richness, but not abundance. This result suggests that competitors, when colonizing earlier, could impede adaptive radiation through other mechanisms such as niche preemption, without significantly diminishing population size of the radiating lineage.

While both population size and niche availability have the potential to influence adaptive radiation, mechanisms regulating their relative importance remain largely unknown (Grant and Grant 2008). Our results indicate that the history of species colonization can strongly influence the role of population size and niche availability in regulating adaptive radiation. In situations where the radiating lineage colonizes a heterogeneous habitat before its competitors, one may expect the size of its population to be of paramount importance as niches would be readily available. This was the case in the SBW25 earlier colonizing treatment of our experiment, where the only significant predictor of SBW25 phenotypic richness was its abundance. On the other hand, in situations where competitors colonize a heterogeneous habitat first, one may expect them to fill some if not all possible niches, making it difficult for a later arriving lineage to split into new forms and diversify. Here the lack of niche opportunities could severely constrain adaptive radiation, even if the population of the radiating lineage is sufficiently large. Accordingly, we found that, when SBW25 arriving later, its niche similarity with

competitors, but not population size, was a significant predictor of its diversification in our experiment. This result echoes those of a number of other experiments demonstrating niche preemption as an important mechanism for competition to reduce adaptive radiation (Brockhurst et al. 2007, Fukami et al. 2007, Bailey et al. 2013, Gomez and Buckling 2013), and is consistent with the fact that well-known examples of adaptive radiations mainly come from habitats harboring few competitor species (e.g., islands) (Losos et al. 1998, Gillespie 2004, Grant and Grant 2008). In the third scenario where the radiating lineage and competitors colonize habitats simultaneously, niche preemption by competitors should be less important given that there is no time advantage for competitors to fill the niches. In this treatment of our experiment, both population size and niche availability accounted for a significant portion of the variation in diversification, suggesting the co-limitation of adaptive radiation by these two factors. Overall, given that lineages rarely colonize environments in which competitors are completely absent, reduced level of diversification associated with niche occupation by competitors may be a common phenomenon.

Our study demonstrates that competition tends to have a negative effect on adaptive radiation, but that the PR between the radiating lineage and competitors and their immigration history interactively regulate the effect of competition on adaptive radiation. We further demonstrate that the mechanisms associated with competitive effects on adaptive radiation vary with species immigration history. These results suggest that making predictions about adaptive radiation in the presence of competitors would be difficult, if not impossible, without exact knowledge on species traits and colonization history. In our experiment, PR was used as an effective surrogate of species niche similarity, which regulates the strength and outcome of competition (Darwin 1859, Chesson 2000). However, when species niches do not show significant phylogenetic signals, which appear to be not uncommon (Webb et al. 2002, Edwards and Donoghue 2006), the predictive power of PR would be compromised. Further, competition strength

and outcome may also be influenced by species fitness differences (Chesson 2000), which also may not be always phylogenetically conserved, adding more complexity to the issue. A thorough understanding of how competition influences adaptive radiation would require identifying species traits important for competition and elucidating how these traits vary across species. While this goal may not be readily achieved in the near future, our study highlights the context dependency of both the role of competition on adaptive radiation and its underlying mechanisms.

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Chapter 4

TEMPORAL NICHE PROMOTES BIODIVERSITY DURING ADAPTIVE RADIATION

Abstract

Understanding mechanisms underlying the origin and maintenance of biodiversity is a central goal of modern ecological and evolutionary research. Ecologists have recognized the potentially important role of temporal niche in promoting species coexistence and diversity, yet little is known about how temporal niche affects the evolution of biodiversity. Here we show that temporal niche strongly influences biodiversity dynamics in rapidly evolving bacteria. An ancestral bacterium quickly diversifies when provided with constant spatial niche opportunities or when experiencing temporal niche dynamics. However, only in communities with temporal niches, which promote frequency-dependent selection and the positive growth of new mutants, is the accumulated phenotypic diversity able to persist. Overall, the presence of temporal niche opportunities eliminates the overshooting dynamics of adaptive radiation typically seen in this and other systems. These results suggest that temporal niche may play an essential role in the maintenance of biodiversity over evolutionary time.

Introduction

For decades, biologists have sought to understand mechanisms underlying high biodiversity observed in many of the Earth's ecosystems (Hutchinson 1961, MacArthur 1972, Tilman 2000). The theory of temporal niche dynamics (TND) suggests that temporal variation in niche availability allows the storage effect to operate, buffering species against extinction (Chesson 2000, Kelly and Bowler 2002). Increased species coexistence in fluctuating environments has been documented for both natural (Caceres 1997, Adler et al. 2006, Angert et al. 2009) and experimental (Descamps-Julien and Gonzalez 2005, Jiang and Morin 2007) communities, and has, in a few cases, been explicitly linked to TND (Kelly and Bowler 2005, Adler et al. 2006). In parallel, diversification under fluctuating selection has been of much interest for evolutionary biologists (Lynch 1987, Hairston and Dillon 1990, Bell 2010). However, while TND has received some theoretical attention in this context (Ellner and Hairston 1994, Abrams et al. 2013), little empirical knowledge exists on how it affects biodiversity dynamics in systems where ongoing evolution contributes to biodiversity (Venail et al. 2011). We hypothesize that because TND modulates ecological interactions that often provide the selective force for evolution, it may affect the emergence and maintenance of biodiversity over evolutionary timescales.

