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Eco-evolutionary interaction between microbiome presence and rapid biofilm evolution determines plant host fitness

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Microbiomes are important to the survival and reproduction of their hosts. Although ecological and evolutionary processes can happen simultaneously in microbiomes, little is known about how microbiome eco-evolutionary dynamics determine host fitness. Here we show, using experimental evolution, that fitness of the aquatic plant *Lemna minor* is modified by interactions between the microbiome and the evolution of one member, *Pseudomonas fluorescens*. Microbiome presence promotes *P. fluorescens'* rapid evolution to form biofilm, which reciprocally alters the microbiome's species composition. These eco-evolutionary dynamics modify the host's multigenerational fitness. The microbiome and non-evolving *P. fluorescens* together promote host fitness, whereas the microbiome with *P. fluorescens* that evolves biofilm reduces the beneficial impact on host fitness. Additional experiments suggest that the microbial effect on host fitness may occur through changes in microbiome production of auxin, a plant growth hormone. Our study, therefore, experimentally demonstrates the importance of the eco-evolutionary dynamics in microbiomes for host–microbiome interactions.

Ecological factors have long been recognized to have strong impacts on evolutionary processes. As evolutionary processes often occur over long periods of time, it has been thought that evolution may not influence short-term ecological processes[1]. This traditional view, however, has been challenged recently by studies that have shown that evolution can happen rapidly over a few generations and have strong feedback effects on ecological dynamics[2–5]. Such rapid interaction between ecological and evolutionary processes (that is, eco-evolutionary dynamics) has been considered important for adaptive radiation[6], species coexistence[7], invasion[8], pollination[9] and many other ecological processes[10]. Although ecological and evolutionary changes could interact in all ecosystems, strong eco-evolutionary dynamics are more likely to be detected in systems with short generation times and strong interspecific interactions[3].

Most organisms, including animals, plants and even microorganisms, host microbial communities as their microbiomes. Eco-evolutionary dynamics within microbiomes could be particularly important in determining the outcomes of host–microbiome interactions[11,12]. That is because microbiome species probably undergo rapid evolution in response to their host or other species in the microbiome, given their large population size and short generation time. Rapid evolution in microbial species may co-occur and interact with rapid ecological changes in species composition within the microbiome, driving eco-evolutionary dynamics in microbiomes with possible influences on hosts[13]. Evolution in microbiomes may directly influence host fitness or influence it indirectly through ecological interactions. In the direct-evolutionary-effect scenario, commensal microbiome species could evolve new mutualistic or pathogenic phenotypes, directly promoting or suppressing host fitness. In the indirect-ecological-effect scenario, a microbe's evolution does not change its impact on the host but changes its competitive ability relative to other microbes that are not evolving. This could alter microbiome community structure, affecting species (for example, mutualists and pathogens) that could directly alter host fitness. Although there is substantial research on the influences of evolution in individual microbial species, especially mutualistic or pathogenic microbes[14–16], community-level host–microbiome interaction studies have mostly focused on changes in microbial species composition, not their evolution[17–19]. Thus, the importance of rapid evolution for both microbiome species composition and host fitness remains unknown.

To robustly test the influences of microbial evolution on host–microbiome interactions, ideally three conditions need to be met: (1) a fast-growing host allowing multigenerational lifetime fitness estimation, (2) a method to effectively control microbiome species composition and (3) a method to stop the evolution of at least one microbial species in one relevant trait. One can thus quantify the ecological impacts of evolution on both the host and other microbiome species by comparing communities with the naturally evolving microbe with those with the non-evolving counterpart. The experimental plant–microbiome system of the duckweed *Lemna minor* and its epiphytic microbiome provides the conditions required to address the empirical gap[20]. First, *L. minor* is a fast-growing aquatic floating plant, reproducing clonally within four days[21]. The lifetime fitness of *L. minor* can therefore be measured across multiple generations quickly. Second, the natural microbiomes of *L. minor* can be eliminated with bleaching (details in Methods). Then, species composition in microbiomes can be manipulated by inoculating microbes of interest. Using this approach, previous studies have shown that certain bacterial species have strong influences on the growth of *L. minor*[22,23]. Third, using genetic knockouts, we can manipulate the evolution of ecologically relevant traits in common plant-associated bacteria. We focus on the biofilm evolution in *Pseudomonas fluorescens*, a common bacterial species in *L. minor* epiphyte. By knocking out the operons in the bacterium that control biofilm formation, we can stop the biofilm evolution in *P. fluorescens*.

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P. fluorescens strain SBW25 is a classic evolutionary diversification model system[24–26]. When grown in a structured environment (for example, a static aqueous microcosm), the ancestral free-living bacterium diversifies within only a few days[24,27,28]. This process can be visualized by plating the bacterial culture from aqueous environments on agar. The ancestral, non-biofilm-producing genotype (smooth morph) forms smooth, regular colonies, and the biofilm-producing genotypes (wrinkly spreaders) form various wrinkly colonies. The presence of a floating plant can provide SBW25 with the spatially structured environment (the broth–solid surface) that facilitates the growth of the wrinkly spreader and the formation of biofilm on the plant surface. The evolutionary diversification of biofilm genotypes occurs through mutations in one of the three key operons (*wsp*, *aws* and *mws*)[29–32]. The genetic basis can be confirmed by knocking out the three operons in the ancestral strain of SBW25, which creates a strain (PBR716; ref. [32]) that cannot evolve to form biofilm for longer than six weeks in duckweed growth medium. The ancestral strain SBW25 and PBR716 are ecologically similar (Extended Data Fig. 1; also see Bailey et al.[33] for using PBR716 as a close competitor for SBW25), while differing in the ability to evolve biofilm. Therefore, we can access the importance of rapid evolution in biofilm formation by comparing the microbiome composition and host fitness in the treatment with SBW25 versus that with PBR716.

Biofilm formation can be highly impactful to plant microbiomes. Biofilm matrices enable bacterial cells to attach to each other and a surface, creating new niches for microbial growth, buffering environmental stress and promoting cooperation between individuals in the biofilm, consequently changing how microbial species interact and function[34,35]. In a plant–microbiome system, the newly evolved biofilm-forming genotypes of *P. fluorescens* could either be mutualists or pathogens, directly affecting *L. minor* fitness or altering the function of other microbiome species, especially those on the surface of *L. minor*.

Here we examined how microbial evolution in biofilm formation interacted with microbiome species presence to influence plant host fitness. We simultaneously manipulated the presence/absence of a synthetic epiphytic microbiome, which comprises nine common, culturable bacterial species collected from *L. minor*'s natural microbiome, and the presence/absence of *P. fluorescens* as well as its ability to evolve biofilm-forming genotypes (no *P. fluorescens*, non-evolving PBR716 or evolving SBW25). We examined their interactive effect on the fitness of *L. minor* over approximately five host generations. Furthermore, to investigate variation in microbial eco-evolutionary interactions across *L. minor*'s genetic backgrounds, we replicated our experiment with three genetically distinct plant clones (hereafter denoted as genotypes A, B and C) collected from three different local ponds near Pittsburgh (Pennsylvania, United States). Finally, we performed bacterial assays on three plant-associated traits to explore possible mechanisms through which the microbiome affected *L. minor*. These traits were the production of auxin (a plant growth hormone), the solubility of phosphorus (the limiting resource for plant growth in freshwater ecosystems) and the production of cyanide (a common microbial secondary metabolite that can suppress plant respiration)[22,36,37]. Our study aimed to answer two questions: how do microbial evolution and microbiome presence affect each other, and how does microbiome evolution interact with microbiome presence to influence *L. minor* fitness?

