Received: 21 March 2023 | Accepted: 6 September 2023

DOI: 10.1111/mec.17142

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Plant neopolyploidy and genetic background differentiate the microbiome of duckweed across a variety of natural freshwater sources

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Funding information
Directorate for Biological Sciences, Grant/Award Number: 1912180, 1935410, 2027604 and 2109452

Handling Editor: Victoria L. Sork

Abstract

Whole-genome duplication has long been appreciated for its role in driving phenotypic novelty in plants, often altering the way organisms interface with the abiotic environment. Only recently, however, have we begun to investigate how polyploidy influences interactions of plants with other species, despite the biotic niche being predicted as one of the main determinants of polyploid establishment. Nevertheless, we lack information about how polyploidy affects the diversity and composition of the microbial taxa that colonize plants, and whether this is genotype-dependent and repeatable across natural environments. This information is a first step towards understanding whether the microbiome contributes to polyploid establishment. We, thus, tested the immediate effect of polyploidy on the diversity and composition of the bacterial microbiome of the aquatic plant *Spirodela polyrhiza* using four pairs of diploids and synthetic autotetraploids. Under controlled conditions, axenic plants were inoculated with pond waters collected from 10 field sites across a broad environmental gradient. Autotetraploids hosted 4%–11% greater bacterial taxonomic and phylogenetic diversity than their diploid progenitors. Polyploidy, along with its interactions with the inoculum source and genetic lineage, collectively explained 7% of the total variation in microbiome composition. Furthermore, polyploidy broadened the core microbiome, with autotetraploids having 15 unique bacterial taxa in addition to the 55 they shared with diploids. Our results show that whole-genome duplication directly leads to novelty in the plant microbiome and importantly that the effect is dependent on the genetic ancestry of the polyploid and generalizable over many environmental contexts.

KEYWORDS
Araceae, microbial community assembly, plant–microbe symbiosis, synthetic polyploidy, whole-genome duplication

1 | INTRODUCTION

Whole-genome duplication ("polyploidy"), which causes organisms to have greater than two sets of each chromosome, is a major force in ecology and evolution, especially for plants (Fox et al., 2020; Hao et al., 2022; Rice et al., 2019; Wood et al., 2009). A key consequence of polyploidy is that it causes phenotypic novelty at all levels of biological complexity, from subcellular traits (e.g. nucleus size) to entire populations (e.g. population growth rate; Anneberg et al., 2023; Doyle & Coate, 2019), and this has long-been appreciated to lead

to novelty in the ecology of organisms (Soltis et al., 2014). Yet, our understanding of the factors that contribute to the success of polyploids is dominated by evidence of their interactions with the abiotic environment. We have comparatively little knowledge about how polyploids interact with their surrounding biotic community, e.g., herbivores, pollinators and microbial symbionts (Forrester et al., 2020; Forrester & Ashman, 2020; Segraves, 2017; Segraves & Anneberg, 2016), although species interactions are likely a major driver of polyploid establishment and persistence (Oswald & Nuismer, 2011). Evidence is accumulating that—compared to their diploid ancestors—polyploids can associate with a different subset of the local biotic community and even associate with novel species, such as mutualists (e.g. pollinators) or antagonists (e.g. herbivores) (Forrester & Ashman, 2020; Van de Peer et al., 2021). These novel associations can, in turn, change the 'macroscopic' biotic community that plants associate with (Segraves, 2017).

A pivotal yet under-explored community-wide effect of polyploidy is the effect on the microbial community they host (Segraves, 2017; Segraves & Anneberg, 2016). Plant microbiomes consist of bacteria, fungi and viruses that live on (ectophytes) and within (endophytes) plant tissues (Cordovez et al., 2019). While studies have demonstrated differential effects of polyploidy on individual root bacterial and fungal taxa (Anneberg & Segraves, 2019; Forrester & Ashman, 2018), characterization of the whole microbial communities of roots (rhizosphere) or leaves (phyllosphere) has lagged behind. Yet, knowledge of ploidy-induced changes in the microbiome can contribute to answering the question of what drives polyploid establishment since the microbiome can influence plant performance and facilitate range expansion (Bai et al., 2022; Gould et al., 2018; Parshuram et al., 2023; Tan et al., 2021; Yan et al., 2022).

Phenotypic changes due to polyploidy may cultivate novel assemblages of microbes (Doyle & Coate, 2019; Fox et al., 2020). For instance, a key phenotypic change is cell and organ enlargement (Doyle & Coate, 2019). Due to a greater surface area, the enlarged organs of polyploids may allow greater microbial colonization and, in turn, increased microbial diversity (Glassman et al., 2017; Lyons et al., 2010; Peay et al., 2010). Larger organs may also relax competition among potentially colonizing microbes (Ghoul & Mitri, 2016), allowing competitively inferior taxa to persist, thus increasing microbial diversity. Other key traits such as stomatal size, chlorophyll content of cells and secondary metabolite production could impact bacterial colonization or persistence. For example, neopolyploids of *Arabidopsis thaliana* have larger stomates with a higher concentration of leaf chlorophyll (Yu et al., 2009), suggesting that neopolyploids could achieve greater metabolic potential than their diploid ancestors, thereby promoting the colonization and growth of microbes that associate with the leaf. Other studies have demonstrated that polyploidy can increase immune gene expression and secondary metabolite accumulation (Lavania et al., 2012; Song & Chen, 2015), suggesting that polyploidy may enhance the ability of plants to suppress or better tolerate pathogen colonization. Taken together, the many phenotypic novelties caused by neopolyploidy might also lead to novelty in the plant microbiome.

A handful of recent studies have shown that whole-genome duplication has variable effects on plant microbial communities. Wipf and Coleman-Derr (2021) sequenced the bacterial rhizosphere of diploid, tetraploid and hexaploid wheat species from a single field site and found that polyploid species hosted more microbial taxa (alpha diversity) and the composition of their root microbiome (beta diversity) was significantly differentiated from diploids. Similarly, synthetic tetraploids from two genotypes of *A. thaliana* were exposed to a single inoculum source and found to host a greater abundance, but similar diversity, of bacterial taxa in the rhizosphere compared to their diploid ancestors (Ponsford et al., 2022). However, another experiment using seven synthetic tetraploid genotypes of *A. thaliana* found that polyploidy did not restructure the microbiome when exposed to a synthetic community of 16 bacterial taxa but the polyploids were more tolerant to pathogenic attack than their diploid progenitors (Mehlferber et al., 2022). However, they also found that diploids inoculated with the synthetic microbiome performed better than the uninoculated diploids when attacked by pathogens (Mehlferber et al., 2022), which may imply some differential recruitment of the microbiome in polyploids versus diploids. These latter two studies used synthetic "neopolyploids" (incipient polyploids), rather than established polyploids, and thus avoided the confounding effects of subsequent evolution following the whole-genome duplication event, and yet they still observed variable responses to polyploidy, suggesting that the genotype of origin can alter the impact of whole-genome duplication. To determine the relative roles of neopolyploidy and inoculum source on the plant microbiome, we need studies that not only include multiple host genotypes but also multiple inoculum sources. Indeed, the set of microbial taxa that frequently colonize their host plants across a broad array of environmental contexts and include the heritable set of microbes, are known as the 'core microbiome' and represent those most likely to have direct effects on plant fitness (Risely, 2020; Wagner et al., 2021). Yet, we have not determined whether the core microbiome of any plant species differs based on ploidy level.