Testing this idea is difficult in many systems, given the generally long period of biodiversity evolution and inadequate knowledge on the niches of evolved lineages. Such tests, however, are feasible using microbial lineages undergoing rapid adaptive radiation, which can give rise to new phenotypes/species adapted to different niches in a short period of time. We investigated biodiversity dynamics in the rapidly diversifying *Pseudomonas fluorescens* SBW25 populations (Rainey and Travisano 1998, Kassen et al. 2000). Previous research has shown that a suite of environmental factors, such as disturbance (Buckling et al. 2000, Massin and Gonzalez 2006) and productivity (Kassen

et al. 2000), affect the diversification of this bacterium. A key advantage of this experimental system is that rapid adaptive radiation produces ecotypes with different niche preferences (Rainey and Travisano 1998, Kassen et al. 2000), which allowed us to directly manipulate the temporal availability of these niches and link coexistence with TND, an approach that has not been taken previously. When introduced into static microcosms, the ancestral *P. fluorescens* ecotype—smooth morph (SM) that occupies the broth phase—diversifies and generates two niche-specialists: the wrinkled spreader (WS) ecotype that colonizes the air-broth interface, and the fuzzy spreader (FS) ecotype that inhabits the bottom of microcosms. Competition for oxygen, whose concentration decreases towards the bottom of static microcosms, is thought to be an important factor in driving this niche differentiation (Rainey and Travisano 1998). Additional variations also exist within each ecotype. Within WS, for example, small-WS, large-WS, wheel-WS and SM-like-WS subtypes may also emerge(Fukami et al. 2007), driven likely by adaptation to micro-niches (Meyer et al. 2011). These *P. fluorescens* phenotypes are genetically determined and can be readily distinguished on agar plates (Rainey and Travisano 1998, Fukami et al. 2007). Each phenotype may be considered as analogous to a biological species since *P. fluorescens* reproduces asexually with a low recombination rate (Rainey and Travisano 1998, Fukami et al. 2007). The spatially structured niches provided by the static incubation are favorable for WS and FS, and crucial for SM diversification. Shaking of microcosms eliminates spatial niches (e.g., the oxygen gradient), making it difficult for SM to diversify. Therefore, temporal shifting between static and shaking conditions provides temporal niche opportunities for *P. fluorescens* communities (Rainey and Travisano 1998).

We examined biodiversity dynamics in a laboratory experiment in which evolving *P. fluorescens* populations were incubated with or without temporal niche (see Methods). We show that the availability of temporal niche is critical for the maintenance of the evolved *P. fluorescens* phenotypic diversity, via the mechanism of promoting negative

frequency-dependent selection. These results suggest that TND have the potential to strongly influence biodiversity dynamics over evolutionary time.

Results

Temporal niches and phenotypic diversity

As in previous studies (Fukami et al. 2007, Meyer et al. 2011), new phenotypes, including small-WS, large-WS, wheel-WS, SM-like-WS, and FS, quickly emerged in static microcosms (all phenotypes detected by day 4; Fig. 4.1a, 4.2a). This rapid increase in phenotypic richness, however, was followed by a slower decline, as some of the emerged phenotypes were later competitively excluded (Meyer et al. 2011) (Fig. 4.1a). Such overshooting dynamics are predicted by theory , and have been previously reported for this experimental system (Fukami et al. 2007, Meyer et al. 2011) as well as adaptive radiations in nature (Gillespie 2004, Adler et al. 2006, Seehausen 2006). By contrast, diversification in microcosms experiencing constant shaking was much slower, presumably due to the lack of spatial niches for new phenotypes (Rainey and Travisano 1998), resulting in the slow accumulation of phenotypes over the duration of the experiment (Fig. 4.1a, 4.2b). The presence of TND, however, led to different biodiversity dynamics. Although rapid diversification also occurred in TND microcosms, most of the derived phenotypes, including FS and multiple WS phenotypes, persisted afterwards in these microcosms (Fig. 4.2c, d). Rather than exhibiting overshooting dynamics, phenotypic richness here approached an asymptote, albeit in an oscillatory fashion, during the second half of the experiment (Fig. 4.1a). As a result, final