Results

The presence of the nine-species microbiome influenced *P. fluorescens* biofilm evolution and abundance. We quantified the degree of *P. fluorescens* evolution as the proportion of the biofilm-forming genotype (that is, wrinkly spreader), following Gómez and Buckling[38]. Microbiome presence promoted biofilm evolution in the evolving *P. fluorescens* (SBW25) populations (Fig. 1a; analysis of variance (ANOVA) followed by Tukey's test, microbiome presence: $P<0.001$). In contrast, no biofilm-forming genotypes evolved in the non-evolving *P. fluorescens* (PBR716) populations regardless of the presence/absence of the microbiome (Fig. 1a and Supplementary Table 1; ANOVA, microbiome presence: $P<0.001$). In addition, the abundance of PBR716 and SBW25 was reduced by microbiome presence, but the abundance of SBW25 was less sensitive to microbiome competition than that of PBR716 (Fig. 1b and Supplementary Table 2; ANOVA, microbiome presence: $P<0.001$).

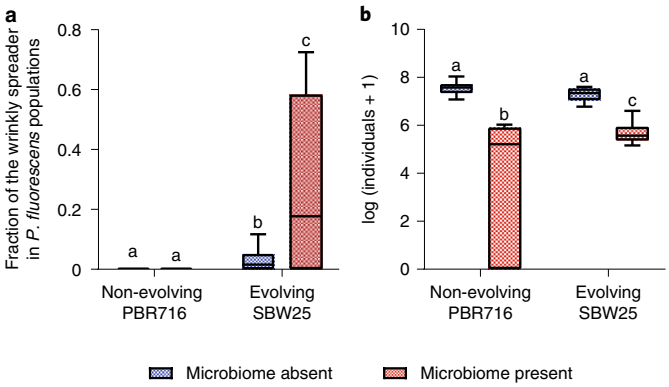

Fig. 1 | The effect of microbiome presence on the biofilm evolution and abundance of *P. fluorescens* populations. a, The degree of biofilm evolution in the non-evolving (PBR716) and evolving (SBW25) *P. fluorescens* strains, which was calculated as the fraction of biofilm-forming genotype, the wrinkly spreader, in *P. fluorescens* populations. **b**, log-transformed *P. fluorescens* abundance. Boxes show medians and interquartile ranges with whiskers for 10th and 90th percentiles ($n=12$). Treatments sharing the same letter are not statistically different ($P>0.05$).

Reciprocally, *P. fluorescens* presence and evolution had strong feedbacks on the microbiome's species composition (Fig. 2 and Supplementary Table 3; multivariate analysis of variance (MANOVA), *P. fluorescens*: $P<0.001$). Although the presence of *P. fluorescens* had a moderate effect (no *P. fluorescens* control versus non-evolving PBR716: $P=0.072$, $\eta^2=0.654$), the biofilm evolution had a strong influence on microbiome species composition (non-evolving PBR716 versus evolving SBW25: $P=0.006$, $\eta^2=0.800$); a result confirmed by discriminant analysis (Supplementary Table 4; no *P. fluorescens* control versus non-evolving PBR716: $P=0.052$; non-evolving PBR716 versus evolving SBW25: $P=0.008$). ANOVA based on individual species abundance revealed that two microbiome species responded significantly to *P. fluorescens* presence or evolution. The presence of *P. fluorescens* increased the abundance of *Rhizobium rosettiformans* by 84.9% and biofilm evolution reduced the abundance of *Agrobacterium tumefaciens* by 64.2% (Extended Data Fig. 2; $P<0.05$).

The reciprocal eco-evolutionary interactions between *P. fluorescens* biofilm evolution and microbiome presence influenced *L. minor* fitness (Fig. 3 and Supplementary Table 5a; generalized linear model (GLM), *P. fluorescens* × microbiome presence: $P<0.001$). *L. minor* fitness was significantly correlated with the abundance of the smooth morph, *A. tumefaciens* and three other species in the microbiome (Supplementary Table 5b; $P<0.01$). The presence of any microbes (the nine-species microbiome and/or the presence of the non-evolving *P. fluorescens* (PBR716) and/or the evolving *P. fluorescens* (SBW25)) improved plant host fitness (Fig. 3; GLM followed by Bonferroni test: $P<0.05$). However, the combined effects of the microbiome presence and *P. fluorescens* on the plant's fitness were not additive. When compared with the treatment with the microbiome alone, the introduction of PBR716 to the microbiome brought no further effect to *L. minor* fitness, whereas the introduc-

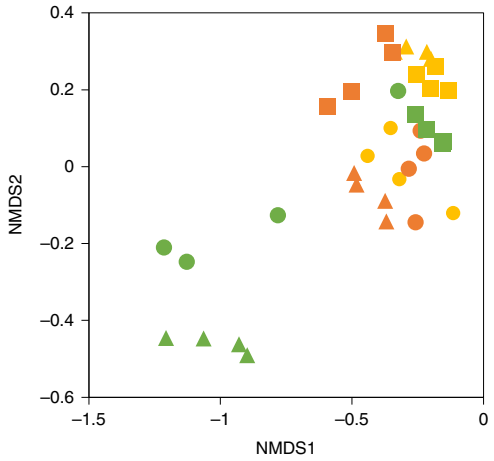

Fig. 2 | The dissimilarity between microbiome communities tested by MANOVA and visualized by non-metric multidimensional scaling with axes NMDS1 versus NMDS2. The distance between two points is the Bray–Curtis dissimilarity between communities. Yellow, no *P. fluorescens*; orange, non-evolving PBR716; green, evolving SBW25. Circles, *L. minor* genotype A; squares, genotype B; triangles, genotype C.

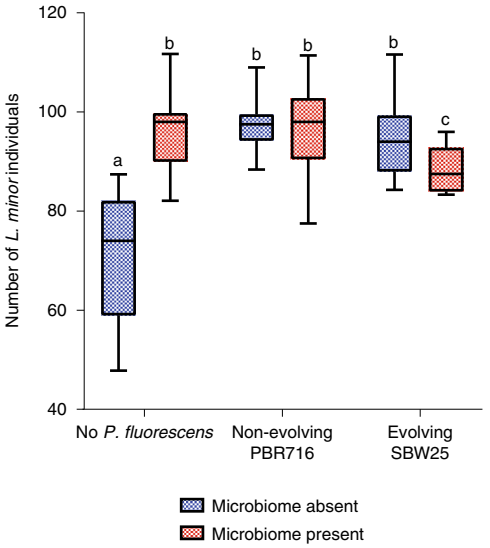

Fig. 3 | The interactive effects of microbiome presence and *P. fluorescens* presence and evolution on *L. minor* fitness. Boxes show medians and interquartile ranges of the final number of *L. minor* individuals after 28 days of growth with whiskers for 10th and 90th percentiles ($n = 12$). Treatments sharing the same letter are not statistically different ($P > 0.05$).

tion of SBW25 reduced *L. minor* fitness (Fig. 3; Bonferroni test: $P < 0.05$). We note that *L. minor* genotypes influenced *P. fluorescens* evolution and microbiome community structure (Supplementary Tables 1–4). Nevertheless, the effects of *P. fluorescens* evolution and microbiome presence on *L. minor* fitness were generally consistent across all three *L. minor* genotypes despite a baseline difference in the abundance of *L. minor* across genotypes (Extended Data Fig. 3).