The effect of neopolyploidy on the plant microbial community likely varies with genetic ancestry since polyploids often arise repeatedly from genetically different diploids (Soltis et al., 2016; Soltis & Soltis, 1999), and these independent maternal origins can strongly influence the degree of phenotypic and genetic divergence from their diploid progenitors (Doyle & Coate, 2019; Oswald & Nuismer, 2011; Pacey et al., 2020; Wei et al., 2020). Having increased genetic diversity among neopolyploid plants could also impact the taxonomic composition of microbial communities since genotype is a strong predictor of microbiome assembly in many studies (Dastogeer et al., 2020). Thus, we expect that the effect of neopolyploidy on microbiome composition will vary across multiple genetic origins and by testing this assertion, we can more accurately measure the repeatable effect of polyploidy on the plant core microbiome.

Variation in environmental setting (i.e. the abiotic and biotic parameters of a site) is likely to influence the effect of polyploidy on microbial communities (Eckert et al., 2022; Li et al., 2023). In particular, the taxonomic diversity within inoculum can co-vary with the

abiotic environment, such as with temperature and nutrient availabilities (Bakker et al., 2015). Therefore, if polyploids preferentially associate with microbial taxa that are not present in certain environments, this could constrain ploidal differences, but if polyploid genotypes vary more than diploids in their response to the environment (e.g. Pacey et al., 2020; Wei et al., 2019), then environmental variation may increase ploidal differences. Finally, if polyploids cultivate a more diverse microbiome, then we may expect that whole-genome duplication will broaden the taxonomic core microbiome (by including novel microbes) across these diverse ecological settings, but no study has addressed the relative roles of whole-genome duplication or genetic background on plant microbial community composition across a variety of ecological settings.

We tested how polyploidy affects plant bacterial communities using experimental inoculation of axenic diploids and synthetic neotetraploids of the aquatic plant "Greater Duckweed" *Spirodela polyrhiza* (L.) Schleid (Araceae). Duckweeds are quickly becoming a model system for studying the microbial ecology of plants (Baggs et al., 2022; Jewell et al., 2023; Lam et al., 2014; O'Brien, Laurich, et al., 2020; O'Brien, Yu, et al., 2020; Tan et al., 2021), due in part to their simple morphology but also because their microbiomes are strongly filtered from their local aquatic habitat (Toyama et al., 2009). Additionally, their compact size and rapid generation time of 4–5 days (Acosta et al., 2021; Ziegler et al., 2015) make duckweed an ideal system for testing how polyploidy affects microbial community assembly in laboratory settings.

We grew four genetically distinct pairs of diploids and their immediate neopolyploid descendants (Anneberg et al., 2023) individually in different water sources collected from 10 different ponds to answer the specific questions: (1) How does neopolyploidy affect the alpha and beta diversity of the duckweed bacterial community? (2) Does the effect of neopolyploidy on bacterial alpha and beta diversity depend more on diploid genetic ancestry or the ecological source of inoculum? (3) Compared to their diploid progenitors, is the core microbiome of neopolyploids taxonomically broadened or restricted? (4) If so, what are the key bacterial taxa that differentiate diploid versus neopolyploid core microbiome?

2 | MATERIALS AND METHODS

2.1 | Study system

Spirodela polyrhiza is one of the largest duckweed species (Acosta et al., 2021), with vegetative fronds floating on the water surface and numerous simple roots growing into the water column. They have a cosmopolitan distribution (Wang et al., 2011), and naturally occur in freshwater habitats. Although they inhabit aquatic habitats, their bacterial community assembly principles are similar to those found in terrestrial plants, and their microbiome is important for population growth and stress tolerance (e.g. Acosta et al., 2020; Iwashita et al., 2020; O'Brien, Laurich, et al., 2020; Tan et al., 2021). We used colchicine-induced neo-autotetraploid and colchicine-exposed but unconverted diploids described in Anneberg et al. (2023) to test our questions. Nevertheless, previous studies following colchicine treatment did not detect a significant effect of colchicine on plant phenotype (Anneberg et al., 2023). We confirmed stable tetraploidization with flow cytometry twice after initial treatment and then again at the time of these experiments—nearly 3 years and many clonal generations later (Wei et al., 2020). These neotetraploids (hereafter 'neopolyploids') came from four distinct diploid genotypes (hereafter 'genetic lineages') of *S. polyrhiza* collected in western Pennsylvania and eastern Ohio, USA (Table S1). These founding genotypes differed in multilocus genotype based on nine microsatellite loci (Table S1, following Kerstetter et al., 2023; Xu et al., 2018). Previous work has shown that these neopolyploid lineages are larger than their diploid ancestors and vary in their growth rates, showing that neopolyploidy has led to phenotypic novelty in this system (Anneberg et al., 2023). To eliminate the microbial taxa that are sourced from the laboratory environment rather than natural settings, we generated axenic cultures of duckweeds 2 weeks prior to the inoculation by following a modified protocol from Barks et al. (2018). Prior to surface sterilization, we activated recalcitrant dormant microbes colonized on tissues by pre-culturing the duckweeds in half-strength Schenk–Hildebrandt media supplemented with 6.7 g/L sucrose, 0.067 g/L yeast extract and 0.34 g/L tryptone powder for 24 h (Schenk & Hildebrandt, 1972). We then submerged all pre-cultured tissues in 0.8% sodium hypochlorite solution in autoclaved deionized water (v/v) with gentle mixing using sterile forceps for 6 min before placing the sterilized plants in fresh quarter-strength Appenroth media (Appenroth et al., 1996).

2.2 | Inoculum sourcing

We sampled inoculum as bulk water from 10 ponds with large natural populations of *S. polyrhiza* spanning from northern New York, USA to northern West Virginia, USA (Figure 1; Table S2). Therefore, our inoculum represents both biotic and abiotic attributes of these ponds. Between August 16 and 28, 2021, we sampled 3 L of raw pond water at each site from directly beneath a patch of wild duckweed near the shore by submerging two sterilized glass screwcap flasks fitted with 0.6 mm pore size mosquito netting over the opening to avoid collecting large solids. To profile the abiotic conditions of the inoculum source environments from where we collected water, we measured pH and took note of the site elevation and GPS coordinates (latitude and longitude). We additionally collected 500 mL of pond water from each pond source in the same way as described above and analysed these samples for a set of abiotically relevant parameters that strongly influence microbial diversity (Dastogeer et al., 2020; Sadeghi et al., 2021; Tang et al., 2020). Specifically, total dissolved solids (TDS), total phosphate, total nitrate, iron, calcium carbonate and sulphate were analysed at the Agriculture Analytical Services Lab at Pennsylvania State University. Last, as a control for contamination due to airborne microbes at sites or transportation of the water back