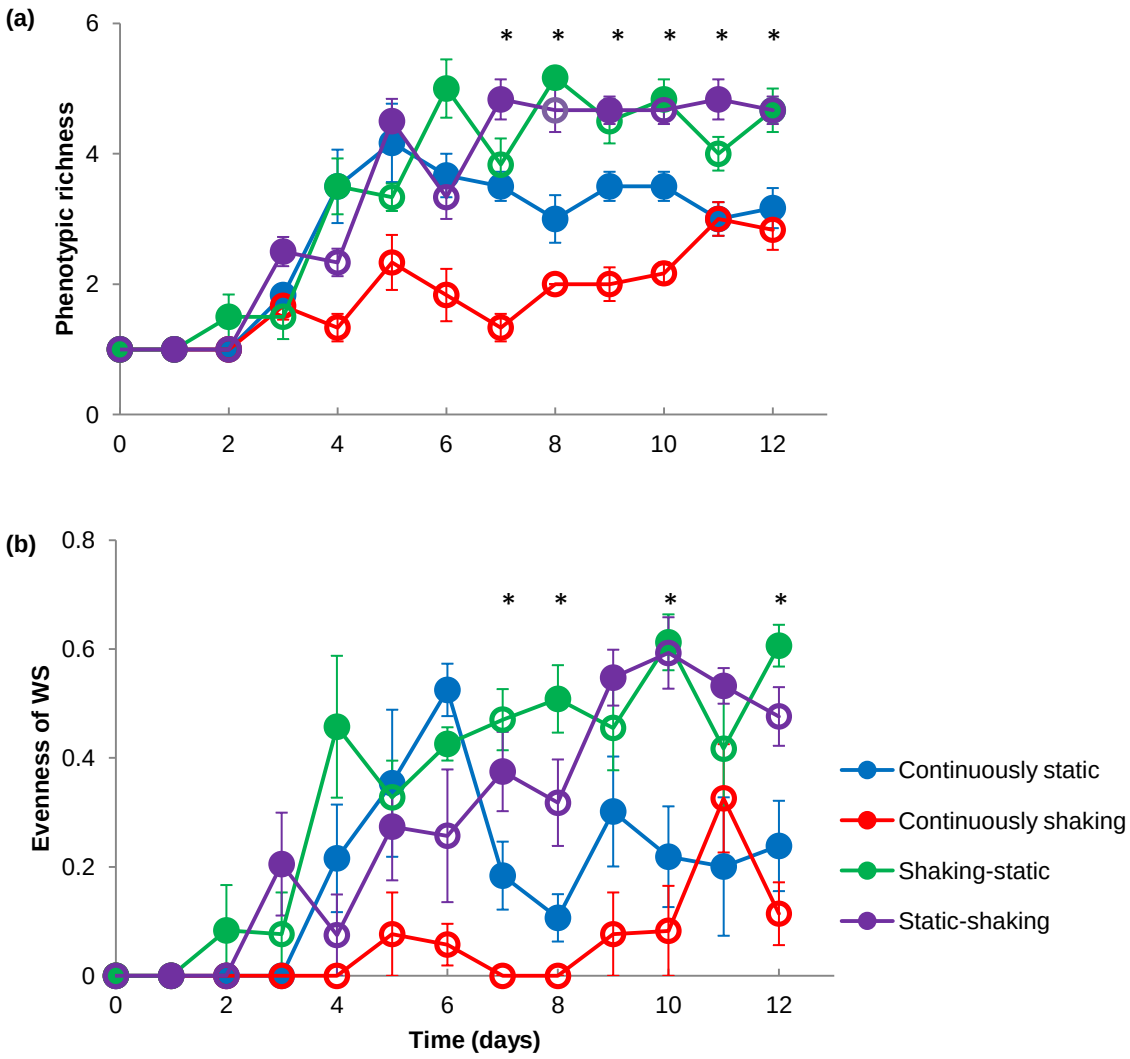

Fig. 4.1 Biodiversity dynamics in the temporal niche experiment. (a) phenotypic richness of *Pseudomonas fluorescens*; (b) evenness of WS phenotypes. Solid and open circles indicate that microcosms were incubated under the static and shaking condition on the day before sampling, respectively. Values are mean ± SE (n = 6). Asterisks indicate that the values in at least one of the two temporal niche dynamics treatments (shaking-static and static-shaking) are significantly greater than the values in the continuously static treatment in a Tukey's HSD test ($P < 0.05$). The effect sizes (η^2) of ANOVA of phenotypic richness and WS evenness on day 12 are 0.62 and 0.53, respectively.

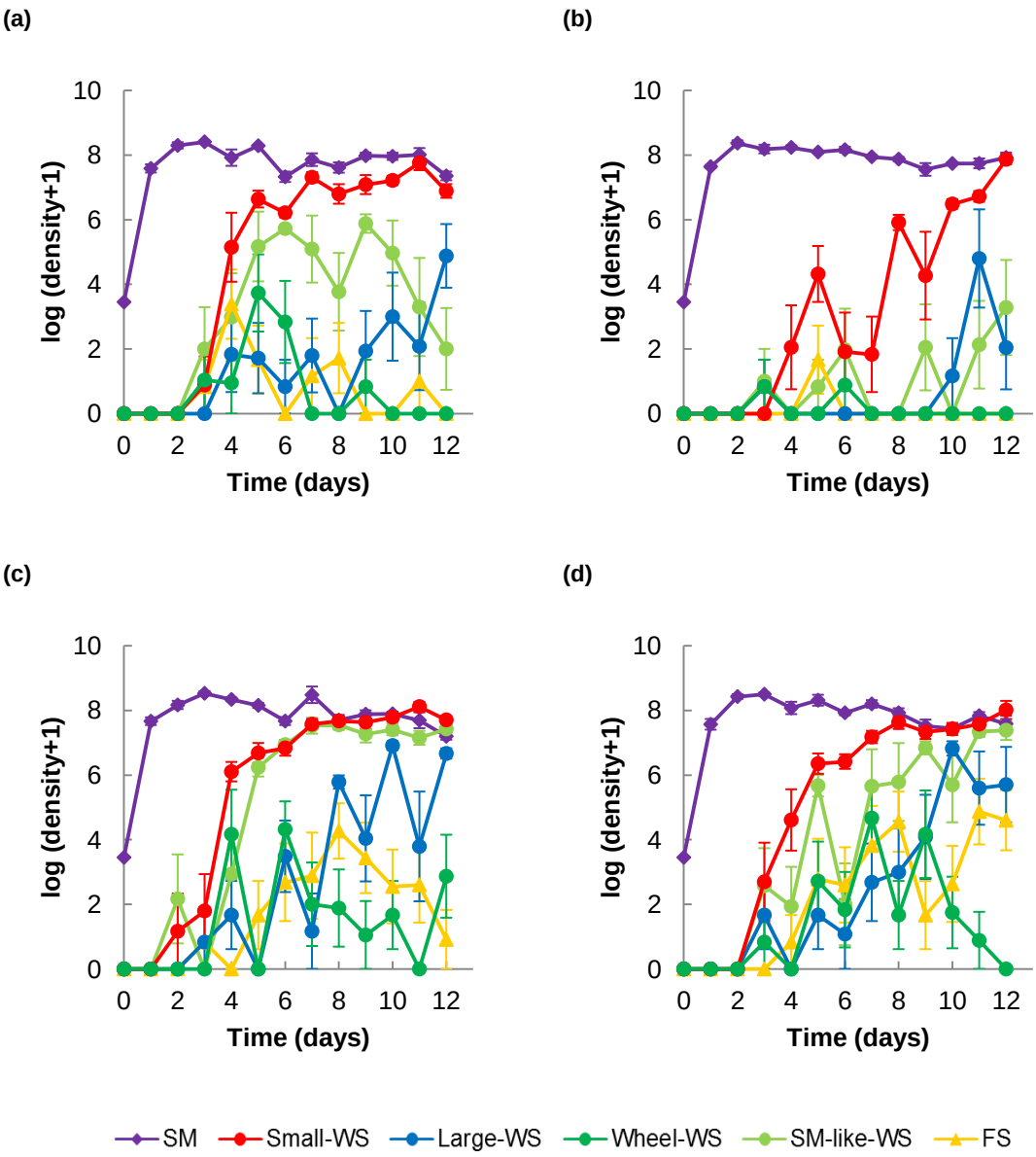

Fig. 4.2 Population dynamics of each phenotype in the four experimental treatments. (a) continuously static; (b) continuously shaking; (c) shaking-static; (d) static-shaking. Population density data (CFU/ml) were $\log_{10}$ (x+1)-transformed. Values are mean ± SE (n = 6).

phenotypic richness was significantly greater with than without temporal niche structure (Fig. 4.1a). The two TND treatments (shaking-static and static-shaking) did not differ in final phenotypic richness.