To elucidate possible mechanisms of how the evolution of *P. fluorescens*' ability to form biofilm and the microbiome presence determined host fitness, we conducted community-level assays to quantify three bacterial traits that are commonly found to impact plant performance[22,36,37]. First, neither *P. fluorescens* alone nor microbiome communities produced cyanide to a detectable level. We therefore ruled cyanide production out as a mechanism that influenced host fitness in our study. Second, the presence of either *P. fluorescens* or the microbiome reduced available phosphorus (Extended Data Fig. 4; ANOVA, the main effects of *P. fluorescens* and the microbiome and interaction: $P < 0.001$). Phosphorus is the limiting resource in our experimental system and in many freshwater ecosystems. Reducing the availability of this nutrient was unlikely to cause an increase in *L. minor* fitness. Third, microbiome presence and *P. fluorescens* evolution had a qualitatively similar interactive effect on the production of auxin as on the fitness of *L. minor* (Fig. 4a and Supplementary Table 6; ANOVA, *P. fluorescens* × microbiome presence: $P < 0.001$, $\eta^2 = 0.438$). Microbiome presence had a positive, non-significant, and marginally negative effect on auxin production, when no, the non-evolving and the evolving *P. fluorescens* were present in the microbiome, respectively. Correlation analysis suggested an asymptotically positive relationship between *L. minor*'s abundance (data collected in the main experiment) and auxin production (data collected in the 24 hour, *L.-minor*-absent, community-level auxin assay; Extended Data Fig. 5). With an auxin-addition experiment we confirmed that when auxin level increased from 0 to 0.3 mg l^{-1} *L. minor* fitness increased, whereas the positive effect of auxin addition saturated at 1.2 mg l^{-1} (Fig. 4b; GLM, auxin addition: $P < 0.001$). We acknowledge that to apply the result of the auxin-addition experiment to explaining the effect of rapid evolution in *P. fluorescens* on auxin production and *L. minor* fitness, we need to include a treatment of auxin concentrations of at least 0.23 mg l^{-1} (in the microbiome presence and SBW25 treatment), 0.49 mg l^{-1} (in the SBW25 treatment) or 0.53 mg l^{-1} (in the microbiome presence and PBR716 treatment). Unfortunately, these data were not available at the present time. Finally, to identify sources of auxin, we incubated each bacterial species/genotype in monoculture and qualitatively examined their ability to produce auxin. Only *A. tumefaciens*, PBR716 and SBW25 (smooth morph alone or combined with the wrinkly spreader) could produce auxin. We note that additional experiments that are beyond the scope of this study are required to confirm that auxin produced by the microbiomes promoted plant fitness.

Discussion

The non-additive effect of *P. fluorescens* evolution and microbiome presence on host fitness was possibly a result of the microbial eco-evolutionary interactions associated with auxin production (summarized in Fig. 5). Microbiome presence reduced the overall abundance of the evolving *P. fluorescens* (SBW25; one of the auxin producers) while increasing the frequency of the biofilm-forming genotype in SBW25 populations (Fig. 1). The enhanced biofilm-forming evolution in SBW25 in turn modified the community composition of the microbiome (Fig. 2) and significantly reduced the abundance of *A. tumefaciens* (another auxin producer). As a result, a microbiome with the evolving *P. fluorescens* strain contained the fewest auxin-producing bacteria, potentially resulting in reduced fitness of the plant host (Fig. 3). Although we could not fully confirm auxin production as the mechanism at play, our results implied that evolution in one microbiome member could alter the performance of other mutualistic species and modify the microbiome community structure, triggering an indirect ecological effect on host fitness.

Although the effect of microbiome presence and *P. fluorescens* biofilm evolution on *L. minor* fitness was generally consistent across three *L. minor* genotypes, *L. minor*'s genotypic identity altered the species composition in the microbiome (Fig. 2). Host genetic variation has long been considered important for microbiome interactions, especially in gut microbiota[39]. Similar effects of host genotypes on the microbiome composition have recently been reported in plants. For example, in maize[40] and cottonwood[41],

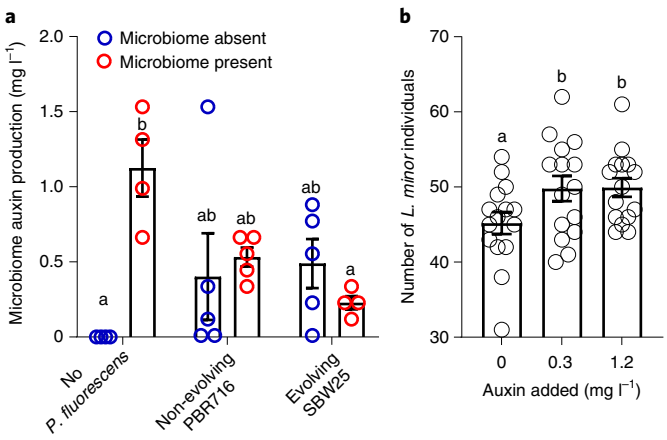

Fig. 4 | Auxin production as a possible determinant for changes in *L. minor* fitness. a, Microbiome ability to produce auxin. Microbiomes were re-assembled with isolates from the main experiment ($n = 4$ or 5; details in Methods). **b**, The number of *L. minor* individuals in three auxin-addition levels ($n = 15$). Bars show means (±1 s.e.). Treatments sharing the same letter are not statistically different ($P > 0.05$).

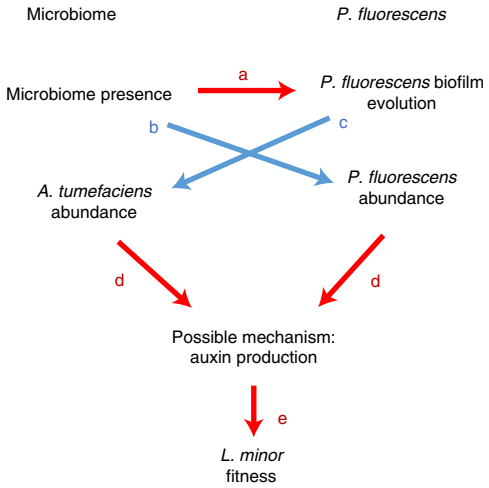

Fig. 5 | Schematic summarizing the results of our study. Red arrows indicate positive relationships and blue arrows indicate negative relationships. Microbiome presence promoted the biofilm evolution in *P. fluorescens* (a) but reduced its abundance (b). Biofilm formation in *P. fluorescens* in turn altered microbiome species composition. It specifically reduced the abundance of the auxin producer, *A. tumefaciens* (c). A decrease in *A. tumefaciens* and *P. fluorescens* abundance hindered microbiome auxin production (d), reducing *L. minor* fitness (e).

host genotypic identity determines the species composition in their rhizosphere microbiome. As the host genotypic effect was not the focus of our study, we are not certain what traits associated with *L. minor* genotypes affected microbiome communities. This could be an interesting avenue for future research.