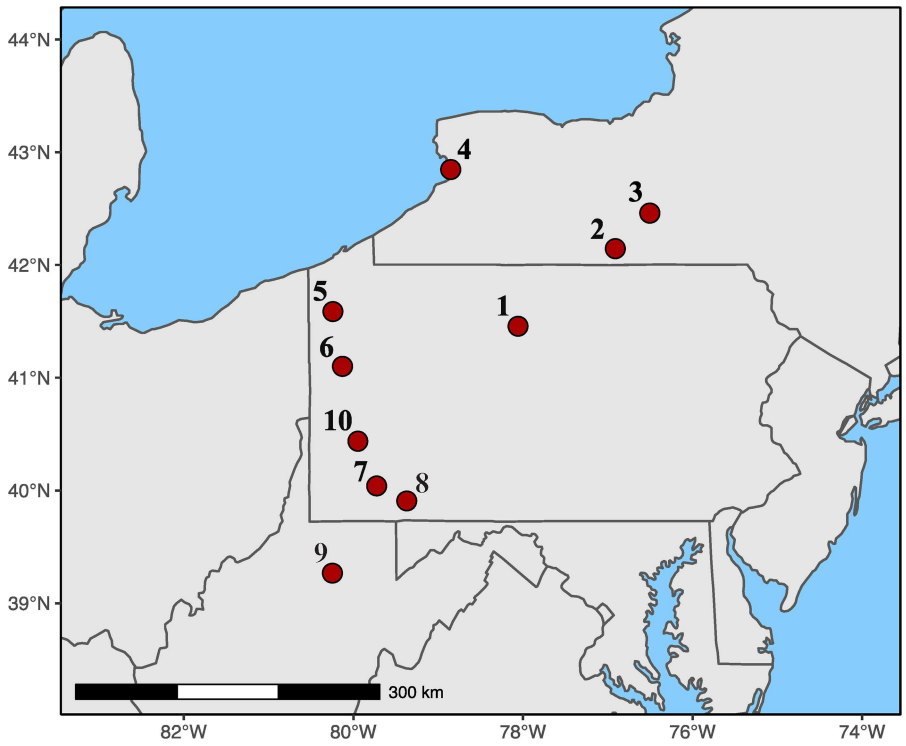


FIGURE 1 Map of the sources of inoculum in the northeastern United States. Source ponds correspond to the numbers Table S1. [Colour figure can be viewed at wileyonlinelibrary.com]

to the lab, we opened a sterile 50-mL falcon tube filled with autoclaved Millipore water and left this tube uncapped and exposed to the air for 2 min. We included one 'field' control for each of the 10 sites. All samples collected from sites were placed into a cooler filled with ice and transported back to the University of Pittsburgh within 24 h of collection.

2.3 | Experimental inoculation

To begin the inoculation experiment, for each pond source, we decanted 20 mL of that pond water into 48 sterile 25-mL glass test tubes (480 tubes total across all sites). Each tube then received three sterile *S. polyrhiza* fronds of a given ploidy level from a given genetic lineage. There were six replicates of each ploidy-genetic lineage-source combination. Due to the large geographic area of sampling, the experimental inoculation was conducted in three temporal blocks (Block 1: four sources; Block 2: three sources; Block 3: three sources), all within a 2-week period. Each lineage of axenic surface-sterilized duckweed was grown in sterile half-strength Appenroth media (hereafter 'media control') for each temporal block (48 tubes per block; 144 tubes total across three blocks). Plants from these media controls were used to evaluate bacterial taxa which are recalcitrant to our sterilization technique to generate axenic duckweed. In total, we grew 624 *S. polyrhiza* samples distributed across two ploidy levels, four genetic lineages and ten inoculum water sources, plus six sterile 'media' controls for each genetic lineage by ploidy level by collection block. To prevent environmental contamination but also allow air exchange, we capped each of the 25-mL glass tubes with an autoclaved gas-permeable sterile plastic cap. The tubes were placed in a completely randomized design in a growth chamber set to 16:8 L:D cycle with 25°C constant temperature and 50% relative humidity and incubated for 2 weeks, which was long enough for approximately three duckweed generations, until harvest.

We collected whole duckweeds for bacterial sequencing analysis by gently collecting plants with forceps via sterile technique and allowing them to drip dry on the sides of tubes. The drip-dried plant was then placed into a labelled 1.5-mL sterile centrifuge tube and stored in a −80°C freezer until DNA could be extracted from whole plant tissue, fronds and roots included. Thus, the bacterial communities reported represent both endo- and ectophytes from both the rhizosphere and phyllosphere.

2.4 | Bacterial DNA sequencing and taxonomic identification

We randomly selected four to five of the six replicates of each ploidy-genetic lineage-inoculum source combination and extracted DNA from approximately 150 mg of tissue. We chose to extract DNA from equivalent amounts of tissue because neopolyploid fronds are heavier than diploid fronds (Anneberg et al., 2023). DNA was extracted using Zymo Quick-DNA Faecal/Soil Microbe Miniprep (Zymo Research) and following the manufacture's protocol with the exception that the DNA elution buffer was increased in volume and time before centrifuging. In total, 352 duckweed samples and 19 media control samples were stored in a −20°C freezer until sequencing for the V5-V6 region of the bacterial 16s rRNA gene via the 799f

and 1115r primer sets (Gilbert et al., 2014). One lane of paired-end sequencing on an Illumina MiSeq platform (Caporaso et al., 2012) was performed by Argonne National Lab (Illinois, USA).

A total of 15,692,711 reads were obtained, quality filtered and processed as follows. Using histograms of forward and reverse read quality scores and the dada2 plugin (Callahan et al., 2016) in Qiime2 (Bolyen et al., 2019), we trimmed forward reads by 20 base pairs and reverse reads by 14 base pairs and truncated based on length (210 in the forward reads and 220 in the reverse reads). We next denoised the sequencing reads with default parameters of the dada2 plugin followed by generating a multiple sequence alignment tree with the fasttree Qiime2 function via mafft (Katoh & Standley, 2013). To assign taxonomy to each amplified sequence variant (ASV), we used the Qiime2 feature classifier plugin (Bokulich et al., 2018) with the Silva small subunit rRNA database release 138.1 (Quast et al., 2013). To merge the ASV counts table, multiple alignment tree, taxonomy table and metadata files, we imported these files into R (R Core Team, 2021) with the phyloseq package (McMurdie & Holmes, 2013). Since one of the kit controls did not amplify any sequences, the dada2 pipeline removed that sample from the data set, and the resulting phyloseq object comprised 10,365 ASVs across 370 samples. We filtered out mitochondrial and chloroplast DNA and this removed 1768 ASVs, leaving 8597 bacterial ASVs in the data set. We further filtered the phyloseq data by removing any ASVs that were detected in either our media controls or field water controls from each collection site. This removed another 189 ASVs, leaving 8408 ASVs from 320 samples. The relative taxonomic abundance of these 189 ASVs that were detected in our sterile duckweed (media controls) is presented in Figure S1, and a full taxonomy table of these taxa is available (Appendix S1). Our final filtering step involved applying a loss function to the ASV table to remove spurious taxa that were rarely detected and could be the product of sequencing error or misclassification. Rather than applying an arbitrary abundance threshold cut-off, we used the PERFect package (Smirnova et al., 2019) in R to permutationally filter out rare ASVs that do not have a detectable effect on the entire ASV covariation matrix (Smirnova et al., 2019). By passing our phyloseq object through PERFect filtering, we removed 5122 ASVs that were in insignificantly low read abundance in the data set, resulting in a final filtered phyloseq object comprised 3286 ASVs across 320 experimentally inoculated duckweed samples. We first used this final phyloseq data set to assess absolute read abundances between diploids and neopolyploids, and also across the 10 inoculum pond water sources and found no differences among these treatment groups (Figure S2). With this phyloseq data set, we then normalized the reads per sample to the overall median (Wei & Ashman, 2018).

2.5 | Statistical analysis

We quantified the effect of ploidy level, genetic lineage and inoculum source on the alpha diversity of the duckweed bacterial community. To calculate bacterial alpha diversity, we used the phyloseq function "estimate_richness" to determine taxonomic (ASV) richness and Shannon diversity. We calculated Faith's phylogenetic diversity with the function "pd". Because we started the inoculation experiment in three separate blocks spread over 2 weeks, we tested for a temporal block effect in our statistical analyses. No block effect was observed in diversity analyses and is therefore not reported further. All statistical analyses were performed with R software (R Core Team, 2021), using the base stats functions as well as the packages ampvis2 (Andersen et al., 2018), edgeR (Robinson et al., 2010), lsmeans (Lenth, 2016), picante (Kembel et al., 2010) and vegan (Dixon, 2003). Each alpha diversity metric was analysed with a linear model, defining ploidy level, genetic lineage and inoculation source as main effects, along with their interaction terms.