We further examined diversity patterns within the group of WS phenotypes, which made up the majority of phenotypic diversity in the population (Fig. 4.2). The dynamics of WS phenotype evenness, calculated as Pielou's *J'* (Pielou 1966), largely mirrored those of overall phenotypic richness (Fig. 4.1b). WS evenness in static microcosms exhibited overshooting dynamics, with the later decline in evenness associated with the extinction or reduced abundance of several WS phenotypes. This can be explained by within-niche competition, where small-WS attained numerical dominance within this group of niche specialists (Meyer et al. 2011) (Fig. 4.2a). In contrast, in TND microcosms, as several WS phenotypes attained appreciable abundance while coexisting with small-WS, overshooting in evenness was not observed (Fig. 4.2c, d).

WS fitness and frequency dependent selection

Our experimental results indicate that TND promoted biodiversity mainly by allowing more phenotypes sharing similar niches (i.e., WS phenotypes) to coexist. Both static and shaking conditions appeared to be important: while the availability of spatial niches under static incubation allowed the emergence of different WS phenotypes, shaking apparently prevented the loss of some of the WS phenotypes that otherwise would be driven to extinction by small-WS. This increased coexistence under temporal niche dynamics may be caused by frequency dependent selection (Chesson 2000, Kelly and Bowler 2002), which is known to operate among some *P. fluorescens* phenotypes (Rainey and Travisano 1998, Brockhurst et al. 2006, Zhang et al. 2009).

To explore the possibility that frequency dependent selection operates among WS subtypes, we conducted a second experiment with four wild-type WS phenotypes and one lacZ-marked SM phenotype, allowing each wild-type WS phenotype to be initially more abundant than others (see Methods). The lacZ-marked SM gives rise to WS mutants whose colonies exhibit a distinct blue color on agar plates with X-gal, which can be used to distinguish them from wild type WS colonies (Fukami et al. 2007). We estimated the fitness of the initially dominant WS phenotypes relative to other WS phenotypes after two-day incubations under either shaking or static conditions (according to Equation 1 in the Methods). We found that while the initially dominant WS phenotypes attained greater dominance in static microcosms, their fitness was significantly reduced in shaken microcosms, such that the relative fitness for none of the four phenotypes was positive (Fig. 4.3a, 4.4). In addition, only in shaken microcosms was the fitness of the initially dominant WS phenotypes negatively correlated with their initial frequency (Fig. 4.3b). These signatures of frequency dependent selection suggest that shaking provided fitness advantage for rare WS phenotypes relative to common ones. Note that the absolute fitness of each WS phenotype was still smaller under shaking relative to static conditions, presumably because shaking eliminated their preferred niche. Moreover, only under shaken incubation was *in situ* mutation from lacZ-marked SM phenotype to WS detected (Fig. 4.5), indicating that shaking favored new WS mutants. Together, these results suggest that shaking, while eliminating the niche of WS phenotypes, promoted their coexistence in the TND microcosms.

Discussion

Using a model organism undergoing rapid adaptive radiation, we examined the hypothesis that TND affects the evolution of biodiversity. Although we did not find

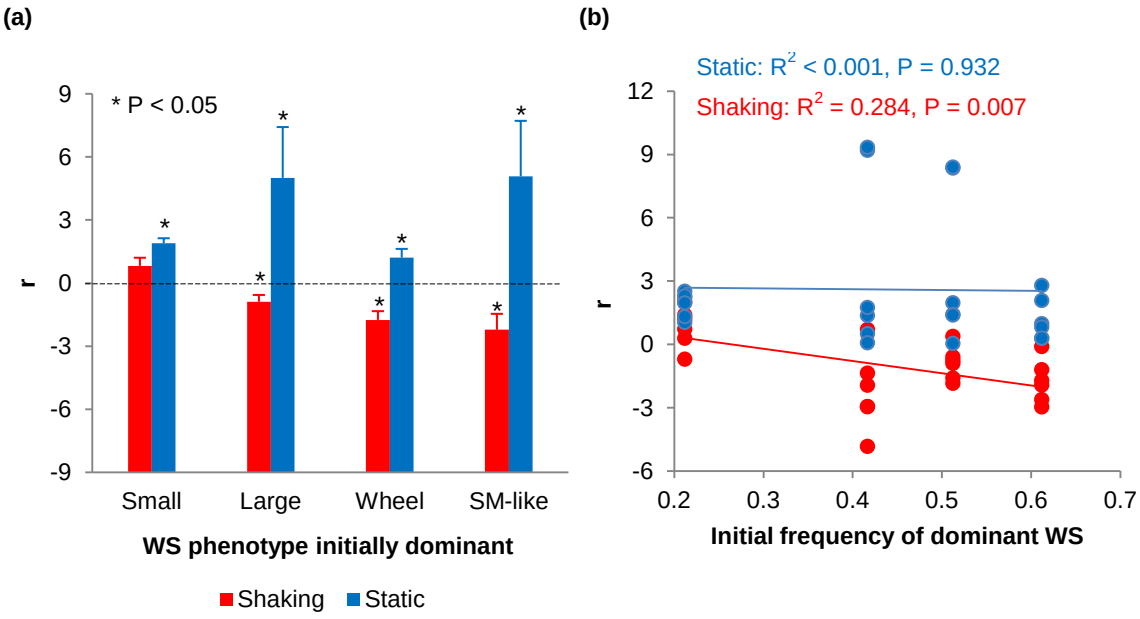

Fig. 4.3 Fitness of the dominant WS phenotype in the shaken and static microcosms. Fitness is calculated as the difference in the Malthusian parameter (*r*) between the initially dominant and other WS phenotypes according to Equation 1. (a) Fitness of each genotype in the shaking and static microcosms. Values are mean + SE (n = 6). Asterisks indicate treatments where the values are different from zero according to one sample t-test ($P < 0.05$). Note that the fitness of small-WS under shaking was not significantly different from zero, whereas fitness in other treatments all differed from zero. (b) The relationship between the frequency of initially dominant WS phenotype and its fitness under shaking and static conditions. The linear regressions are shown with data.