P. fluorescens biofilm evolution drove community-level ecological changes in the microbiome (Fig. 2). The ecological impact of rapid evolution from our study complements a growing number of experimental studies that have also quantified the connection between evolution and community-level ecological changes[42–44]. For example, Pantel et al.[43] adapted the water flea, *Daphnia magna*, to environments with predatory fish and/or artificial macrophytes. In a subsequent common garden experiment, they revealed strong influences of *D. magna* evolution, compared with non-adapted populations, on the zooplankton community structure. Our study, on the other hand, examined the importance of rapid evolution in a host–microbiome context. By comparing the community-level effects of two almost isogenic strains of *P. fluorescens* that differ in their potential to evolve biofilm, our study provided evidence for the impact of the evolution of one critical trait (biofilm formation) on the concurrent ecological dynamics of other members of its community and host. The continued development of gene-editing technology in many taxa can make our manipulative experimental evolution approach more feasible for examining eco-evolutionary dynamics in numerous systems[45–47].

We acknowledge two limitations of our study. First, *P. fluorescens* is a common bacterial species in the microbiome of plants, including duckweed[48–52], but strain SBW25 originates from the phyllosphere of a sugar-beet leaf[53]. We used SBW25 instead of a *P. fluorescens* strain from the natural duckweed microbiome because the molecular mechanisms of SBW25 evolution have been well studied, making it a tractable experimental model of biofilm evolution[24]. However, our results are relatable to biofilm formation in the microbiomes of duckweed and other plants in nature. Most *P. fluorescens* species (including SBW25), along with many other bacterial species, contain the same genetic pathways (*wsp, aws* and *mws*[30,31,54,55]) that direct biofilm formation. Therefore, other bacterial species in duckweed microbiomes have the potential to evolve biofilm and affect host fitness through the same mechanisms observed in our study. Nevertheless, future research should consider the effect of the origins of bacterial species on the eco-evolutionary dynamics in microbiomes and their fitness impacts. Second, our synthetic microbiome community contained only nine epiphytic bacterial species, cultivated in a stable environment. Although these bacterial species were found to be the most abundant cultured species in the microbiome and are phylogenetically distantly related (representing a broad range of the bacterial domain), microbial communities in natural microbiomes are more complex. Particularly, we did not consider endophytic bacterial species, which were found in in vitro trait assays to be able to produce auxin[52]. Also, many abiotic and biotic factors, such as herbivory, parasitism and fluctuation in nutrient availability, were not explicitly tested in our experiment.

In recent years, interest in studying the eco-evolutionary interactions between hosts and microbiomes has been increasing[56,57]. Despite ample evidence for the various roles of microbes in plant growth, few studies have considered the role of evolution within microbiomes in mediating the natural lifetime fitness of host species. Using experimental evolution, with controls in which the trait of interest does not evolve, we experimentally demonstrate that the presence of the microbiome directs the biofilm evolution in one microbiome member, which, in turn, modifies the community structure of the whole microbiome. Such eco-evolutionary dynamics, possibly acting through the production of a plant growth hormone, strongly influence plant-host fitness (Fig. 5). These results highlight the important role of microbial evolution on host fitness in contemporary timescale and underscore the importance of knowledge of the eco-evolutionary dynamics in the microbiome and between the microbiome and host in predicting the outcomes of host–microbiome interactions.

Methods

Study organisms. The host plant was the duckweed *L. minor*, a widespread floating angiosperm[58]. Each *L. minor* individual is composed of one floating frond and rootlet. They can reproduce within three to four days via asexual budding into clusters of up to eight fronds that break apart into smaller clusters. Due to its fast growth rate, *L. minor* has become a candidate for biofuel and feedstock production[59]. Multiple clonal genotypes exist in natural *L. minor* populations. To assess whether the treatment effects depended on the host genotype, we replicated our experiment with three *L. minor* genotypes (denoted as A, B and C) collected

from Boyce Mayview Park, Deer Lakes State Park and Schenley Park, respectively, all within 30 km of Pittsburgh, Pennsylvania, United States. These three genotypes are genetically distinct in two microsatellite loci[7] and have been maintained in laboratory cultures for 12 months.

We used a bleaching methodology to eliminate the natural microbiome of these three genotypes. We first rinsed the *L. minor* clusters from the sample sites with tap water and propagated them in the lab. After that, we transferred the clusters into test tubes with 0.025 M phosphate buffer saline (PBS) and sonicated the test tubes for five minutes. These clusters were further treated with 1% sodium hypochlorite for 20 seconds individually and transferred into sterile microcosms with fresh medium. After the bleaching treatment we kept *L. minor* cultures in a growth chamber at 27 °C under a 16/8 photoperiod. We examined whether the *L. minor* cultures were axenic by testing the presence of bacterial 16S and fungal internal transcribed spacer (ITS) DNA in *L. minor*'s epiphyte and endophyte. To extract epiphytic microbes, we rinsed *L. minor* with PBS and concentrated the cell pellets using a centrifuge. To extract endophytic microbes, we snap-froze the rinsed *L. minor* with dry ice, ground the *L. minor* and suspended cell pellets in PBS. The genomic DNA was extracted from both *L. minor* epiphyte and endophyte extracts, followed by the amplification of 16S and ITS, using PCR. No PCR products from either the epiphyte or endophyte were produced, suggesting the *L. minor* cultures were free of bacteria or fungi. Each *L. minor* clone was tested with two technical replicates of two biological replicates.

We isolated nine bacterial species from the epiphytic microbiome of an *L. minor* population from the same pond where genotype B was sampled in Deer Lakes State Park, Pennsylvania, United States. We first rinsed *L. minor* fronds twice in sterile water to remove bacteria in water, then transferred the fronds to 0.025 M sterile PBS and sonicated them for five minutes to suspend the microbes from the microbiome to PBS. We serially diluted the PBS and plated it on Reasoner's 2A agar (R2A). Bacterial species formed colonies with distinct morphology on agar. We picked nine of the most common colonies, propagated them in Reasoner's 2B medium (R2B) and preserved them in 15% glycerol at −80 °C. We sequenced the 16S ribosomal RNA of each bacterium for identification (bacterial species' names in Extended Data Fig. 2). We acknowledge that duckweed also hosts a diverse endophytic microbiome[52]. However, culturing endophytic bacteria and re-inoculating them into duckweed is logistically challenging. Thus, they were not included in our synthetic communities.