To estimate beta diversity of duckweed bacterial communities, we analysed Bray–Curtis dissimilarities. We calculated Bray–Curtis dissimilarities with the distance function in the phyloseq package and then used the Adonis2 function from the vegan package to fit a permutational analysis of variance (PERMANOVA) model to the data. In the PERMANOVA, we defined the model as Bray–Curtis dissimilarities explained by ploidy level, genetic lineage, inoculation source and their interaction terms. We then tested for homogeneity of variance among the groups in the PERMANOVA model by measuring beta dispersion for each factor via the betadisper function in the vegan package. We further assessed whether bacterial community compositions of neopolyploids are more likely to resemble each other instead of diploids by conducting a nearest neighbour analysis of the Bray–Curtis dissimilarities of samples. We did so by constructing a minimum spanning tree (Friedman & Rafsky, 1979) which aligns samples based on their Bray–Curtis dissimilarities. To test whether nearest neighbours of samples belong to the same groups or not, we conducted a permutational graph-based test by using the graph_perm_test function from the phyloseqGraphTest package (Fukuyama, 2020) in R with 10,000 permutations.

To infer which bacterial ASVs contribute to the differentiation of the diploid and neopolyploid communities, we built a random forest model (Breiman, 2001) with the randomForest function and specifying 2000 trees. Using this model, we classified diploid versus neopolyploid microbiomes given the differences in their ASV communities. We recorded the out-of-bag error rate of the overall model and the specific class errors between diploids and neopolyploids, respectively, with the confusion matrix. From the random forest model and using the "importance" function in R, we derived a list of the 30 most important ASVs that discriminate diploid versus neopolyploid microbiomes based on the mean decrease in the Gini impurity index (Nembrini et al., 2018) for each ASV. We further tested for key taxa differentiating neopolyploids from their diploid progenitors by quantifying the $\log_{10}$-fold change of the most differentially abundant ASVs between diploids and neopolyploids. To do so, we first selected only the ASVs with variance in their counts below a threshold of $1*10^{-6}$ and used the phyloseq_to_edgeR function from McMurdie and Holmes (2013) to carry out an exact test of ASV abundance between diploids and neopolyploids using the exacttest function in the edgeR package (Robinson et al., 2010). We then adjusted p-values

from multiple testing following Benjamini and Hochberg (1995) while implementing the topTags function in the edgeR package.

Last, we assessed the effect of polyploidy on the composition of the core microbiome via the "amp_venn" function. We used a minimum 0.1% relative abundance as a cut-off for being included in the core. After accounting for the relative abundance cut-off, we followed other similar studies in their determination of the core bacterial microbiome by using a frequency cut-off of 50% (Ainsworth et al., 2015; Vidal-Verdu et al., 2022), meaning that any "core" ASV must be colonized in at least 50% of all samples within any one of the three possible ploidy groupings (2× exclusive, 2× and 4× combined and 4× exclusive core microbiome). However, we also report on a second, more conservative relative frequency cut-off of ≥75% to evaluate how restricted the core microbiome estimation can be to relative frequencies within groups.

3 | RESULTS

3.1 | Polyploidy increases the alpha diversity of the duckweed bacterial microbiome

Neopolyploids had significantly greater bacterial alpha diversity than their diploid progenitors across all three diversity measures (Table 1): Polyploidy increased taxonomic diversity by 4%–11% (Shannon diversity and species richness respectively) and phylogenetic diversity by 8% (Faith's PD) (Figure 2). Despite the alpha diversity of the bacterial microbiome of duckweed significantly depending on the inoculum source water, it did not interact with ploidy level (Table 1).

3.2 | The effect of polyploidy on the duckweed microbiome composition depends on environment and genetic background

Unsurprisingly, bacterial communities were strongly differentiated among inoculum sources, as they were collected from ponds hundreds of kilometres apart which differ in numerous measured (Table S2) and unmeasured biotic and abiotic conditions (Wagner et al., 2021). The effect of polyploidy on beta diversity of duckweed bacterial communities was influenced by genetic lineage and inoculum source (Figure 3). This three-way interaction between ploidy, genetic lineage and inoculum source explained approximately 4% of the total variation in Bray–Curtis dissimilarities among samples ($F_{27,240}=1.14$, $p=.029$; Table 2; Figure S4). Beyond this complex three-way interaction, there was a polyploidy by inoculum source interaction that explained approximately 2% of the variation in the model as well as an interaction between polyploidy and genetic lineage explaining an additional 1% of variation (Table 2).

The minimum spanning tree showed that neopolyploid bacterial communities resemble each other significantly more than they resemble the bacterial communities of their diploid ancestors ($p<.0001$; Figure 4a). This is apparent in that the most similar samples (nearest neighbours in Bray–Curtis dissimilarity) are more often the same ploidy (Figure 4a, filled circles sharing the same colour), rather than not (i.e. nearest circle having a different colour). Similarly, samples inoculated with the same pond water resembled each other significantly more than a random neighbour in Bray–Curtis dissimilarity ($p<.0001$ Figure 4b, closer dots share colours).

3.3 | Key taxa differentiate diploid and neopolyploid duckweed microbiomes

Several bacterial taxa uniquely colonize neopolyploids relative to diploids. The random forest model that classified the ploidy level of samples based on the Bray–Curtis dissimilarities among samples had an out-of-bag error rate of 27%, meaning that the model could correctly identify whether a sample bacterial community belongs to either a diploid or neopolyploid 73% of the time. This random forest model was not biased in the ability to correctly identify a diploid bacterial community from a neopolyploid community, as indicated by the accompanying confusion matrix showing that the class-wise

TABLE 1 Results of ANOVAs of the effects of ploidy, genetic lineage, inoculation source and their interactions on taxonomic diversity (Species richness and Shannon diversity) and phylogenetic diversity (Faiths' PD) of bacterial communities of duckweed.

		Shannon diversity		Species richness		Faith's PD	
Factor	***df***	***F* value**	***p* value**	***F* value**	***p* value**	***F* value**	***p* value**
Ploidy	1	6.97	**.009**	6.22	**.013**	5.79	**.017**
Lineage	3	2.03	.111	1.38	.249	1.78	.152
Inoculum source	9	2.59	**.007**	1.71	.088	3.95	**<.001**
Ploidy: Lineage	3	0.24	.866	0.16	.924	0.14	.935
Ploidy: Inoculum source	9	0.86	.562	0.30	.975	0.44	.910
Lineage: Inoculum source	27	0.66	.903	0.72	.840	0.73	.830
Ploidy: Lineage: Inoculum source	27	0.77	.793	0.68	.880	0.81	.740

Note: Significant factors are in bold. There were 240 residual degrees of freedom across all three alpha diversity models. For model coefficients of each linear model, see Tables S3, S4 and S5.