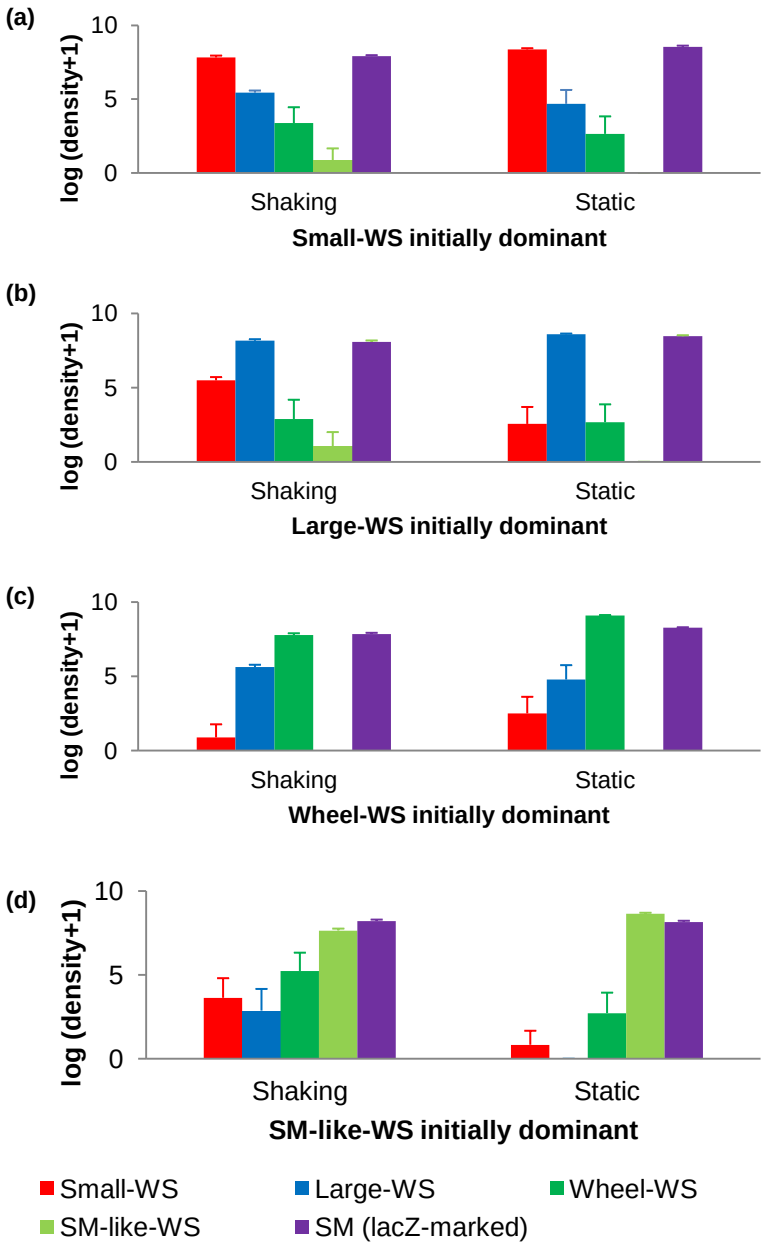

Fig. 4.4 Population density of each phenotype in the WS fitness experiment. (a) small-WS initially dominant; (b) large-WS initially dominant; (c) wheel-WS initially dominant; (d) SM-like-WS initially dominant. Population density data (CFU/ml) were $\log_{10}(x+1)$-transformed. Values are mean + SE (n = 6).