P. fluorescens SBW25 is a classic experimental model for biofilm evolution[24]. PBR716 is the non-evolving strain, which is isogeneic to SBW25 except having the three operons knocked out[32]. Through a mutual invasion experiment, we found that the smooth-morph ancestors of SBW25 and PBR716 used in our main experiment were ecologically highly similar (Extended Data Fig. 1). Our observations confirmed that all individuals in PBR716 remained as the smooth morph during our experiment (Fig. 1a).

Experimental protocols. We used 20 mm by 150 mm loosely capped glass test tubes as the microcosms. Each microcosm contained 15 ml of medium made of 607 mg l^{-1} KNO_3, 6.12 mg l^{-1} KH_2PO_4, 177 mg l^{-1} $Ca(NO_3)_2{\cdot}4H_2O$, 3.04 mg l^{-1} $FeCl_3$, 5.48 mg l^{-1} EDTA, 90.2 mg l^{-1} $MgSO_4$, 0.232 mg l^{-1} H_3BO_3, 0.0726 mg l^{-1} $Na_2MoO_4{\cdot}2H_2O$, 1.23 mg l^{-1} $MnCl_2$ and 250 mg l^{-1} protozoan pellets (Carolina Biological Supply). The inorganic salts supported *L. minor* growth while protozoan pellets made from plant extracts were used to facilitate the establishment of bacteria in the microbiome by releasing a minuscule amount of organic carbon to the medium.

Our experiment used a full three-way factorial design. We manipulated the presence/absence of the microbiome (comprised of nine bacterial species; species' names in Extended Data Fig. 2), *P. fluorescens* presence and ability to evolve biofilm (no *P. fluorescens*, PBR716 or SBW25), and *L. minor* genotype (A, B or C). Each treatment combination was replicated four times. We therefore used 72 microcosms in our experiment: microbiome presence/absence (2 levels) × *P. fluorescens* treatment (3 levels) × *L. minor* genotype (3 levels) × 4 replicates.

We introduced nine *L. minor* fronds of one genotype into one autoclaved microcosm with the growth medium. We replicate our experiment with three *L. minor* genotypes to examine the consistency of microbiome effects across host genetic variation. The microcosms were incubated in an incubator under the 16/8 photocycle. The photosynthetic photon flux levels during the day were set at 180 μmol m^{-2} s^{-1}. The incubation temperature was set at 27 °C during the day and 21 °C at night. We incubated the axenic *L. minor* for four days to confirm that each microcosm was free of contamination before introducing bacteria.

We purified the microbiome species and smooth morph of both SBW25 and PBR716 by streaking them on agar three times. We incubated agar plates between streaking for 2 days (~20 generations). We note that colonies of smooth morph, as well as wrinkly spreaders, maintained their morphology over multiple rounds of streaking, suggesting that the smooth-morph and wrinkly spreader phenotypes are heritable. The purified colonies of SBW25 and PBR716 were propagated separately in R2B broth by shaking overnight. We transferred 5 μl of the culture into the corresponding treatments.

We mixed nine microbiome species in a microcosm with axenic *L. minor*, allowing them to equilibrate for three days. Before the inoculation, we vigorously vortexed and sonicated the microcosm for five minutes to release and mix the microbiome community, and then transferred 20 μl of this culture from the microcosm into each experimental microcosm to seed the microbiome community. We repeated the procedure twice (a total of 40 μl) to ensure that microbiome species were successfully inoculated into the experimental microcosms. The total initial abundance of bacteria was ~10^6 colony-forming units. We acknowledge that due to logistic limitations, we were unable to test the effect of each microbiome species on *L.* minor's fitness, though these results could be useful for a better interpretation of our results. In treatments with the microbiomes and *P. fluorescens*, all bacterial species were introduced at the same time. After we inoculated the bacteria, we ran the experiment for 24 days. We ended our experiment on day 28.

We counted the number of *L. minor* individuals in each microcosm as the measure of its fitness. To estimate the abundance of each bacterial genotype/species, we transferred *L. minor* from each microcosm to a test tube with 10 ml of 0.025 M sterile PBS. We extracted bacteria by vigorously vortexing the test tubes for one minute and then sonicating them for five minutes. As this sampling procedure could disrupt the formation of biofilm, we sampled the microcosms only once at the end of the experiment. We serially diluted bacterial extracts and plated them on agar plates. We counted the number of colonies of each bacterial species/genotype in the microbiome and calculated bacterial abundance as the number of individuals (colony-forming units) in the microbiome extracts. We also plated the bacterial culture directly collected from the broth of each microcosm. The results of the bacterial communities in the broth were qualitatively similar to those in the microbiome.

Bacterial traits. We tested the ability of bacterial communities with multiple genotypes/species to express three plant–microbe interaction traits (auxin production, phosphate solubilization and hydrogen cyanide production), following Ishizawa et al[22]. We tested five bacterial communities: the microbiome with nine species, the smooth morph of PBR716, the microbiome with the smooth morph of PBR716, the smooth morph and wrinkly spreader of SBW25 and the microbiome with the smooth morph and wrinkly spreader of SBW25. We also included a bacteria-free control.

From agar plates used for quantifying bacterial abundance, we isolated the microbiome species, re-assembled microbiome communities for the six experimental treatments (two microbiome presence × three *P. fluorescens* treatments) and allowed the communities to equilibrate for one week. These microcosms for microbiome propagation contained *L. minor*. We then transferred the communities with a 1:1000 dilution into microcosms with fresh medium to test the effect of microbiomes on phosphate availability. We biologically replicated each treatment three times. The microcosm set up and incubation conditions were identical to those of the main experiment except we excluded *L. minor*, which might excrete or uptake phosphate and hinder us from assessing bacterial activities. After incubating the test tubes for five days, we removed the bacteria by 0.45 μm syringe filtering. The total phosphorus in the filtered culture (the available phosphorus) was measured at the Regional Stable Isotope Lab for Earth and Environmental Science Research of the University of Pittsburgh.

For cyanide testing, we spread the microbial communities on R2A plates with 0.44% glycine and a circular disk of Whatman's filter paper No. 1 with 2% sodium carbonate and 0.5% picric acid solution placed in the lid. We biologically replicated each treatment four times. We sealed the plates for cyanide testing with parafilm and incubated them at room temperature for 24 hours. We considered the bacterium-produced cyanide when observing the development of yellow to brown on the filter paper by referring to the control plate.

For auxin testing, we transferred the communities with a 1:1000 dilution into microcosms with fresh medium and 0.1% tryptophan. We biologically replicated each treatment five times. These microcosms contained no *L. minor*. After the 24 hour incubation, we removed the bacteria by filtering the culture through a 0.45 μm syringe filter. One microcosm from the control, the microbiome only and the microbiome and SBW25 treatment were excluded from testing, as we suspected the presence of fungal contamination. We mixed 0.15 ml of filtered culture with 0.3 ml of Salkowski's reagent. All the mixtures were then added to a 96-well microplate and the optical density of all the samples was taken at 520 nm, using a BioTek HTX Microplate Reader (BioTek Instruments Inc.). We estimated auxin concentration according to standard graph values produced with synthetic indole-3-acetic acid (RPI Corp.). The minimum detectable level of auxin using our protocol is 0.098 mg l^{-1}. Note that we were unable to quantify auxin concentration in our experimental microcosms after they were processed for measuring *L. minor* fitness and microbiome species composition since auxin was degraded, probably due to light exposure.