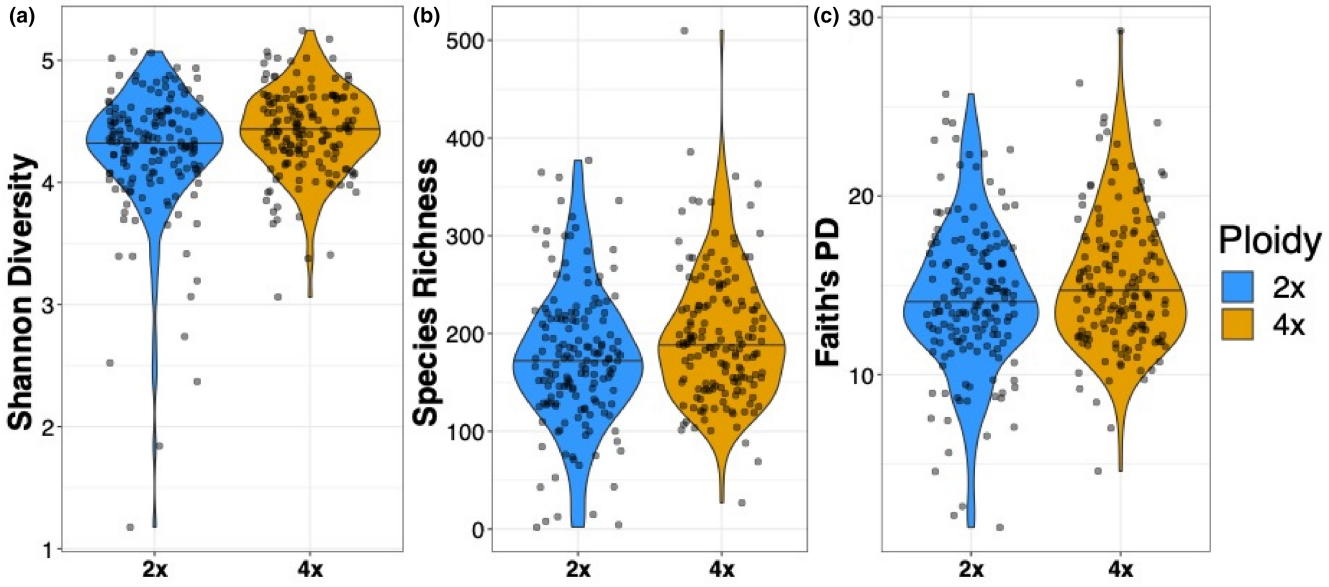
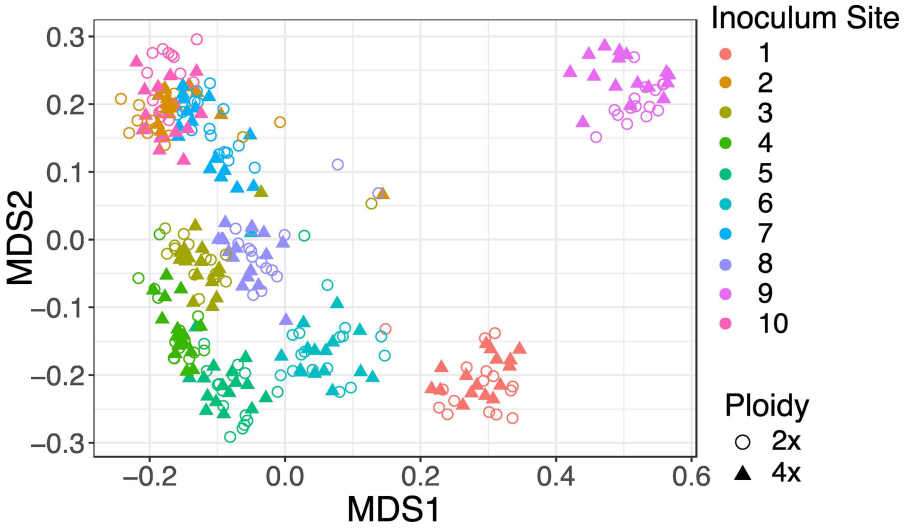


FIGURE 2 Violin plots showing the median and distribution of alpha diversity of bacterial communities for diploids (2×) and neopolyploids (4×). Neopolyploids had greater alpha diversity than their diploid progenitors for (a) Shannon diversity, (b) Species richness and (c) Faith's phylogenetic distance. The median is denoted by a lateral black line in each violin plot and independent samples are represented by each black point plotted over the violin plots. [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 3 PCoA plot of bacterial Bray–Curtis dissimilarities colonized on diploid (2×) and neopolyploid (4×) duckweeds. Each point represents an independent duckweed plant and is coloured by the site of the inoculum source (number represents the locations in Figure 1) and shapes represent ploidy level. [Colour figure can be viewed at wileyonlinelibrary.com]



error rates were approximately equal for both diploid and neopolyploid bacterial communities (Table S6). The 30 indicator ASVs that discriminate diploid and neopolyploid *S. polyrhiza* were primarily members of the families Rhizobiaceae ($n=7$) and Comamonadaceae ($n=6$) in the Alphaproteobacteria and Gammaproteobacteria respectively (Figure 5; Table 3).

For the bacterial taxa shared by diploid and neopolyploid duckweed, we found 90 ASVs that were significantly differentially abundant between the two ploidy levels (Figure 6). Of these 90 ASVs, 63 of them were members of the Proteobacteria. The most common taxonomic families of the differentially abundant ASVs were 23 members of the Comamonadaceae and 11 members of the Rhizobiaceae. Neopolyploids tended to have greater abundance of these ASVs than diploids (Figure 6).

TABLE 2 Results from PERMANOVA to evaluate the effect of ploidy, genetic lineage, inoculation source and their interactions on beta diversity of duckweed bacterial communities (Bray–Curtis dissimilarities).

Factor	*df*	R^2	*F* value	*p* value
Ploidy	1	0.005	3.93	**<.001**
Lineage	3	0.010	2.77	**<.001**
Inoculum source	9	0.576	52.10	**<.001**
Ploidy: Lineage	3	0.006	1.66	**.003**
Ploidy: Inoculum source	9	0.021	1.86	**<.001**
Lineage: Inoculum source	27	0.050	1.52	**<.001**
Ploidy: Lineage: Inoculum source	27	0.038	1.14	**.029**
Residual	240	0.295		

Note: Significant effects in the model have a bolded *p* value.

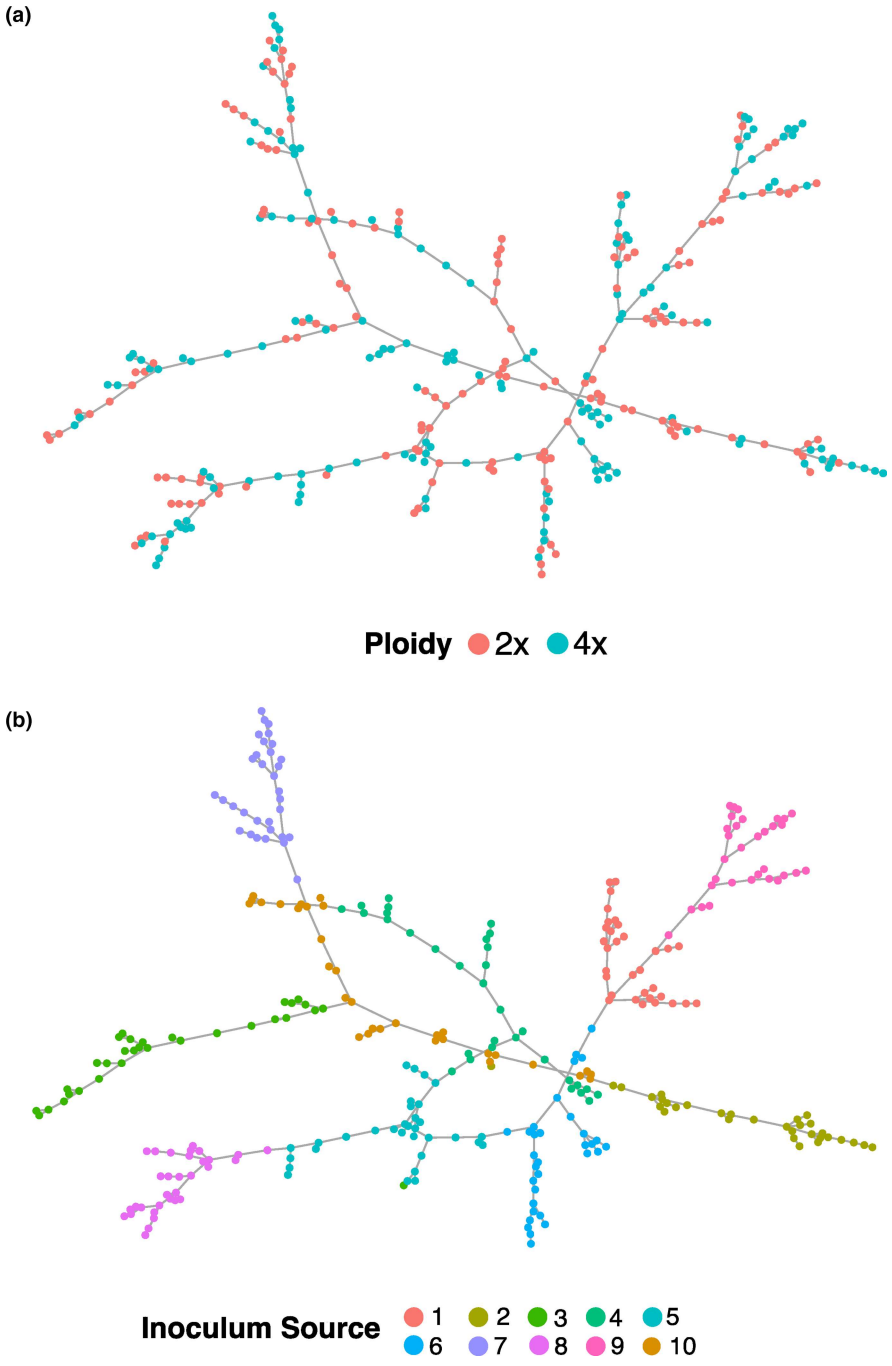


FIGURE 4 Minimum spanning trees that align samples of bacterial communities according to their nearest neighbour in Bray–Curtis dissimilarity, showing when they are coloured by (a) the ploidy level of the *S. polyrhiza* host plant (diploid-2×; nepolyploid-4×), and (b) the inoculum source (number represents the locations in Figure 1) that duckweeds were inoculated with at the beginning of the experiment. [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Polyploidy broadens the core microbiome

When the core microbiome was characterized by a 50% frequency cut-off, there were 55 bacterial ASVs shared between diploids and neopolyploids, 15 ASVs uniquely hosted by neopolyploids and none were unique to diploids (Table 4, Appendix S2). Increasing the stringency of the frequency cut-off defining the core to 75% yielded 15 shared core ASVs between diploids and neopolyploids, and 5 ASVs that were unique to neopolyploids (Table 4). At this level of stringency, the majority of the shared bacteria were members of the Proteobacteria belonging to either the Burkholderiales or Rhizobiales order (Appendix S2). Although we were unable to resolve the taxonomy of the five ASVs belonging to the neopolyploid-exclusive core bacterial more specifically than the family level, one was a member of the Actinobacteriota phylum belonging to the Illumatobacteriacea family, and the other four were members of the Proteobacteria belonging to the families: Comamonadaceae, Rhizobiales (*Incertae sedis*) Solimonadaceae, Xanthomonadaceae (Appendix S2).

4 | DISCUSSION

Our culture-independent test of how neopolyploidy affects the duckweed microbiome across a broad set of naturally sourced

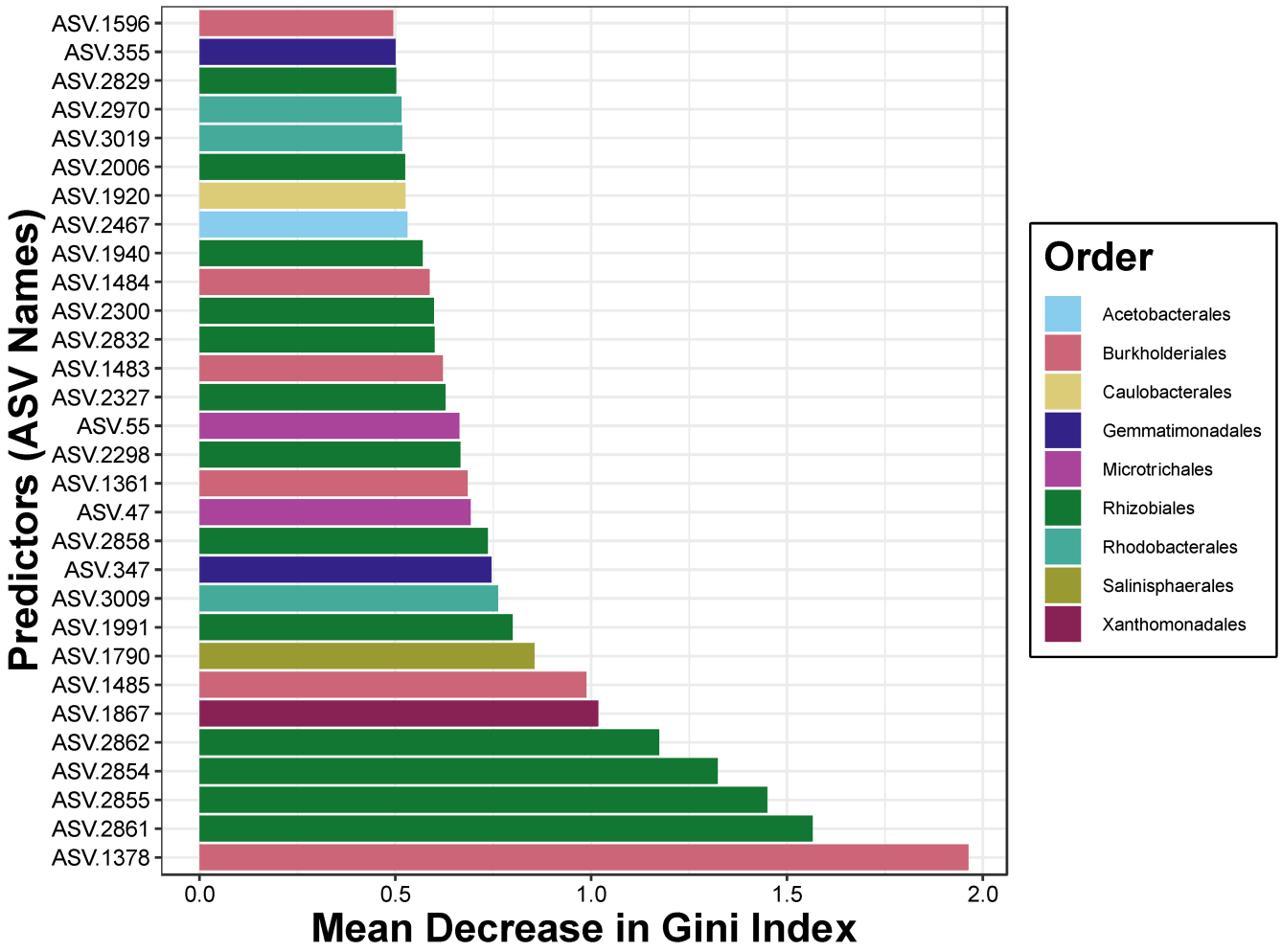


FIGURE 5 Gini index plot which shows the most important bacterial ASVs delineating the bacterial communities of diploids from neopolyploids in the random forest model. The greater the mean decrease in the Gini index, the more important that ASV is in discriminating diploids from neopolyploids. Individual bars are coloured by their taxonomic order and the ASV names correspond to the ASV number in the taxonomy table in Table 3. [Colour figure can be viewed at wileyonlinelibrary.com]