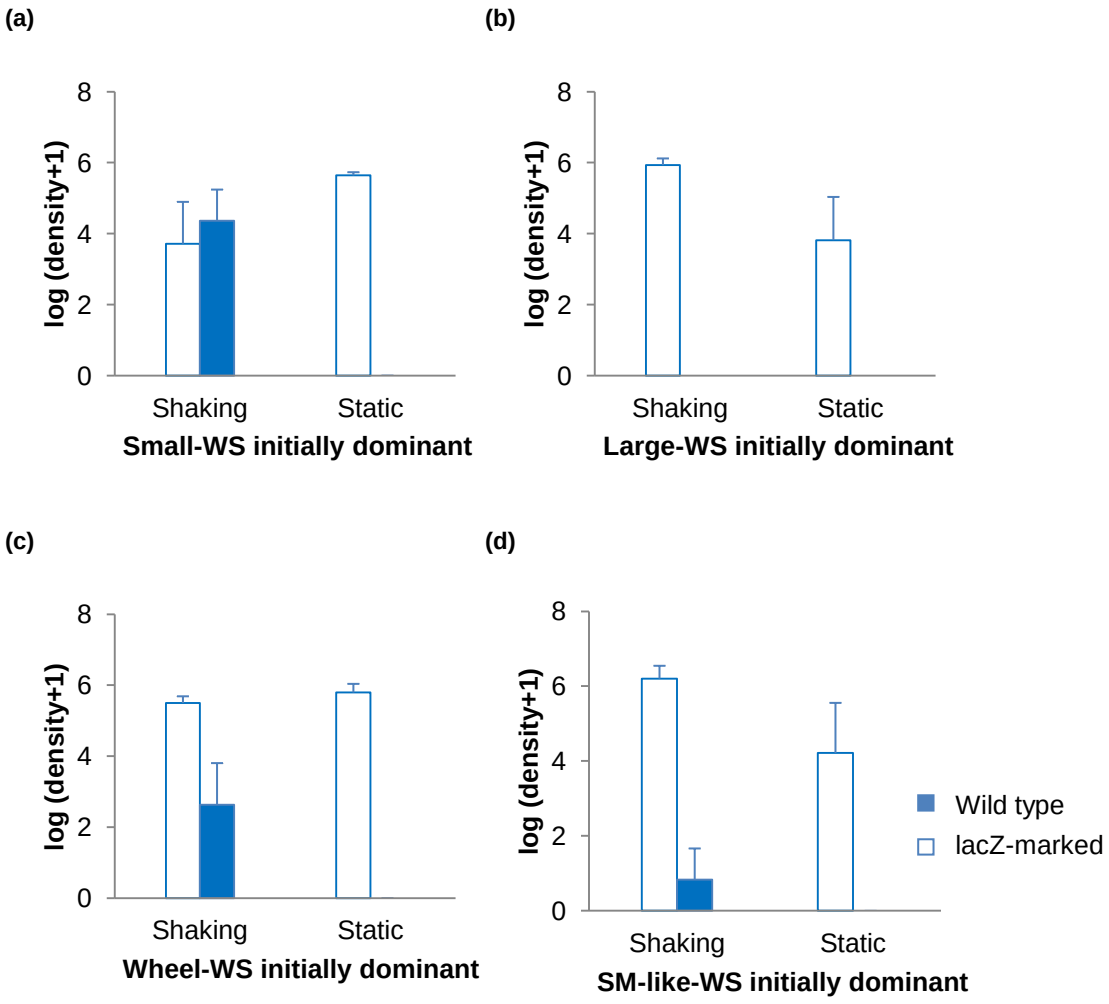

Fig. 4.5 The density of nondominant WS phenotypes of wild and lacZ-marked types in the shaken and static microcosms. Data (CFU/ml) were $\log_{10}(x+1)$-transformed. Values are mean + SE (n = 6).

evidence of TND altering the emergence of diversity, our experiments clearly show that it functioned to maintain evolved diversity and stabilize its dynamics. To our knowledge, this is the first experimental demonstration of TND promoting biodiversity over evolutionary time.

One notable characteristic of TND in our experiment is that static conditions facilitated the generation of diversity by offering spatial niches, whereas shaking prevented the loss of evolved diversity by promoting negative frequency dependent selection between the WS phenotypes. Previous studies have revealed that negative frequency-dependent selection also operated between SM and WS phenotypes under static conditions, facilitating their coexistence (Rainey and Travisano 1998, Zhang et al. 2009, Meyer et al. 2011). This scenario differs from the more commonly considered situations where the TND effect operates as the result of different species being favored by different niches associated with different environmental conditions. Nevertheless, both static and shaking conditions were essential for the persistence of evolving diversity, in a similar manner as alternations in environmental conditions that favor different species were essential for species coexistence over ecological time. The negative frequency dependent selection among the WS phenotypes under shaken conditions warrants some explanation. Frequency dependent selection arose probably because subordinate WS genotypes faced strong competition in their niche (air-broth interface) housing concentrated small-WS populations under static conditions, but experienced weakened competition from diffused small-WS populations under shaking conditions. In addition, for essentially the same reason, shaking may have facilitated the coexistence of WS genotypes via increasing the fitness of newly evolved nondominant WS individuals. In the context of the storage effect (Chesson 2000), the first scenario would generate covariance between competition and environment, and the second scenario would contribute to buffered population growth (i.e., the supply of new mutants buffering

populations against extinction) in addition to that afforded by overlapping generations (Warner and Chesson 1985, Ellner and Hairston 1994).

Several features of our experimental regime are worth noting. First, alternation between static and shaking conditions in our experiment not only created temporal niche opportunities, but also altered the average environmental conditions. Since it is impossible to create a constant environment equivalent to the average of static and shaking conditions, the possibility that increased diversity may be partly driven by changes in average conditions cannot be completely excluded. The demonstrated role of static conditions for diversity generation and of shaking conditions for diversity maintenance, however, point to the importance of TND. Second, our TND microcosms were shifted between static and shaking conditions every 24 h, which allowed frequency dependent selection to exert its force under shaking conditions while mitigating competitive exclusion of nondominant WS populations under static conditions. In a related experiment, Massin and Gonzalez (2006) studied the effects of short-term (2 minute duration) pulse disturbance, in the form of shaking the otherwise static microcosms, on *P. fluorescens* diversification. Contrasting with our results, their experiment showed that periodic disturbance slowed diversification, presumably because the short-term shaking eliminated spatial niches without incurring frequency dependence. Third, daily dilution was used to propagate *P. fluorescens* populations in our experiment. Buckling et al. (2000) reported that the frequency of dilution affected *P. fluorescens* phenotypic diversity, such that diversity exhibited a unimodal relationship with dilution frequency in static microcosms. Obviously, experiments with different dilution frequencies are needed to further test the robustness of our results. Note that daily dilution in our experiment (see Methods) may have also resulted in different diversity dynamics than previously reported for *P. fluorescens* batch cultures (Rainey and Travisano 1998).

Our results demonstrate that TND can strongly influence evolutionary dynamics of biodiversity. Given the prevalence of environmental fluctuations that offer temporal niche opportunities at various timescales in nature (Gavrilets and Losos 2009, Reddy et al. 2012), this result has important implications for understanding diversification patterns in many natural systems. For example, whereas overshooting dynamics are frequently encountered when studying adaptive radiation in nature (Gillespie 2004, Seehausen 2006), many lineages have diversified without showing an apparent decline in diversity (Baldwin and Sanderson 1998, Reddy et al. 2012). Although alternative hypotheses exist (Fukami et al. 2007, Gavrilets and Losos 2009), our results suggest that TND may potentially explain the maintenance of the accumulated biodiversity over evolutionary time.

Methods

Bacterial cultivation

We cultivated *P. fluorescens* SBW25 (wild type and lacZ-marked) in 25 ml loosely capped test tubes containing 6 ml King's Medium B (KB) on a shaker (250 rpm) at 28℃. After 48 h, we plated the cultures on KB agar plates and incubated the plates at 28℃ for another 48 h. One wild type SM colony was isolated for use in the experiments; one lacZ-marked SM colony was isolated and cultivated in KB overnight and stored in 15% glycerol at -80℃. Prior to setting up the SM stock culture, we thawed the *P. fluorescens* culture previously frozen at -80℃, and plated it on agar. We then isolated one two-day-old SM colony on the agar plate, and introduced it into a test tube with 6 ml KB. After incubating the culture for 2 h, we introduced 10 μl of this culture into each experimental microcosm.