Because all microbiome communities showed the ability to produce auxin, we performed a qualitative single-species assay to identify which species/genotypes produced auxin. We inoculated one bacterium in one microcosm with fresh medium and 0.1% tryptophan, incubated the microcosm for 24 hours, removed the bacteria by filtering the culture through a 0.45 μm syringe filter and mixed 0.15 ml of filtered culture with 0.3 ml of Salkowski's reagent. We performed two biological replicates for each species/genotype. We recorded a positive result when Salkowski's reagent turned from yellow to orange or red. Both replicates of *A. tumefaciens*, PBR716, smooth morph and wrinkly spreader of SBW25 showed positive results.

To confirm the causal relationship between auxin production and an increase in *L. minor* fitness, we conducted a short-term growth experiment. We set a control (with no auxin) and two auxin-addition (0.3 and 1.2 mg l^{-1} indole-3-acetic acid added) treatments. The experiment was replicated five times with three genotypes. We introduced three axenic individuals of each *L. minor* genotype into the microcosms, added auxin to the designated microcosms, incubated them for 14 days and measured the number of *L. minor* individuals.

Statistical analysis. We quantified the degree of biofilm evolution in *P. fluorescens* by calculating the number of wrinkly spreader individuals over the total number of individuals in *P. fluorescens* populations. We tested the effects of *P. fluorescens*, the microbiome and *L. minor* genotype on biofilm evolution, using ANOVA (with a Type III sum-of-squares method) with *P. fluorescens* (no *P. fluorescens*, PBR716 and SBW25), the presence/absence of the microbiome and *L. minor* genotypes (A, B and C) as the independent variables and the fraction of wrinkly spreader in *P. fluorescens* populations as the dependent variable. A similar ANOVA was performed to examine the effects of *P. fluorescens*, the microbiome and *L. minor* genotype on *P. fluorescens* abundance. Note that all bacterial abundance data were log $(x+1)$ transformed to improve the normality of residuals. To assess the effect of *P. fluorescens* and *L. minor* genotype on the microbiome community structure, we performed a MANOVA with *P. fluorescens* (no *P. fluorescens*, PBR716 and SBW25) and *L. minor* genotypes (A, B or C) as the independent variables and microbiome species abundance as the dependent variable. The abundance data of each microbiome species was a vector of the dependent variable in the MANOVA. Note that *P. fluorescens* abundance was not included in this analysis. We visualized the MANOVA results by the nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity. In addition, we performed three separate MANOVAs (interactions with *L. minor* genotypes considered) and discriminant analyses (interactions with *L. minor* genotypes not considered) to determine the pairwise differences between the three *P. fluorescens* treatments.

To test for the drivers of *L. minor* fitness, we implemented a GLM with *P. fluorescens* (no *P. fluorescens*, PBR716 and SBW25), the presence/absence of microbiome and *L. minor* genotypes (A, B or C) as the fixed effects and the number of *L. minor* fronds as the dependent variable with Poisson distribution (goodness-of-fit test: $\chi^2 = 42.195$, d.f. = 54, $P = 0.781$). We considered all three-way interactions in the GLM. We used a similar GLM to test the relationship between bacterial abundance and *L. minor* fitness across all treatments. In the model, the abundance of each bacterial species, including each *P. fluorescens* genotype, was the independent variable and the number of *L. minor* individuals was the dependent variable. In addition, we used ANOVA to examine the effects of *P. fluorescens* and the microbiome on auxin and phosphorus concentrations in the follow-up experiment. We implemented both a linear and asymptotic model in R to determine the relationship between auxin production and *L. minor* fitness (Extended Data Fig. 5). ANOVA was performed on the auxin-addition production data to determine the significance of the difference between the fitness in the control and auxin-addition treatments. All statistical analyses, except the correlation between auxin production and *L. minor* fitness, were performed with SPSS (version 26, IBM Corp.).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the finding of this study are available on Figshare (https://doi.org/10.6084/m9.figshare.13644161). Source data are provided with this paper.