inocula revealed that ploidy level, genetic lineage, pond water source and their interactions play major roles in the diversity and composition of the plant microbiome (Table 2). As is common in microbiome assembly, inoculum source (environment) played a stronger role than host genetics (reviewed in Wagner et al., 2021), but even in spite of this, the effect of polyploidy on its own or in combination with source water or lineage had a measurable signature. For instance, despite the composition of the bacterial microbiome of *S. polyrhiza* generally remaining the same across the 10 field sites (Figure S3), there were certain bacterial taxonomic groups that were only present from some field sites, such as members of the Chloroflexia which were most abundant in source ponds 4 and 9 (Figure 1). These nuanced differences among the pond water sources led to an interaction between source and ploidy level on microbiome community composition (Table 2; Figure S4). Furthermore, our minimum spanning tree analysis showed that the nearest neighbours in bacterial community composition were of the same ploidy level more often than not (Figure 4). This suggests that the signature of polyploidy on bacterial community composition is observable across diverse natural aqueous environments which encompasses variability in both abiotic and biotic diversity. Consequently, we found that polyploidy increases the taxonomic and phylogenetic diversity of the bacterial community as well as broadening the taxonomic core microbiome of *S. polyrhiza*. The neopolyploid core microbiome comprises novel taxa that did not colonize their diploid progenitors while retaining the taxonomic core members of their diploid progenitors. Thus, our results show that polyploidy can immediately broaden the biotic niche of plants, and importantly that this effect is dependent on both genetic ancestry as well as the ecological setting that neopolyploids are in.

Our manipulative experiment found that the effect of polyploidy on the plant bacterial microbiome is generalizable over multiple genetic origins and across a variety of pond water sources. While our work also corroborates similar previous work (Ponsford et al., 2022; Wipf & Coleman-Derr, 2021) in that neopolyploidy increased bacterial alpha diversity (Figure 2) and differentiated the plant bacterial community structure (Figure 3), we additionally found that this effect varied among the sources of inoculum as well as the multiple

TABLE 3 The 30 most important bacterial genera (or family) that discriminate microbiome composition of diploid and neopolyploid duckweeds based on a random forest model of ASVs.

ASV Name	Phylum	Class	Order	Family	Genus
ASV.47	Actinobacteriota	Acidimicrobiia	Microtrichales	Ilumatobacteraceae	NA
ASV.55	Actinobacteriota	Acidimicrobiia	Microtrichales	Ilumatobacteraceae	Ilumatobacteraceae
ASV.347	Gemmatimonadota	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas
ASV.355	Gemmatimonadota	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas
ASV.1361	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	NA
ASV.1378	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	NA
ASV.1483	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	NA
ASV.1484	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	NA
ASV.1485	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	NA
ASV.1596	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	Rhizobacter
ASV.1790	Proteobacteria	Gammaproteobacteria	Salinisphaerales	Solimonadaceae	Nevskia
ASV.1867	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Silanimonas
ASV.1920	Proteobacteria	Alphaproteobacteria	Caulobacterales	Hyphomonadaceae	Hirschia
ASV.1940	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia
ASV.1991	Proteobacteria	Alphaproteobacteria	Rhizobiales	Pleomorphomonadaceae	Chthonobacter
ASV.2006	Proteobacteria	Alphaproteobacteria	Rhizobiales	Pleomorphomonadaceae	Chthonobacter
ASV.2298	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Pedomicrobium
ASV.2300	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium
ASV.2327	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium
ASV.2467	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	Roseomonas
ASV.2829	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales	Uncultured
ASV.2832	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales	Uncultured
ASV.2854	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium-Neorhizobium
ASV.2855	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	NA
ASV.2858	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	NA
ASV.2861	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Allorhizobium-Neorhizobium
ASV.2862	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	NA
ASV.2970	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA
ASV.3009	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA
ASV.3019	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA

Note: ASV designation corresponds to the ASV predictors' Gini importance index values in Figure S1. NA under the genus column represents ASVs that were not identifiable at the genus level.

genetic lineages of neopolyploids that we used (Table 2). Since the total diversity within the bacterial microbiome increased with neopolyploidy and this effect did not interact with inoculum source or genetic background (Table 1), this suggests that polyploidy has a universal effect on the alpha diversity of the plant bacterial microbiome. In contrast, both the source of inoculum water as well as genetic lineage strongly interacted with polyploidy in structuring the beta diversity of the plant microbiome (Table 2), revealing that genetic ancestry and the ecological setting in which polyploids arise can have a deterministic effect on whether polyploidy differentiates bacterial community compositions from their diploid progenitors. The fact that some pond water sources did not lead to compositional differences in neopolyploid microbiomes from their diploid progenitors implies that these habitats could have an increased risk of neopolyploid extinction due to stronger niche overlap with diploids (Fowler & Levin, 2016; Rodriguez, 1996). Conversely, the pond environments in which neopolyploids had strongly differentiated bacterial microbiomes show a broadened biotic niche which could improve the odds of neopolyploid establishment through relaxing the competitive forces with their diploid ancestors.

Our analysis of the taxonomic core microbiome—the bacterial taxa that frequently colonize host plants across broad ecological contexts (Neu et al., 2021; Risely, 2020)—showed that neopolyploidy broadened the core microbiome of *S. polyrhiza* (Table 4). Following previous

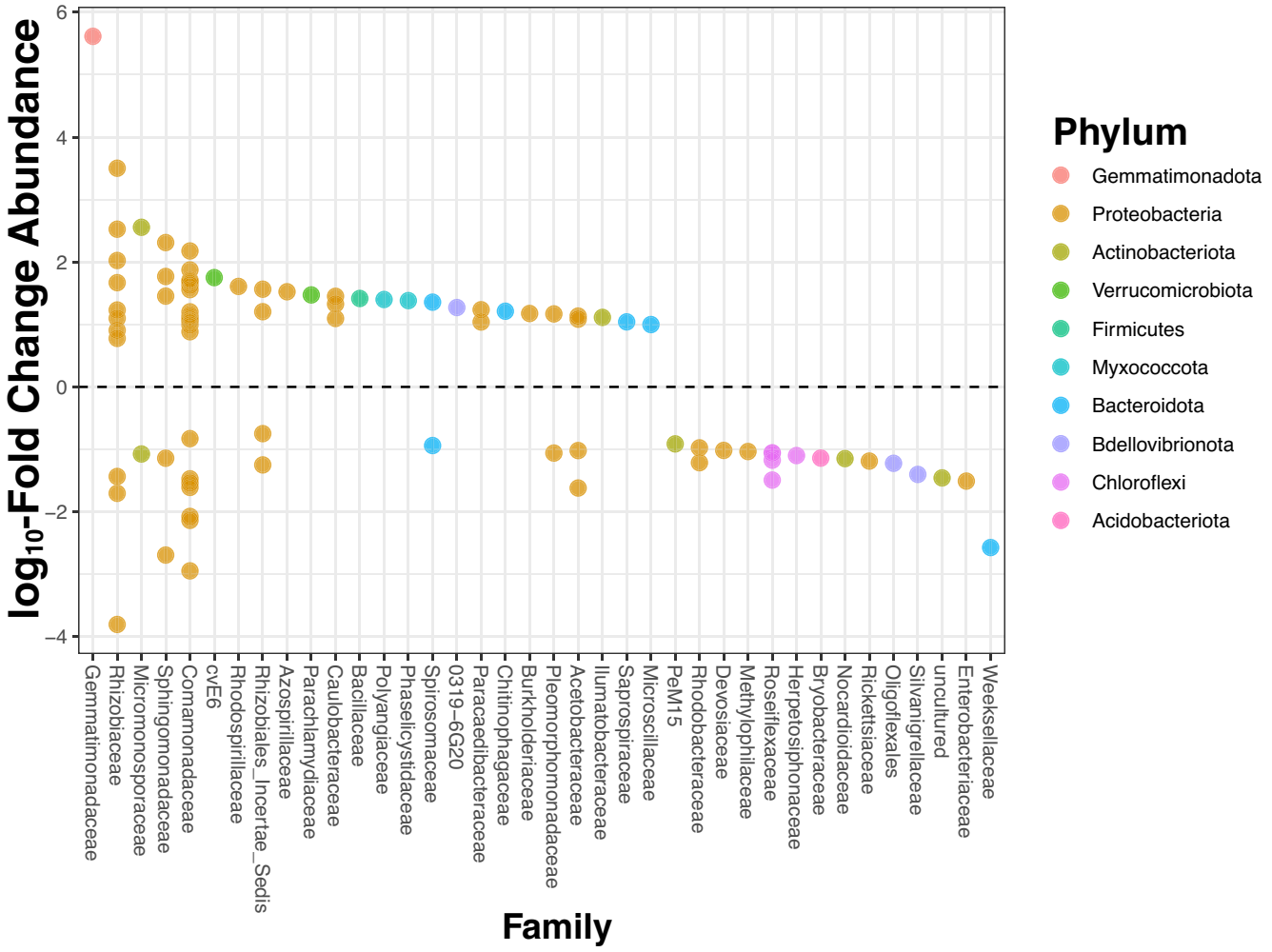


FIGURE 6 Log_{10}-fold change in the most differentially abundant bacterial ASVs (grouped by family on the *x*-axis) between diploid and neopolyploid *S. polyrhiza*. Any ASV denoted with a coloured dot above zero means that was found more often on neopolyploids than diploids, and the reciprocal applies to any ASV plotted below zero. [Colour figure can be viewed at wileyonlinelibrary.com]

studies that have used a 50% frequency cut-off in determining the core versus non-core microbiome taxa (Ainsworth et al., 2015; Vidal-Verdu et al., 2022), we found that neopolyploid core microbiome comprised an additional 15 taxa on top of the 55 taxa they share with the diploid core microbiome (Table 4). This result reveals that neopolyploidy in *S. polyrhiza* causes a broader biotic niche by associating with 27% more bacterial taxa. We can draw analogies of our findings to previous work that characterized how pollinator communities are influenced by polyploidy, such as in *Chamerion angustifolium*, in which diploids and polyploids strongly overlap in the pollinator communities that they host, but the polyploids are visited by two additional pollinator species that do not visit diploids (Kennedy et al., 2006). Taken together, our results show that neopolyploidy leads to immediate novelty in the biotic niche of plants.

Neopolyploids cultivated a greater abundance of key bacterial taxa in their microbiome compared to diploids. Similar to previous work that has characterized the bacterial microbiome of duckweeds, we found that members of the Proteobacteria were dominant across all duckweed samples (Acosta et al., 2020; Bunyoo et al., 2022; Iwano et al., 2020; Iwashita et al., 2020). However, polyploidy not only increased the relative abundance of these key bacterial taxa (Figure 6), but it also increased the phylogenetic diversity of bacteria that colonize duckweed (Figure 1), again demonstrating that polyploidy broadens the biotic niche. Although our 16s sequencing data could not resolve taxonomy below either the family or genus level, we found that polyploids host particularly more Rhizobiaceae and Comamonadaceae than their diploid progenitors (Table 3; Figure 6). The Rhizobiaceae are noteworthy for their diverse function in influencing plant growth, as some taxa within this family are growth-promoting for provisioning nitrogen to host plants while others can hinder plant growth through enhancing pathogen attack (Carareto Alves et al., 2014). Two key previous studies that have characterized the microbiome of duckweed have specifically found that duckweed have an increased representation of nitrogen-fixing bacterial members of the genus *Rhizobium* within the Rhizobiaceae compared to the surrounding environment (Acosta et al., 2020; Zhao et al., 2015). Similarly, Bunyoo et al. (2022) found that members of the genera *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* increased in abundance on duckweed in

TABLE 4 The number of core bacterial ASVs either shared between diploid and neopolyploid duckweeds or uniquely hosted by them.

	Frequency cut-off (%)	Number of ASVs	Relative abundance (%)
Diploid core	50	0	0
Neopolyploid core	50	15	4.9
Shared core	50	55	43.2
Non-core	50	3216	51.9
Diploid core	75	0	0
Neopolyploid core	75	5	2.4
Shared core	75	15	20.2
Non-core	75	3216	77.4

Note: Darker shaded rows denote the core bacterial ASVs when we used a frequency cut-off of 50% across communities of diploids, neopolyploids or both, and the lighter shaded rows denote a 75% frequency cut-off. The relative abundance column refers to the relative abundance of the corresponding number of ASVs in their respective rows.

response to stress conditions, with a notable enrichment of nitrogen metabolism gene pathways from metagenomic sequencing. While our data set did not have the taxonomic precision to test for differences between diploids and neopolyploids in the colonization of nitrogen-fixing bacteria, the significantly greater colonization of members of the Rhizobiaceae on polyploids warrants future metagenomic studies that could achieve that level of precision.

By using multiple genetic lineages of axenic diploids and neopolyploids inoculated with a variety of pond water sources, we revealed that whole-genome duplication can immediately expand the plant biotic niche. In particular, polyploidy not only increased the diversity and restructured the composition of the bacterial microbiome, but it also caused many of the dominant taxa in the duckweed microbiome to increase in relative abundance, suggesting that neopolyploidy enhances the quality of the host plant for these dominant taxa or decreased the quality of the host for subordinate taxa. Future work should seek to understand how changes in the microbiome associated with host polyploidy could influence competitive interactions among diploids and their polyploid derivatives.

AUTHOR CONTRIBUTIONS

Thomas J. Anneberg, Tia-Lynn Ashman and Martin M. Turcotte conceptualized and designed the experiment. Thomas J. Anneberg collected field and laboratory samples, carried out the experiment, analysed the data and wrote the first draft of the manuscript. Thomas J. Anneberg, Martin M. Turcotte and Tia-Lynn Ashman edited subsequent manuscript drafts.

ACKNOWLEDGEMENTS

We thank Elizabeth O'Neill for the generation and maintenance of the synthetic neopolyploid lineages, Jason Simmons for assistance in field collection and media preparation and Nevin Cullen for feedback on experimental design and data analysis. This project was supported by the Dietrich School of Arts & Sciences and grants from the National Science Foundation (#2109452 to T.J.A., #2027604 & 1912180 to T-L.A. and #1935410 to M.M.T.).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest with this work.

DATA AVAILABILITY STATEMENT

All 16s rRNA gene sequences generated from this work are deposited into the NCBI SRA database under the accession ID PRJNA946155. We also deposited all underlying data in the production of this article to be made freely available from the Zenodo data repository (DOI: 10.5281/zenodo.7750374).

BENEFIT-SHARING

The benefits generated from this work accrue from our freely available data on public data repositories described above.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Anneberg, T. J., Turcotte, M. M., & Ashman, T.-L. (2023). Plant neopolyploidy and genetic background differentiate the microbiome of duckweed across a variety of natural freshwater sources. *Molecular Ecology*, *32*, 5849–5863. https://doi.org/10.1111/mec.17142