Experimental protocols

Microcosms were 25 ml capped test tubes containing 6 ml KB. The initial SM density in each microcosm was ~10^3 colony-forming units (CFU)/ml. Microcosms without temporal niche were incubated under continuously static or shaking conditions. Microcosms with temporal niche were alternated between shaking and static conditions every 24 h, a period that permits *P. fluorescens* phenotypes to grow and interact for multiple (10-12) generations. Two different regimes of temporal niche were used: one under shaking incubation on odd days and static incubation on even days (shaking-static), and the other with the reverse sequence (static-shaking). The two regimes allowed us to discern if initial environmental conditions (static or shaking) matter for biodiversity evolution. We propagated *P. fluorescens* populations by transferring 1% of the content of each microcosm into a fresh microcosm daily, and quantified the abundance of each phenotype afterwards. Under the static condition, microcosms were kept at 28℃ without shaking; under the shaking condition, microcosms were placed on a shaker (250 rpm) at 28℃. Each treatment was replicated six times. The experiment ended after 12 days.

Quantifying phenotypic density and diversity

The density of each phenotype was measured after the daily transfer. The sample from each microcosm was spread onto KB agar plates after serial dilutions. A total of six *P. fluorescens* phenotypes, SM, FS, small-WS, large-WS, wheel-WS and SM-like-WS, were identified and the number of colonies of each phenotype was recorded. Phenotypic richness was the number of phenotypes detected in the sample, and the evenness of WS phenotypes was calculated as Pielou's *J*'(Pielou 1966). We assessed the treatment effects on phenotypic richness and WS evenness using ANOVA, followed by Tukey's HSD tests.

WS fitness experiment

From the temporal niche experiment, we isolated four WS phenotypes derived from the wild-type SM and preserved them in 15% glycerol at -80℃. Prior to the experiment, we established separate stock cultures for the four WS phenotypes and lacZ-marked SM in microcosms with 6 ml KB and incubated them overnight. We initiated the experiment with highly uneven WS phenotype densities such that the ratio of the dominant WS, three nondominant WS and lacZ-marked SM was 1000:1:1:1:1000 (initial density: 10^6:10^3:10^3:10^3:10^6 CFU/ml). The experiment had four treatments each with a different WS phenotype being initially dominant. Each treatment was replicated 6 times. Microcosms were under either static or shaking incubation for 2 days at 28℃. Thereafter, we sampled each microcosm to quantify the final abundance of each phenotype. We calculated the relative fitness of the initially dominant WS using the ratio of Malthusian parameter (*r*), according to the following equation:

$$r = \ln \frac{\text{final frequency of dominant WS}}{\text{initial frequency of dominant WS}} - \ln \frac{\text{final total frequency of nondominant WS}}{\text{initial total frequency of nondominant WS}} \quad \text{Equation 1}$$

We conducted a two-tailed t-test to compare the difference in *r* between shaking and static incubations for each of the four experimental treatments.

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Appendix A

SUPPLEMENT TO CHAPTER 2

Table A.1. Species composition of the resident communities.

PD level		Phylogenetic distance between the invader and most closely related species		
		Low	Intermediate	High
Low	1	*Bacillus pumilus 1* *Bacillus pumilus 4* *Enterobacter cloacae 1*	*Deinococcus sp.* *Staphylococcus sp.* *Vogesella indigofera*	*Micrococcus luteus* *Staphylococcus haemolyticus 2* *Staphylococcus pasteuri*
	2	*Enterobacter cloacae 1* *Staphylococcus sp.* *Deinococcus sp.*	*Vogesella indigofera* *Bacillus pumilus 2* *Bacillus pumilus 4*	*Bacillus pumilus 3* *Bacillus subtilis 2* *Staphylococcus pasteuri*
	3			*Lysinibacillus sphaericus* *Staphylococcus pasteuri* *Bacillus pumilus 3*
Inter-mediate	1	*Acinetobacter baylyi 1* *Bacillus pumilus 4* *Enterobacter cloacae 2*	*Bacillus subtilis 1* *Pseudomonas mosselii* *Micrococcus luteus*	*Staphylococcus haemolyticus 2* *Bacillus pumilus 3* *Micrococcus luteus*
	2	*Enterobacter cloacae 2* *Vogesella indigofera* *Deinococcus sp.*	*Vogesella indigofera* *Pseudomonas mosselii* *Staphylococcus haemolyticus 2*	*Bacillus pumilus 3* *Pseudomonas resinovorans* *Staphylococcus haemolyticus 1*
	3			*Pseudomonas resinovorans* *Deinococcus sp.* *Bacillus subtilis 1*
High	1	*Enterobacter cloacae 2* *Rheinheimera texasensis* *Staphylococcus pasteuri*	*Bacillus subtilis 2* *Pseudomonas resinovorans* *Vogesella indigofera*	
	2	*Enterobacter cloacae 2* *Pseudomonas resinovorans* *Bacillus subtilis 1*	*Rheinheimera texasensis* *Vogesella indigofera* *Staphylococcus pasteuri*	

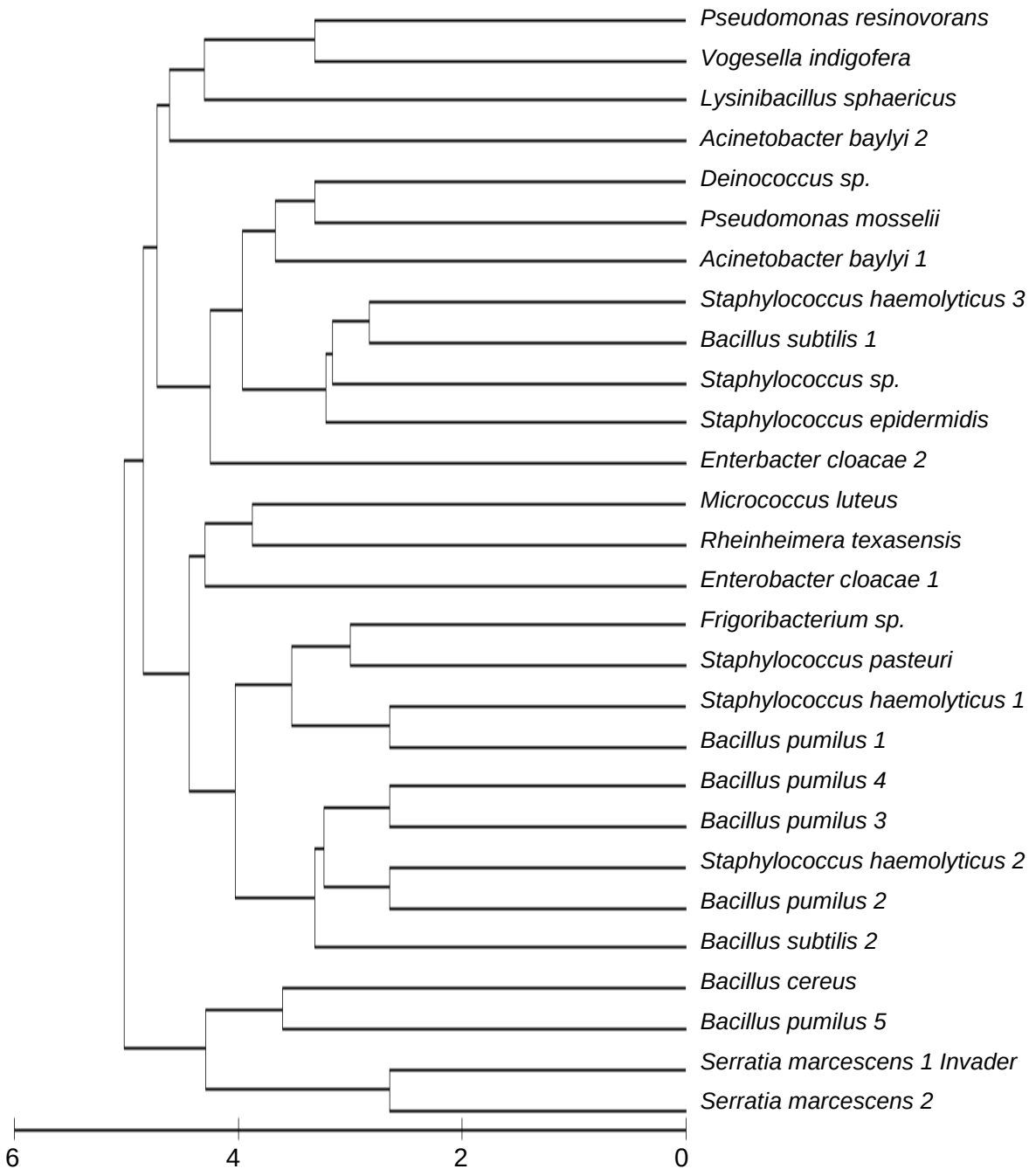

Fig. A.1 The bacterial functional dendrogram, constructed based on a UPGMA-based cluster analysis of the 55 functional traits. The scale for branch lengths is shown beneath the functional dendrogram.

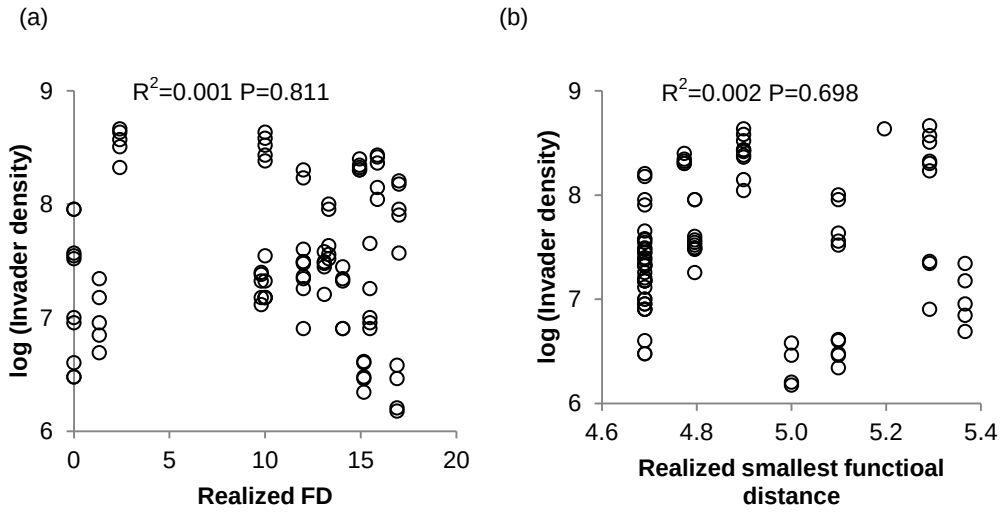

Fig. A.2 Relationships between invader population densities and (a) realized FD and (b) realized function distance (the inverse of FS) in communities containing three resident species.

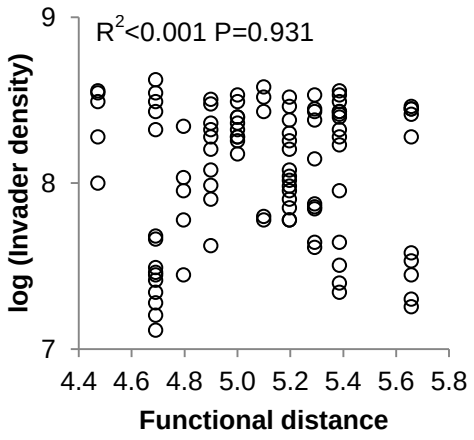

Fig. A.3 The relationship between invader-resident functional distance (the inverse of FS) and invader population density in monocultures.

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