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Author contributions

J.T. and M.M.T. conceived the idea and designed the study. J.T. and J.E.K. performed the experiments. J.T., J.E.K. and M.M.T. analysed the data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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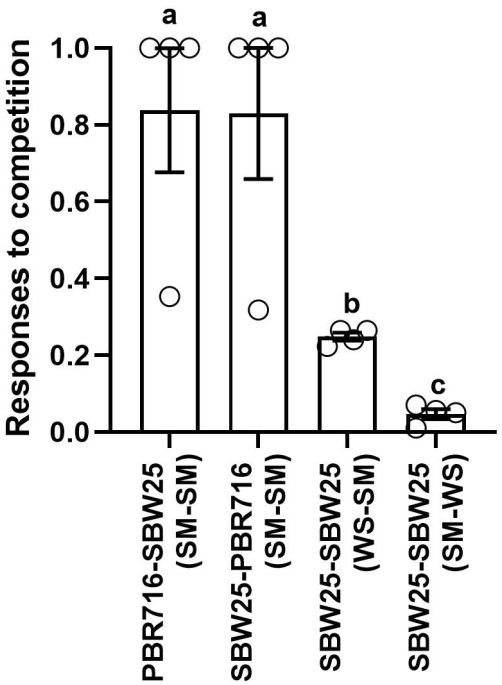
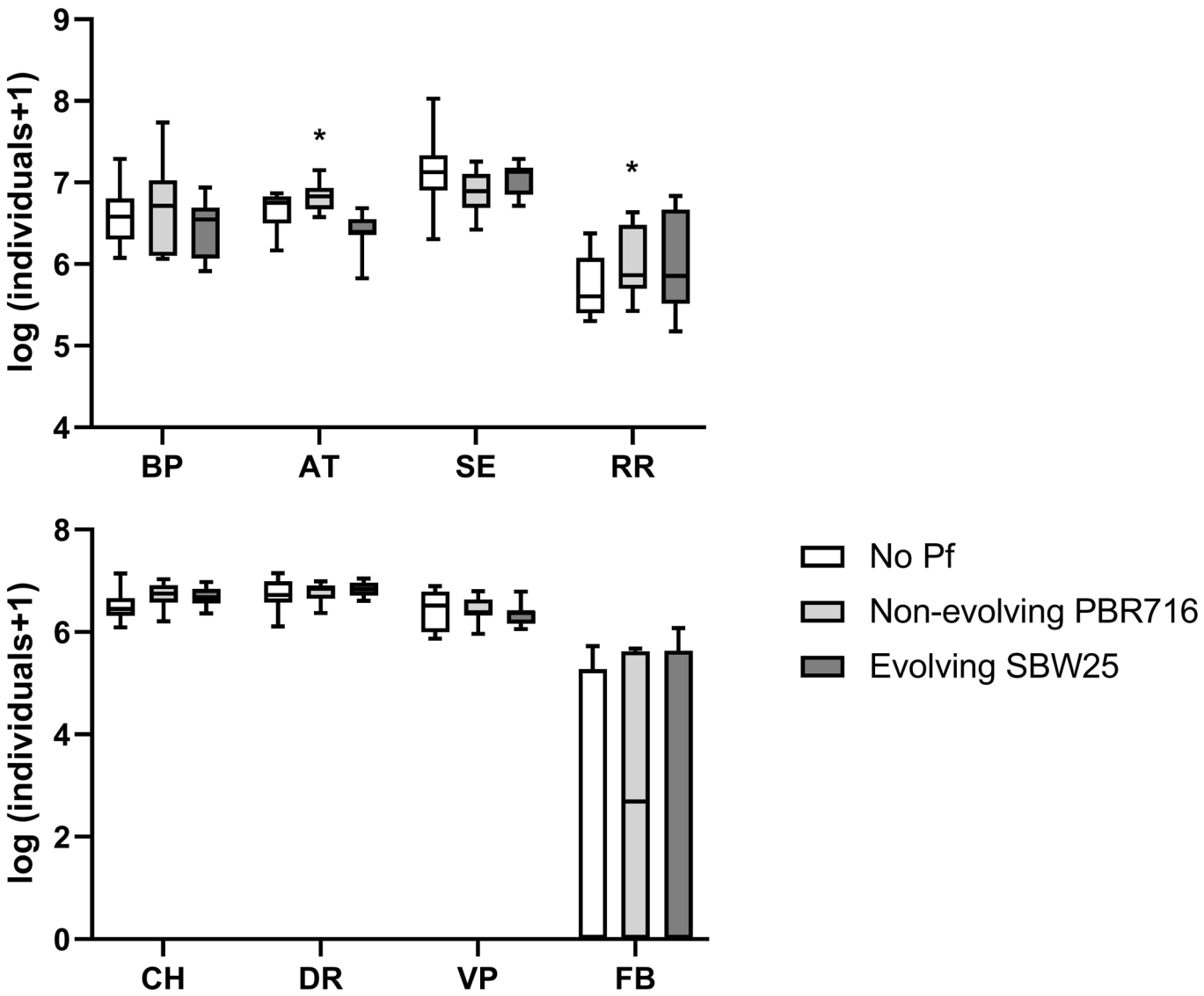
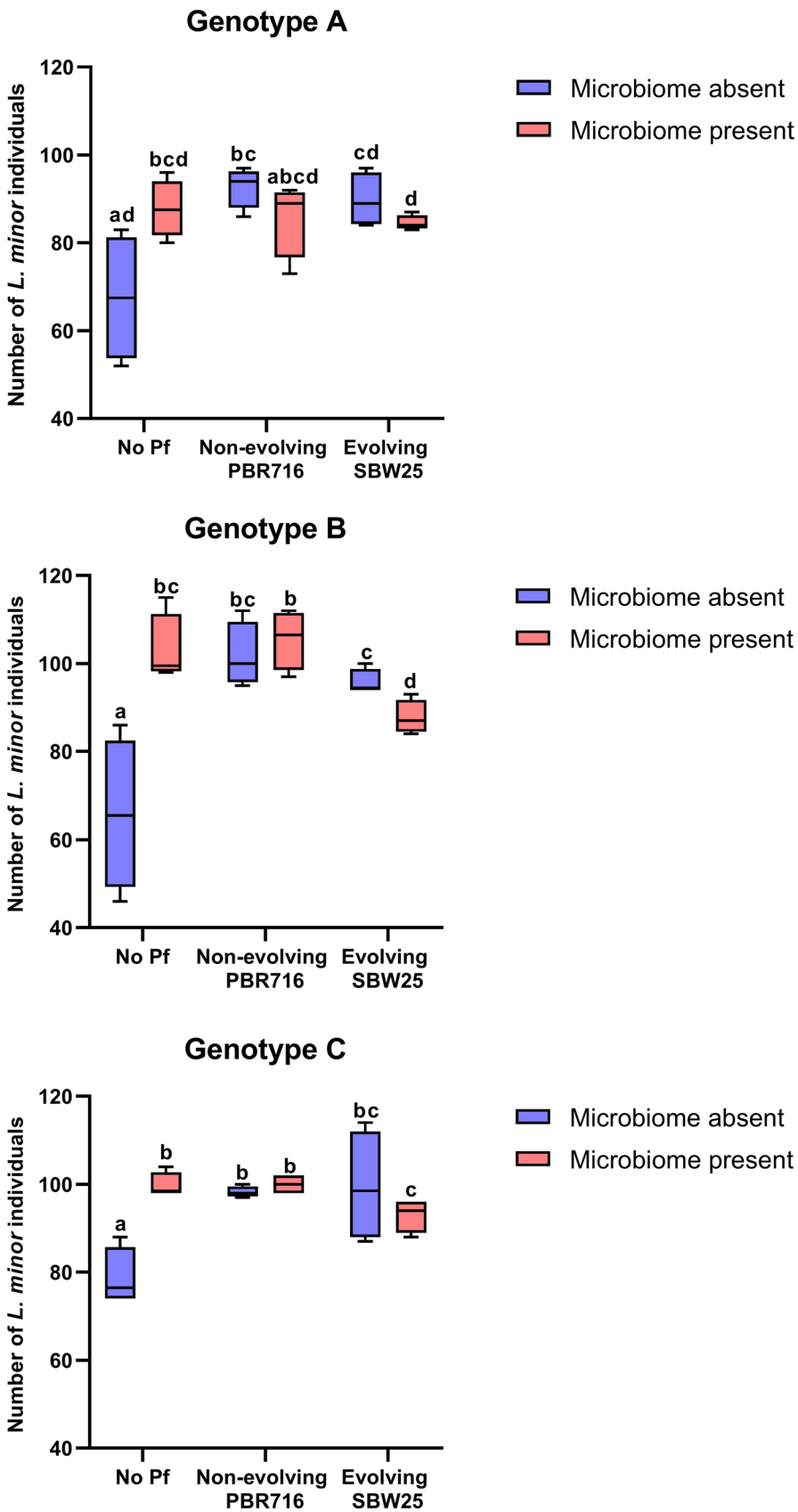


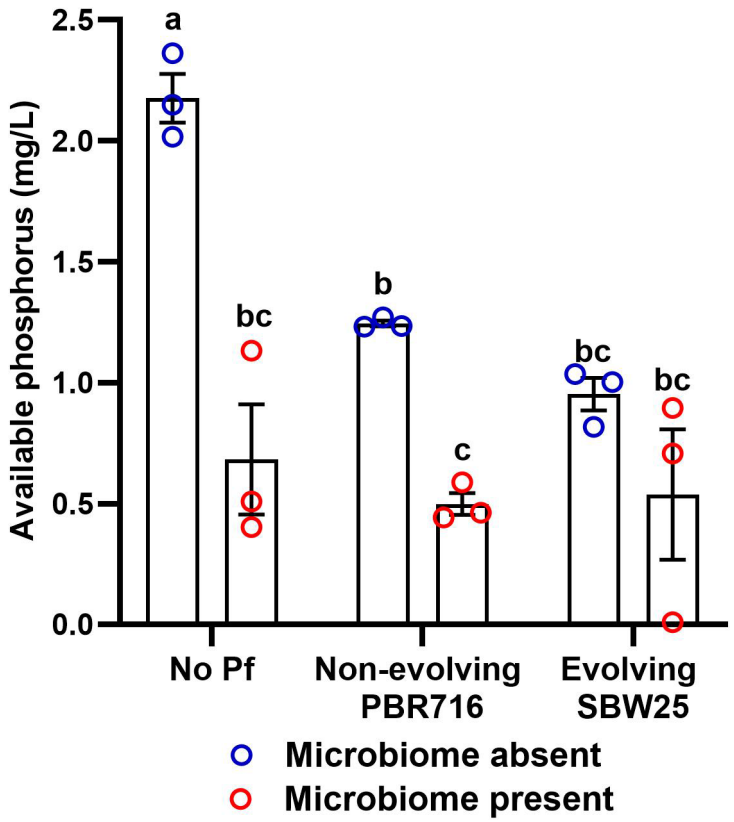
Extended Data Fig. 1 | Results of the mutual invasion experiments between the various *P. fluorescens* lineages. The goal of these experiments is to test whether the ancestral free-living SBW25 (smooth morph, SM) is ecologically similar to the isogenic PBR716 (SM) with the three operon knock-outs. We also quantified the ecological differences between wrinkly spreader (WS) and SM of SBW25 for comparison. Between SM of PBR716 and SM of SBW25, we first quantified their growth in monoculture (r_{mono}) in aqueous microcosms. We set the initial abundance at ~10^6 CFU (0.01x of carrying capacity). We quantified bacterial abundance after 24 h of growth and calculated r_{mono} as ln (abundance). We quantified their invading growth ($r_{invading}$) in a culture of the other strain that had been already growing to high abundance for 24 h. The growth of the invading strain was calculated based on its final abundance after 24 h static incubation. Please note that SBW25 strain we used carried a neutral lacZ marker while PBR716 did not. Therefore, we distinguished the SM of the two strains based on their colony color on with X-gal (SBW25, blue; PBR716, white). All treatments were replicated four times. We quantified the competitive response (S) of one strain to the other as 1- $r_{invading}/r_{mono}$. S close to 1 indicates stronger interaction and potentially higher ecological similarity between two strains, while that close to 0 indicates weaker interaction and lower ecological similarity. We showed that higher competitive responses between SM of PBR716 and SM of SBW25 than those between SM and WS of SBW25. Values are means (± 1 s.e.; n = 4). Treatments sharing the same letter are not statistically different.



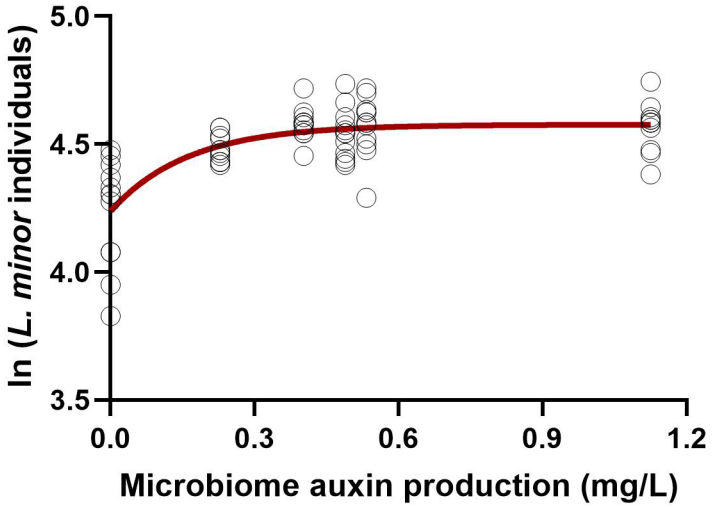
Extended Data Fig. 2 | The abundance of the microbiome species subject to three *P. fluorescens* treatments (No Pf, non-evolving PBR716, and evolving SBW25). The microbiome contained *Bacillus pumilus* (BP), *Agrobacterium tumefaciens* (AT), *Sphingomonas elodea* (SE), *Rhizobium rosettiformans* (RR), *Chryseobacterium hispalense* (CH), *Duganella radices* (DR), *Variovorax paradoxus* (VP), and *Flavobacterium buctense* (FB) (n for each species = 12). *Bacillus aryabhattai* was isolated from *L. minor* epiphyte, but appeared only in the medium, and was therefore not shown here. Boxes show medians and interquartile ranges with whiskers for 10th and 90th percentiles. Stars indicate that the abundance of *A. tumefaciens* and *R. rosettiformans* was significantly influenced by *P. fluorescens* presence or evolution.



Extended Data Fig. 3 | The abundance of *L. minor* in each *L. minor* genotype treatment. Boxes show medians and interquartile ranges with whiskers for 10th and 90th percentiles (n = 4). Treatments sharing the same letter are not statistically different (P > 0.05). Pf stands for *Pseudomonas fluorescens*. *L. minor* was genotyped with two microsatellite primers R5C (F: TGATGCCAGTAGATCCGGC R: ACGCCTGAACACGATTGATG) and R15B (F: GTGACAGCGTATCCTTGTGC R: TCAGCGGCAAGATCATCAAG).



Extended Data Fig. 4 | The concentration of available phosphorus. Values are means (± 1 s.e.; n = 3). Pf stands for *Pseudomonas fluorescens*. Treatments sharing the same letter are not statistically different ($P > 0.05$).



Extended Data Fig. 5 | The correlation between auxin production and *L. minor* fitness. After the main experiment ended, we plated the culture from each microcosm on agar plates to quantify the abundance of bacterial species/genotypes. We isolated each present species/genotype and re-assembled the microbiome in a new microcosm with *L. minor*. The microbiome was incubated for a week to allow them to propagate and equilibrate. Then, we added them into a new microcosm without *L. minor* to quantify auxin concentration after 24 h. The average level of auxin production of the six experimental treatments (microbiome presence/absence × *P. fluorescens* treatments) were calculated and presented in Fig. 4. Here, we plot the number of *L. minor* individuals of each treatment (data collected from the main eperiment, y-axis data, ln-transformed before the analysis) against auxin production (x-axis; N = 72). We implemented both a linear (y=a – bx) and asymptotic (y=a – [a – b] exp [–cx]) model in R. The asymptotic model had a lower AIC value (asymptotic 529.410, linear 557.702; P values for both models is smaller than 0.001) and thus better predict the relationship between auxin production and *L. minor* fitness.

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Data collection	Duckweed and microbiome data were manually collected by the authors and undergraduate assistants. No software is used.
Data analysis	Data analysis was performed using SPSS 26.0.

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Sample size	72
Data exclusions	No data were excluded from the analysis.
Replication	For the main experiment, four biological replicates for each treatment were performed. For the follow-up trait assays, the number of replications (3-5) varied. For details please see the Methods.
Randomization	Plant hosts were randomly selected for the experiment. Microcosms were randomly placed in the incubators.
Blinding	Data collection involved multiple undergraduate assistants who had no knowledge of the treatment information.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging