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Research

Polyploid plants obtain greater fitness benefits from a nutrient acquisition mutualism

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Summary

- Polyploidy is a key driver of ecological and evolutionary processes in plants, yet little is known about its effects on biotic interactions. This gap in knowledge is especially profound for nutrient acquisition mutualisms, despite the fact that they regulate global nutrient cycles and structure ecosystems. Generalism in mutualistic interactions depends on the range of potential partners (niche breadth), the benefits obtained and ability to maintain benefits across a variety of partners (fitness plasticity). Here, we determine how each of these is influenced by polyploidy in the legume–rhizobium mutualism.
- We inoculated a broad geographic sample of natural diploid and autotetraploid alfalfa (*Medicago sativa*) lineages with a diverse panel of *Sinorhizobium* bacterial symbionts. To analyze the extent and mechanism of generalism, we measured host growth benefits and functional traits.
- Autotetraploid plants obtained greater fitness enhancement from mutualistic interactions and were better able to maintain this across diverse rhizobial partners (i.e. low plasticity in fitness) relative to diploids. These benefits were not attributed to increases in niche breadth, but instead reflect increased rewards from investment in the mutualism.
- Polyploid plants displayed greater generalization in bacterial mutualisms relative to diploids, illustrating another axis of advantage for polyploids over diploids.

Introduction

Polyploidy, or the condition in which an organism contains more than two complete sets of chromosomes, is an important driver of ecological and evolutionary processes (Levin, 1983; Husband *et al.*, 2013; Soltis & Soltis, 2016). Polyploidy occurs in every major eukaryotic lineage but is particularly common in plants; all angiosperms share ancestral polyploid events and 24% of extant plant species are recent polyploids (Husband *et al.*, 2013; Barker *et al.*, 2016; Soltis & Soltis, 2016). Polyploidy can have profound effects on plant genomes, phenotypes and abiotic tolerances (Levin, 2002; Husband *et al.*, 2013), yet few studies have explored how polyploidy impacts biotic interactions, especially mutualisms (Segraves & Anneberg, 2016; Spoelhof *et al.*, 2017).

Plants engage in a variety of mutualisms that serve reproductive (e.g. pollinators, seed dispersers; Segraves & Anneberg, 2016) and nutrient acquisition (e.g. mycorrhizae, nitrogen-fixing bacteria; Shantz *et al.*, 2016) functions, which may be altered by plant polyploidy. Specifically, increases in cell size, enhancements in genetic diversity and physiological changes that occur after polyploidy events might permit polyploid plants to establish mutualisms with a broader range of partners or obtain greater fitness benefits from them (Segraves & Anneberg, 2016; Forrester & Ashman, 2018, 2019). The few studies testing these hypotheses have largely focused on reproductive mutualisms and have produced variable results (Thompson & Merg, 2008; reviewed by Segraves & Anneberg, 2016). Thus, it remains unclear whether polyploidy alters generalization along the axes of niche breadth and fitness benefits obtained from nutrient acquisition mutualisms, despite the fact that these drive global nutrient cycles and structure communities in natural, agricultural and urban environments (Bascompte *et al.*, 2003; Poisot *et al.*, 2011; Shantz *et al.*, 2016; Sprent *et al.*, 2017).

A model nutrient acquisition mutualism is the legume–rhizobium symbiosis, in which rhizobia fix atmospheric nitrogen (N) into a plant-usable form in exchange for photosynthetic resources (carbon) provided by plants (Wang *et al.*, 2012). From the plant perspective, generalization in rhizobial interactions can be defined by the taxonomic niche breadth of partners (Harrison *et al.*, 2018) and the extent of fitness benefits obtained from diverse partners (Forrester & Ashman, 2018). Plants showing more generalized rhizobial interactions might have the ability to establish mutualisms with diverse rhizobial partners, maintain high fitness across rhizobial strains (i.e. exhibit low plasticity in fitness) or reduce costs of associating with lower quality partners, resulting in more consistent and greater benefits obtained from the mutualism (Rodríguez-Echeverría *et al.*, 2008) than those with specialized rhizobial interactions.

Generalization could be enhanced by plant polyploidy if it increases the amount and diversity of resources available to invest in supporting rhizobial symbionts (Powell & Doyle, 2015; Forrester & Ashman, 2018). Polyploid plants often have greater photosynthetic rates (Warner & Edwards, 1993) and a greater diversity of compounds that function in mutualism establishment (e.g. flavonoids, nod factor receptors; Levy, 1976) and maintenance (e.g. nodule-specific cysteine-rich peptides, leghemoglobins; Young *et al.*, 2011; Li *et al.*, 2013). In addition, polyploid plants have larger cells, which might allow them to host a greater quantity of rhizobia, thereby increasing the amount of N obtained (Forrester & Ashman, 2018). These changes might enable polyploid plants to establish mutualisms with a broader range of rhizobial partners and/or host more or higher quality symbionts relative to diploids (reviewed by Forrester & Ashman, 2018; Forrester & Ashman, 2019). However, it remains unclear whether these differences translate to greater generalization in taxonomic niche breadth and host benefits obtained by polyploids across diverse rhizobial environments (Forrester & Ashman, 2018).

We conducted an inoculation experiment using a geographically widespread sampling of diploid (2*x*) and autotetraploid (4*x*) lineages of the plant species complex *Medicago sativa* and a diverse panel of *Sinorhizobium* bacterial symbionts. While synthetic neopolyploid plants can be used to isolate the immediate effects of polyploidy (Husband *et al.*, 2008; Ramsey, 2011), with some caveats (see Münzbergová, 2017), established polyploid lineages shed light onto the evolutionary consequences of genome duplication (Spoelhof *et al.*, 2017; Baduel *et al.*, 2018; Forrester & Ashman, 2019) and thus contribute to our understanding of successful polyploids. Ancient polyploidy is hypothesized to have enhanced legume mutualisms with rhizobia by duplicating genes that function in the establishment and maintenance of mutualism (Young *et al.*, 2011; Li *et al.*, 2013), yet no studies have tested biotic niche divergence in a polyploid (e.g. *M. sativa*; Spoelhof *et al.*, 2017) or fitness gains conferred to natural polyploids in relation to it.

We sought to determine whether autotetraploid plants: establish mutualisms with a broader range of rhizobial symbionts, obtain greater fitness benefits from rhizobial mutualisms, exhibit reduced plasticity in fitness across rhizobial environments and show reduced costs of specialization in rhizobial interactions relative to diploids.

Materials and Methods

Plant host selection

Medicago sativa is a perennial, outcrossing plant native to central Asia but now geographically widespread due to its agricultural importance (Muller *et al.*, 2005; Havananda *et al.*, 2011). *Medicago* has experienced multiple whole genome duplication events throughout its history, including an ancient event within the Rosid I clade (Cannon *et al.*, 2006) and a more recent event *c.* 58 million years ago when *Medicago* separated from *Glycine* (Young *et al.*, 2011). Within the *M. sativa* complex, plant lineages from two independent autopolyploidy events were used to avoid confounding the effects of polyploidy with the effects of hybridization (Havananda *et al.*, 2011). *Medicago sativa* subsp. *caerulea* ($2n = 2x = 16$) is the diploid progenitor of autotetraploid *M. sativa* subsp. *sativa* ($2n = 4x = 32$), and *M. sativa* subsp. *falcata* contains both diploid and autotetraploid populations (Havananda *et al.*, 2011). Ploidy of these accessions was previously determined using flow cytometry (Brummer *et al.*, 1999; Sakiroglu & Brummer, 2011) and seeds from 10 accessions were obtained from the USDA National Genetic Resources Program. Accessions were used as a proxy for independent replicates within taxa, as previous studies found significant genetic variation among subspecies and accessions within *M. sativa* (Sakiroglu *et al.*, 2010; İlhan *et al.*, 2016). Specifically, genetic analyses of *M. sativa* accessions from the USDA National Genetic Resources Program revealed distinct clusters for each of the subspecies included here. Furthermore, these studies identified strong isolation-by-distance patterns within subspecies, suggesting that geography plays a role in explaining genetic differentiation among accessions (Sakiroglu *et al.*, 2010; İlhan *et al.*, 2016). Diploid and autotetraploid accessions within taxa (*M. sativa* subsp. *caerulea*/*sativa*, or *M. sativa* subsp. *falcata*) were matched by geographic origin when possible (http://www.ars-grin.gov/; Supporting Information Table S1). Given the geographic dispersion of accessions, they will be referred to as 'lineages' throughout the remainder of the text for clarity.

Seed scarification and planting

Seeds were scarified with 72% (w/w) sulfuric acid for 10 min, rinsed with sterile double distilled H_2O (ddH_2O) and sterilized with 10% bleach for 10 min following standard protocols (Heath & Tiffin, 2007). Sterilized seeds were placed in small Petri dishes on sterilized filter paper with 1 ml of sterile ddH_2O. Plates were sealed with parafilm, wrapped in aluminum foil and placed in a 4°C refrigerator for 2–4 d to synchronize germination. Seeds were then transferred to a dark cabinet at room temperature for 1–3 d to induce germination. Once seeds developed radicles, they were planted into sterilized growth pouches (CYG; Mega International, Newport, MN, USA) containing 20 ml of sterile, nitrogen-free Fahraeus solution, as described in the *Medicago truncatula* Handbook (https://www.noble.org/medicago-handbook/).

For each lineage, eight seeds were planted for each rhizobial or water-inoculated control treatment (four seeds per pouch, two pouch replicates per lineage per treatment). Pouches were sorted by treatment and replicate and then placed into plastic containers (18058606 Large Flip Top, Clear; Sterilite, Townsend, MA, USA). To prevent cross-contamination, each container held a single rhizobium treatment or water-inoculated control. Containers were sterilized before housing pouches by soaking them in a 10% commercial bleach solution for 5 min. Each container held 10 pouches (one pouch per lineage per treatment) and each treatment had two replicate containers (two pouches × 10 lineages × 25 treatments (21 rhizobial strains + four water-inoculated controls) = 500 pouches). Containers were randomly placed *c.* 6 in. apart in a growth room set to 25°C and 60% humidity, and with supplemental lighting to achieve 16-h days.

Rhizobial strains

Twenty-one strains of *Sinorizobium* (*Ensifer*) were used to evaluate nodulation traits and host growth response of diploid and autotetraploid *M. sativa*. These included one strain of *S. terangae*, two strains of *S. fredii*, one strain of *S. saheli*, six strains of *S. medicae* and 11 strains of *S. meliloti*. These strains span the *Sinorhizobium* phylogeny (Fig. S1), have genetic resources available and exhibit diverse symbiotic phenotypes with *M. truncatula* (Sugawara *et al.*, 2013). Twenty strains were obtained from M. Sadowsky at the University of Minnesota and one strain (*S. meliloti* USDA1002) was obtained from P. Elia at the National Rhizobium Germplasm Resource Collection.

Experimental design and treatments

The experiment was divided into four temporal blocks that occurred from May to October 2017 and overlapped by 1 wk. Each block lasted 7 wk from the time of seed scarification to harvesting and comprised four to six unique rhizobial strains and a water-inoculated control treatment. The first temporal block had four rhizobial strains, the second block had five strains, and the third and fourth blocks had six strains each. Before planting, seedlings were sorted into size groups to avoid effects of initial plant size at the time of inoculation and each seedling within a size group was randomly assigned to a rhizobial treatment or the water-inoculated control.

Inoculation and plant growth

For the first block, rhizobial strains were grown in 30 ml of tryptic-soy media with biotin (TY), with four replicate flasks per strain. Cultures were transferred to 50 ml Falcon tubes, centrifuged to pellet cells and remove media, and resuspended in 10 ml of sterile ddH_2O. Due to the limited growth in liquid culture for two of the strains (KH16b and KH36c), cells were scraped from TY plates to achieve the desired concentration of 10^9 cells ml^{-1} (based on OD_{600}). For the three additional blocks, rhizobial strains were cultured on TY plates, scraped and resuspended in 10 ml sterile ddH_2O to achieve 10^9 cells ml^{-1}.

Seven or 8 d after planting, treatment plants were inoculated with 1.0×10^9 cells in 50 µl ddH_2O by slowly applying inocula directly along the plant root surface using a pipette. Control plants were given 50 µl ddH_2O applied following the same protocols as the rhizobial treatments. Plants were given 9 ml of N-free Fahraeus solution 8 d after inoculation. Three weeks after inoculation, non-nodulating and control plants appeared nitrogen-deficient and had reduced survival. To ensure a sufficient number of control plants could be analyzed, all plants in each temporal block were harvested 3 wk after inoculation and within 2–4 d.

Plant harvest and data collection

Plants were removed from pouches, numbers of leaves and nodules were counted, and nodule color was recorded by a single observer as pink, white, brown and/or green. Pink nodules show the presence of leghemoglobin and are probably fixing nitrogen and providing it to their plant hosts, whereas white nodules probably do not fix nitrogen (Imaizumi-Anraku *et al.*, 1997). Green nodules may fix nitrogen but are in the early stages of senescence, whereas brown nodules are completely senesced but may have fixed nitrogen at some point. Plants were then dissected into shoot, root and nodule tissue, and dried in an oven at 55°C for at least 4 d. Root and shoot tissue samples were weighed to the nearest 0.1 mg on a Mettler AE-200 Analytical Balance to assess growth benefits from rhizobial mutualisms. Total nodule biomass per plant was estimated by weighing all nodules to the nearest 0.1 µg on a Cahn C-31 Microbalance. These data were used to test for differences in the quantity of symbionts, as nodule biomass is correlated with rhizobial abundance within the nodule (Kiers *et al.*, 2003; Heath & Tiffin, 2007; Regus *et al.*, 2015).

Plant biomass and nodule traits were collected for all plants that survived in the experiment ($n = 1139$). For plants in rhizobial treatments, 959 of the 1680 seeds planted germinated and survived to the end of the experiment. For control plants, 180 of the 320 seeds planted germinated and survived to the end of the experiment. Of the 180 control plants, only one plant produced a single nodule and was excluded from analyses. To minimize nonindependence, an average value was calculated for pouches that contained more than one plant, making pouch the unit of replication for this experiment.

Host benefit analyses

To test whether autotetraploid plants benefitted more from rhizobial symbioses than diploids, mean shoot biomass, total biomass and host growth response (HGR) were calculated for each lineage and rhizobial treatment combination. Analyzing shoot biomass and total biomass provides insight into whether overall plant size differs between diploid and polyploid plants, and whether these differences lead to greater benefits obtained from rhizobial symbioses. By contrast, HGR controls for ploidy effects on plant size to evaluate whether benefits obtained by diploid and polyploid plants were not solely due to initial size differences. HGR was quantified as the mean percentage difference in shoot biomass between inoculated and uninoculated controls within each lineage ((average shoot biomass inoculated plants − average shoot biomass uninoculated plants)/average shoot biomass uninoculated plants) × 100; Regus *et al.*, 2015). All 21 rhizobial strains were included in these analyses because non-nodulating strains have been shown to modulate host fitness benefits (Gano-Cohen *et al.*, 2016).

Shoot biomass, total biomass and HGR metrics were approximately normally distributed and fitted model assumptions. Individual linear mixed-effects models were used to test for effects of ploidy, strain and their interaction (all fixed) accounting for lineage nested within subspecies (random effect) on shoot biomass, total biomass and host benefit using the LME4 (v.1.1) and LMERTEST (v.3.0-1) packages in R (v.1.1.453).

Plasticity analyses

To test whether autotetraploid plants were better able to maintain fitness benefits across all 21 rhizobial strains than diploids,

we estimated plasticity in fitness (relative distance plasticity index, or RDPI, of HGR; Valladares *et al.*, 2006) for diploid and autotetraploid *M. sativa* lineages. Within each lineage, we calculated the average pairwise distance in HGR for all combinations of rhizobial environments using the Canberra method, which measures the absolute distance between pairs of points in a vector space. RDPI values range from zero to one, with values closer to zero reflecting the maintenance of quality over rhizobial environments and is associated with greater generalism.

In addition to assessing plasticity in fitness, exploring whether autotetraploid *M. sativa* plants have a greater ability to maintain benefits closer to the maximum benefit provides insight into potential costs associated with specializing on particular rhizobial symbionts. To explore costs of specialization, we calculated the relative distance from the maximum HGR of shoot biomass for diploid and autotetraploid lineages across all 21 rhizobial strains. While this metric can be correlated with RDPI, and is correlated in this case ($r = 0.76$, $P < 0.01$), the point of comparison differs and, as a result, provides additional insight into the factors driving generalization in host benefits obtained.

RDPI and cost of specialization were calculated in R using the VEGAN package (v.2.5-4), and *t*-tests were used to test for significant differences between ploidy levels (STATS package v.3.5.2). We reran host benefit and plasticity analyses using only the 17 nodulating strains, and the results were the same as the 21 strain models (data not shown). Data were visualized using GGPLOT2 (v.3.0.0).

Nodule trait analyses

Functional traits that reflect the quantity (nodule number and biomass) and quality (nodule color as a proxy for N fixation) of the rhizobial symbionts hosted might underlie the differences in fitness benefits obtained by diploid and autotetraploid *M. sativa*. Plants of this species form indeterminate root nodules that have a persistent meristem and exhibit continuous growth. Only strains that produced nodules with more than five plants were included in these analyses. Four strains were excluded – three did not nodulate any plants in the experiment and one strain only nodulated one diploid and four autotetraploid plants – resulting in 17 strains included (Fig. S1).

To analyze differences in nodule color for diploid and autotetraploid plants, nodule color was converted from the qualitative metrics recorded during harvest to a quantitative scale that ranged from zero to one. White nodules were given a score of 0, brown nodules 0.5, green nodules 0.75 and pink nodules 1. For plants that had multiple nodule colors recorded during harvest, an average quantitative score was calculated. To evaluate potential bias in our quantitative scale, we reran analyses with a different scale in which green nodules were given a score of 0.5 and brown nodules were given a score of 0.25, but the results did not change.

Nodule traits were approximately normally distributed, and models fitted assumptions. Mean nodule number per plant, total nodule biomass and quantitative nodule color were calculated for each lineage and rhizobial treatment combination for the 17 nodulating strains. Individual linear mixed effects models were used to test for effects of ploidy, strain and their interaction (all fixed) accounting for lineage nested within subspecies (random effect) on mean number of nodules produced per plant, total nodule biomass and quantitative nodule color using the LME4 (v.1.1) and LMERTEST (v.3.0-1) packages in R. Models were rerun using root biomass as a covariate to account for effects of plant size on nodule traits. To evaluate whether diploid and autotetraploids plants differed in the quantity of benefits obtained per unit investment in rhizobial symbioses, we ran correlations of total nodule biomass and shoot biomass as well as total nodule biomass and HGR.

Data availability

The data and code used to analyze data are publicly available via Dryad (doi: 10.5061/dryad.5tb2rbp1r).

Results

Diploid and autotetraploid *M. sativa* exhibited similar breadth in the taxonomic range of rhizobial partners with which they could establish mutualisms. Specifically, all diploid and autotetraploid lineages of *M. sativa* (Table S1) were nodulated by the same 17 of 21 possible rhizobial strains that span the *Sinorhizobium* phylogeny (Fig. S1).

By contrast, autotetraploid *M. sativa* exhibited greater host benefits from rhizobial mutualisms than diploid plants. Autotetraploid *M. sativa* produced almost twice as much shoot biomass as diploids on average (5.72 mg shoot biomass vs 3.00 mg), but the degree of this increase differed across rhizobial strains (ploidy by strain interaction, $F_{21,216} = 2.58$; $P < 0.001$; Fig. 1a; Table 1). These patterns were also evident in total biomass production ($F_{21,216} = 2.25$; $P < 0.01$; Fig. S2; Table 1). When controlling for the effects of polyploidy on plant size, autotetraploid *M. sativa* exhibited a greater positive growth response of shoot biomass on average compared to diploids across all 21 rhizobial strains (1.75-fold increase in shoot biomass vs 1.2-fold increase; $F_{1,206} = 5.69$; $P = 0.04$; Fig. 1b; Table 1). These patterns held across rhizobial environments even though strains differed significantly in their effects on HGR ($F_{20,206} = 12.81$; $P < 0.001$), ranging from costly to highly beneficial (−7 to 406% averaged across autotetraploid lineages and from −23 to 439% averaged across diploid lineages). Individual *M. sativa* lineages exhibited extensive variation in HGR values, which accounted for 7% of the variation in the model. These values ranged from −54 to 574% for autotetraploid lineages and from −91 to 733% for diploid lineages.

Autotetraploid *M. sativa* had a significantly lower RDPI of HGR compared to diploids (0.63 vs 0.72; $t = 3.55$, $P = 0.008$; Fig. 2a), consistent with lower fitness plasticity across rhizobial partners. Furthermore, autotetraploid *M. sativa* plants had a significantly lower cost of specialization in rhizobial interactions compared to diploids, as they achieved benefits closer to their maximum HGR across a broad range of symbionts (0.65 vs 0.82; $t = 5.02$, $P = 0.001$; Figs 2b, S3).

Ploidy level influenced several functional traits associated with nodulation. While some nodule traits were correlated, including

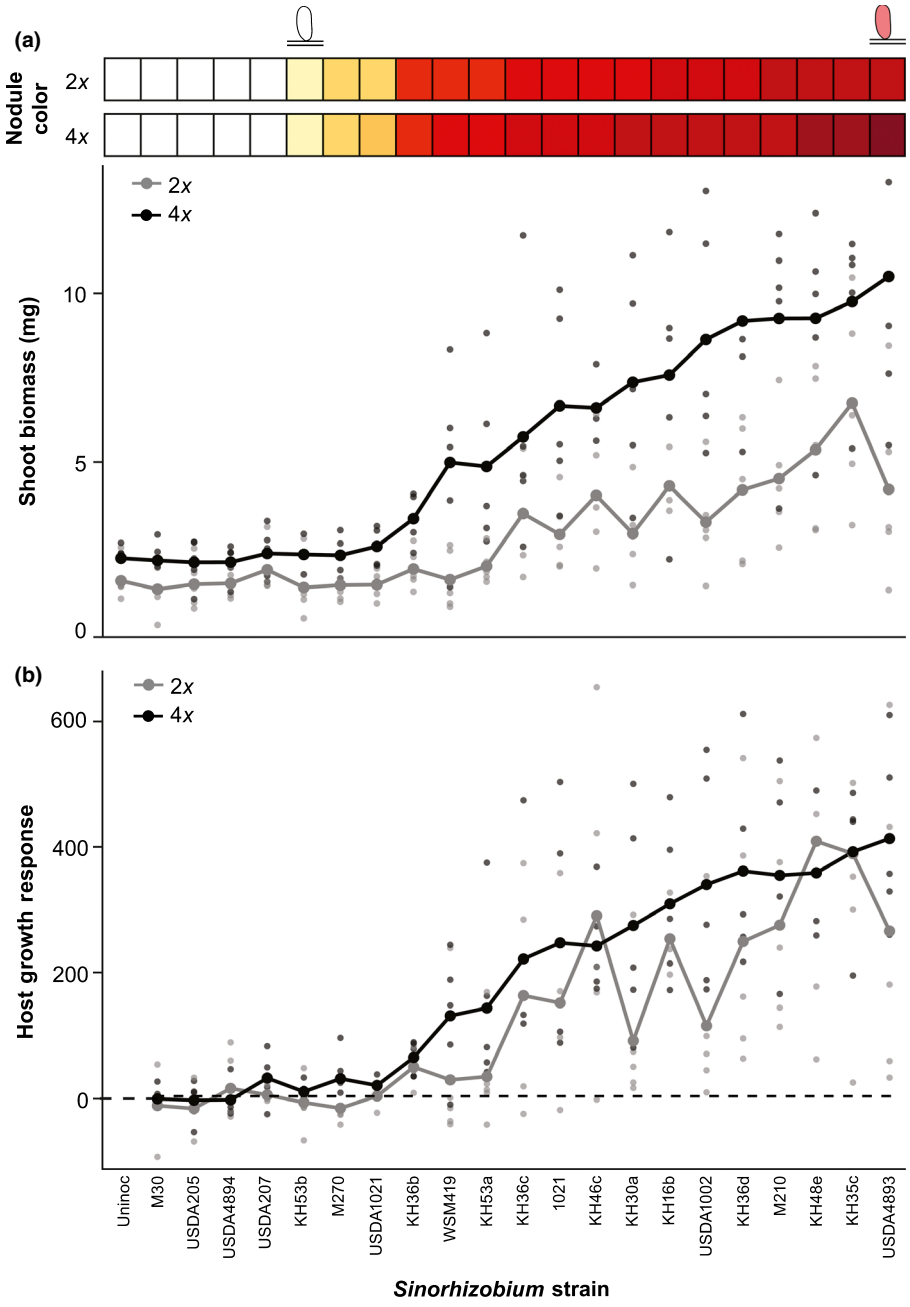

Fig. 1 (a) Mean shoot biomass of diploid (2*x*, gray) and autotetraploid (4*x*, black) lineages of *Medicago sativa* associated with 21 *Sinorhizobium* strains and without rhizobia ('Uninoc'). (b) Mean host growth response of diploid (2*x*, gray) and autotetraploid (4*x*, black) lineages of *M. sativa* associated with 21 *Sinorhizobium* strains. Host growth response quantifies the percentage change in shoot biomass of inoculated plants relative to water-inoculated control plants (dashed line) within a lineage. Mean shoot biomass and host growth response per strain are shown by small circles for each plant lineage and large circles for each ploidy level. *Sinorhizobium* strains are ordered by nodulation ability and then by average nodule color, a metric of nitrogen fixation function (see Methods) here illustrated as ranging from white (non-nodulating) to yellow (ineffective) to dark red (highly effective), produced by 2*x* (upper bar) and 4*x* (lower bar) plants.

Table 1 ANOVAs for shoot biomass, total biomass, host growth response and nodule traits of *Medicago sativa* diploid and autotetraploid plants when grown with single strains of *Sinorhizobium*.

	df	Shoot biomass	Total biomass	df	Host growth response of shoot biomass	df	Nodule number	Total nodule biomass	Nodule color
Ploidy	1	11.71*	11.14*	1	5.69*	1	1.52	14.82**	7.83*
Strain	21	16.38***	12.20***	20	12.81***	16	3.55***	5.94***	26.83***
Ploidy : Strain	21	2.58***	2.25**	20	0.85	16	0.92	2.39**	0.72

ANOVAs of shoot biomass and total biomass included water-inoculated control plants. ANOVAs of nodule traits were analyzed for the subset of rhizobial strains that produced nodules (17 of the 21 strains). *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Numerator df and *F* values are shown for each effect. Denominator df = 216 for shoot and total biomass, 206 for host growth response of shoot biomass, and 168 for nodule number, total nodule biomass and nodule color.

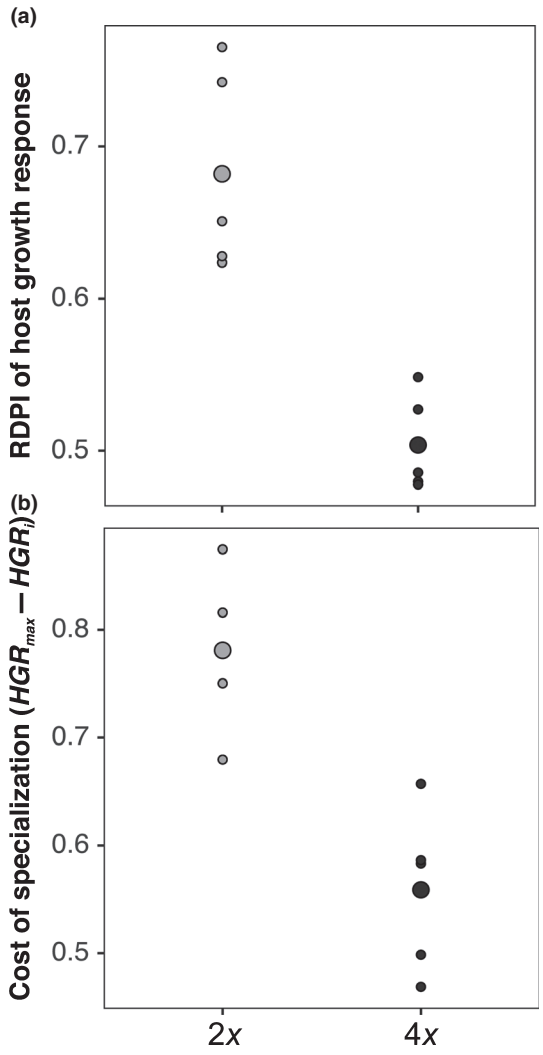

Fig. 2 Plasticity of fitness and cost of specialization of diploid (2*x*, gray) and autotetraploid (4*x*, black) lineages of *Medicago sativa* inoculated with 21 *Sinorhizobium* strains. (a) Relative distance plasticity index (RDPI) for host growth response of shoot biomass of 2*x* and 4*x* lineages. (b) Cost of specialization as estimated by the average of the individual distances (HGR_i) from the maximum growth response (HGR_{max}) of shoot biomass for 2*x* and 4*x* plants. Average RDPI and cost of specialization are shown as small circles for each plant lineage and large circles for each ploidy level.

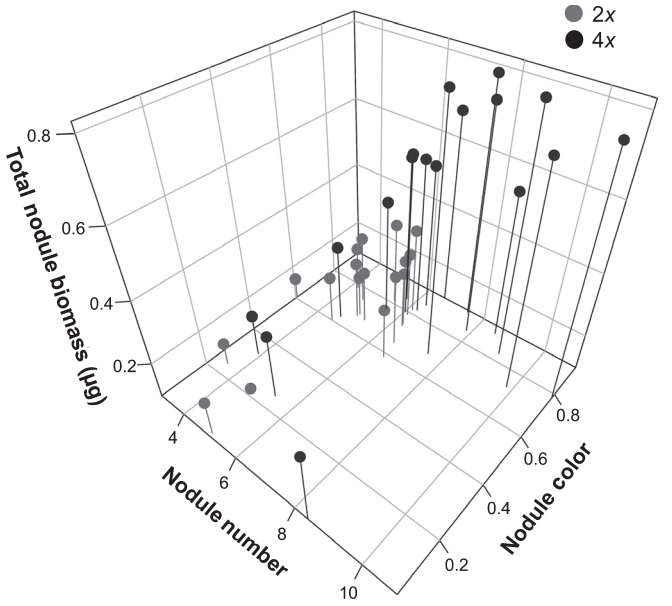

Fig. 3 Nodule traits (total biomass, number and color) of diploid (2*x*) and autotetraploid (4*x*) *Medicago sativa* associated with 17 nodulating strains of *Sinorhizobium*. Each data point represents the average nodule trait value produced by diploid (gray) or autotetraploid (black) plants associated with a given *Sinorhizobium* strain.

nodule number per plant and total nodule biomass (diploids: $r = 0.30$, $P < 0.001$; autotetraploids: $r = 0.55$, $P < 0.001$), others were not, such as nodule color and nodule number. Autotetraploid *M. sativa* produced, on average, more than twice as much total nodule biomass than diploids, but the degree of this increase differed across rhizobial strains (ploidy by strain interaction, $F_{16,168} = 2.39$, $P < 0.01$; Figs 3, S4; Table 1, S2). While autotetraploid *M. sativa* plants produced more total nodule biomass than diploids across every nodulating rhizobial strain, the most pronounced differences were observed in strains that provided intermediate host growth benefits. The effect of ploidy on total nodule biomass was not solely due to the larger size of polyploid plants, as this interaction remained significant ($F_{1,168} = 1.87$, $P = 0.03$) when root biomass was included as a covariate (Table S3). Moreover, the effect of polyploidy was evident even though rhizobial strain influenced total nodule biomass ($F_{16,168} = 5.94$, $P < 0.001$).

Ploidy level did not affect the number of nodules produced per plant ($F_{1,168} = 1.52$, $P = 0.25$; Table S2). Instead, nodule number was influenced by rhizobial strain ($F_{1,168} = 3.55$, $P < 0.001$) and root biomass ($F_{1,168} = 13.33$, $P < 0.001$). Although nodule color also varied across rhizobial strains ($F_{16,168} = 26.83$, $P < 0.001$), autotetraploid *M. sativa* consistently produced significantly darker nodules (i.e. higher scores on the quantitative scale of nodule color) than diploids ($F_{1,168} = 7.83$, $P = 0.03$; Figs 1, 3; Table S2), suggesting effective N fixation by rhizobial symbionts (Imaizumi-Anraku *et al.*, 1997; Burghardt *et al.*, 2018). As expected by the relationship between color and N fixation, shoot biomass and HGR were positively correlated with average nodule color for both diploid (shoot: $r = 0.57$, $P < 0.001$; HGR: $r = 0.55$, $P < 0.001$) and autotetraploid plants (shoot: $r = 0.58$, $P < 0.001$; HGR: $r = 0.61$, $P < 0.001$) across the 17 nodulating rhizobial strains.

Shoot biomass was also positively correlated with total nodule biomass for both diploid ($r = 0.67$, $P < 0.001$) and autotetraploid *M. sativa* ($r = 0.70$, $P < 0.001$), suggesting similar benefits obtained per unit investment in the mutualism (Fig. 4a). While HGR was positively correlated with total nodule biomass for diploids ($r = 0.42$, $P < 0.001$) and autotetraploids ($r = 0.64$, $P < 0.001$), the correlation coefficients were significantly different ($z = 1.99$, $P = 0.05$; Fig. 4b).

Discussion

In a model nutritional mutualism, we demonstrate that autotetraploid *M. sativa* obtained greater benefits from rhizobial

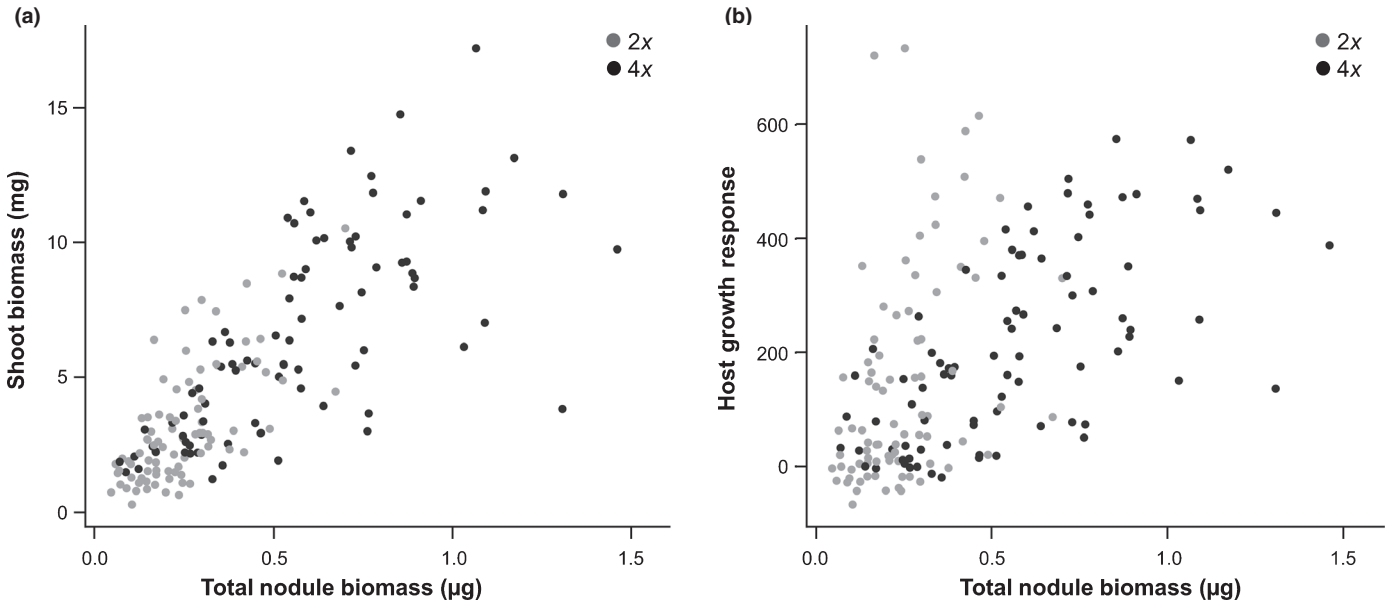

Fig. 4 Quantity of benefits obtained per unit investment in rhizobial symbioses for diploid (2*x*, gray) and autotetraploid (4*x*, black) *Medicago sativa* plants associated with 17 nodulating strains of *Sinorhizobium*. (a) Correlation of total nodule biomass and shoot biomass. (b) Correlation of total nodule biomass and host growth response.

partners than diploids and maintained these high benefits across a wide range of interactions (i.e. reduced plasticity in fitness). Furthermore, autotetraploid plants showed reduced costs of specialization in rhizobial interactions relative to diploids. Although diploid *M. sativa* lineages obtained high benefits from a few strains, they exhibited higher plasticity in fitness and rarely obtained benefits close to their maximum growth response when associated with other strains, therefore revealing that specialized interactions come at a fitness cost when hosts are partnered with less effective symbionts. Differences in growth benefits of diploid and autotetraploid plants were not due to increased niche breadth (i.e. ability to interact with a broad taxonomic range of patterns). Instead, autotetraploid *M. sativa* plants appeared to invest more in the mutualism, as demonstrated by greater nodule production relative to diploids. These results imply that autotetraploid *M. sativa* obtain more N than diploids from the same rhizobial strains.

Several mechanisms may permit autotetraploid *M. sativa* plants to enhance the quantity and quality of rhizobial interactions, thereby obtaining more N from them (Forrester & Ashman, 2018). A common consequence of polyploidy is an increase in cell size (Balao *et al.*, 2011), which can lead to greater shoot biomass and root biomass relative to diploids (Forrester & Ashman, 2018). Increases in root biomass might permit polyploids to host a greater quantity of symbionts (i.e. host more or larger nodules) and increases in shoot biomass might provide more photosynthetic resources to invest in bacterial mutualisms (Forrester & Ashman, 2018). The effects of polyploidy on plant size were evident, as autotetraploid *M. sativa* plants were consistently larger than diploids both with and without rhizobial associations. Due to the observed greater nodule biomass of autotetraploid *M. sativa*, they probably hosted more rhizobial symbionts than diploids because this metric is positively correlated with rhizobial abundance within the nodule (Kiers *et al.*, 2003; Heath & Tiffin, 2007; Regus *et al.*, 2015). In addition, autotetraploid *M. sativa* plants consistently produced more pink, N-fixing nodules when associated with the same strains as diploids, potentially reflecting enhanced quality of bacterial symbioses. Furthermore, although not tested directly here, a separate analysis of internal nodule traits of diploid and neotetraploid *M. sativa* subsp. *caerulea* found that polyploidy directly increased the size of rhizobia (bacteroids) hosted within nodules (Forrester & Ashman, 2019), a metric positively correlated with the amount of N provided to plant hosts (Oono & Denison, 2010). Thus, multiple mechanisms may have led to the polyploid advantage observed here.

The similarity of fundamental biotic niche breadth (i.e. the number of taxonomic partners in the absence of partner competition) of diploids and polyploids that we observed is consistent with previous studies exploring the effects of plant polyploidy on the range of potential mutualistic partners. Previous studies have found that diploid and polyploid plants often share similar pollinator communities (Castro *et al.*, 2010; Nghiem *et al.*, 2011; Borges *et al.*, 2012; but see Thompson & Merg, 2008) and mycorrhizal fungal associations (Tesitelova *et al.*, 2013; Sudova *et al.*, 2018). Within the legume–rhizobia mutualism, four legume species did not differ in their ability to associate with 31 single strains of rhizobia in a glasshouse experiment (i.e. fundamental niche breadth; Ehinger *et al.*, 2014). However, in the wild, these species specialized on distinct subsets of rhizobial strains, displaying differences in realized niche breadth (Ehinger *et al.*, 2014). Competition between rhizobial strains is known to be an important factor influencing the establishment of mutualistic interactions as well as the benefits obtained from them (Gano-Cohen *et al.*, 2016; Burghardt *et al.*, 2018), so it is possible that diploid and autotetraploid *M. sativa* exhibit differences in realized niche breadth of rhizobial associations in natural populations or when

co-inoculated. Competition among rhizobial strains might also differ for diploid and polyploid plants, particularly if polyploids have more resources to the invest in the mutualism (e.g. increased nodule biomass).

Numerous studies have also addressed whether plant polyploidy is associated with increases in abiotic niche breadth, yet no clear patterns have emerged (Husband *et al.*, 2013; Glennon *et al.*, 2014; Brittingham *et al.*, 2018). Although some polyploid plant taxa occupy larger abiotic niches than their diploid progenitors (Lowry & Lester, 2006; Coughlan *et al.*, 2017), others occupy different or smaller niches (Ramsey, 2011; Brittingham *et al.*, 2018). Additional studies testing how polyploidy shapes niche breadth of both biotic and abiotic interactions are needed to elucidate broad patterns and clarify underlying mechanisms. Taken together, these studies highlight that fitness advantages frequently observed in extant polyploid plants might not be attributed to expansion in the taxonomic range of mutualistic partners (or habitats), but the ability of polyploids to obtain greater benefits from interactions and/or maintain fitness across biotic (or abiotic) environments once partnerships are established.

Here, we demonstrate that autotetraploid *M. sativa* not only obtain greater benefits from rhizobial symbionts, but also maintain higher fitness across biotic environments, thus displaying greater generalization in bacterial mutualisms relative to diploids. To our knowledge, this is the first test of diploid and polyploid fitness plasticity across biotic environments (i.e. the different rhizobia taxa) and supports the characterization of polyploid plants as 'jacks-of-all-trades' and 'masters-of-some' (*sensu* Richards *et al.*, 2006; Wei *et al.*, 2019). Previous work has identified a fitness cost of generalization in rhizobial mutualisms, such that maximum host benefit was lower for generalist legume species compared to specialized legume species (Ehinger *et al.*, 2014). Our results are consistent with this finding in that one diploid *M. sativa* lineage achieved the greatest maximum HGR (733%) when associated with one rhizobial strain. However, specialization also appears to come at a fitness cost. Diploid *M. sativa* received lower mean benefits per strain than autotetraploids for 15 of the 17 nodulating rhizobial strains, and diploids rarely achieved HGR values close to their maximum when associated with less beneficial rhizobia.

The ability of autotetraploid *M. sativa* to obtain and maintain greater benefits from rhizobial symbionts seems to be due, in part, to increased plant size. Diploid and autotetraploid *M. sativa* displayed similar relationships between total shoot biomass obtained per unit investment in the mutualism. Autotetraploid plants were able to host more nodule biomass than diploids and, as a result, obtain greater shoot biomass benefits when associated with rhizobia. However, when the effects of ploidy on plant size were controlled for using HGR, diploid and autotetraploid *M. sativa* differed in the relationship between benefits obtained per unit investment in the symbiosis, suggesting that other mechanisms beyond plant size shape rhizobial interactions. Specifically, diploid *M. sativa* had less nodule biomass and lower HGR than autotetraploids; however, for a few strains, diploids obtain high benefits from relatively little investment in the mutualism (i.e. low nodule biomass), displaying a more specialized strategy. By contrast, autotetraploid *M. sativa* had more nodule biomass and greater HGR across strains, but for all strains, increased host benefits required increased investments in the mutualism, fitting a more generalist strategy. The mechanisms underlying the specialization and generalization strategies observed here may reflect differences in the regulation of rhizobial interactions (e.g. autoregulation of nodulation, host sanctions, resources provided to rhizobial symbionts); however, additional studies are needed to uncover these mechanisms.

The patterns observed here are consistent with previous studies demonstrating higher mean fitness and reduced plasticity in fitness of polyploid plants across abiotic environments (Petit *et al.*, 1996; McIntyre & Strauss, 2017; Wei *et al.*, 2019). Autotetraploid *M. sativa* plants might obtain greater benefits and exhibit reduced plasticity in fitness across biotic environments due to increased investment in the mutualism by plant hosts and their bacterial symbionts (i.e. increased nodule biomass and high quantitative scores for nodule color) relative to diploids. Additional studies are needed to uncover the specific mechanisms permitting polyploid plants to invest more in bacterial mutualisms; however, the present study suggests that generalization is beneficial and can be an important component of polyploid fitness advantages. Enhancements in genomic, transcriptomic and phenotypic plasticity that result from polyploidy are known contributors to polyploid fitness advantages across abiotic environments (Bretagnolle & Thompson, 2001; Leitch & Leitch, 2008; Shimizu-Inatsugi *et al.*, 2017), and might also explain why polyploids exhibit greater generalization in biotic interactions. Empirical studies evaluating these mechanisms would be particularly insightful for understanding how niche breadth and the fitness benefits of mutualistic interactions contribute to polyploid success.

By quantifying the degree of generalization in and fitness benefits obtained from a broad range of partnerships, this work supports the role of polyploidy as an important ecological and evolutionary driver of variation in mutualistic interactions. Polyploid plants that obtain high benefits from a broad range of mutualistic partners might outcompete diploids and facilitate the occurrence of diverse bacterial symbionts within or across environments (Heath & Stinchcombe, 2014). By contrast, more specialized diploids might enrich the environment with a few highly beneficial strains and, in doing so, outcompete polyploid plants and reduce the presence of other symbionts. At a larger scale, variation in the degree of generalization in mutualistic interactions between intraspecific diploid and polyploid plants might maintain high diversity of symbiotic partners (Batstone *et al.*, 2018) as well as ploidy level diversity within and among plant populations. Depending on the scale of environmental variation, diploid and polyploid plants might coexist in mixed-ploidy populations if each associates with distinct rhizobial strains, potentially increasing the population-level breadth of mutualistic partners. Even if diploid and polyploid plants outcompete one another within populations depending on the availability of beneficial rhizobia strains, these interactions could enhance species-level partner breadth across the geographic range (Batstone *et al.*,

2018). These processes might occur in autopolyploid species, such as *M. sativa* used here; however, it is possible that allopolyploid plants exhibit even greater generalization in species interactions, which could lead to greater variation in mutualistic partners. Studies using established diploid and polyploid plants in separate and mixed-ploidy populations would be particularly informative for addressing these hypotheses.

Comparing established diploid and polyploid plants of the same species provides insight into how polyploidy might impact the evolutionary trajectory of plant species in natural populations (Spoelhof *et al.*, 2017; Baduel *et al.*, 2018; Forrester & Ashman, 2019). *Medicago* experienced multiple polyploidization events throughout its history, including an event 58 million years ago (Cannon *et al.*, 2006; Young *et al.*, 2011). Although the origin and timing of polyploidization events among the *M. sativa* subspecies and lineages tested here are uncertain (Havananda *et al.*, 2011), it is possible that extensive heterogeneity exists in the evolutionary changes that occurred following polyploidization in these lineages. Testing the patterns detected here in young and old polyploid lineages would inform how time since origin affects mutualism traits and benefits obtained from legume–rhizobial interactions.

Studying legume–rhizobial interactions with established polyploids, however, does not isolate the direct effects of polyploidy on plant traits or abiotic and biotic interactions (Maherali *et al.*, 2009; Forrester & Ashman, 2019). The immediate effects of polyploidy can be identified by comparing diploid and synthetic neopolyploid plants, with some caveats (see Münzbergová, 2017). Greater understanding of the long-term evolutionary consequences of polyploidy as well as the underlying direct mechanisms can be gained by conducting complementary studies using neopolyploid and established polyploid plants (Forrester & Ashman, 2019). The results presented here and those on neopolyploid *Medicago* offer confirmatory insight into this interaction. For instance, both neoploids and extant polyploids showed patterns of larger nodule size. Within nodules, increases in size at the suborganellar and cellular levels were revealed as direct effects of polyploidy (Forrester & Ashman, 2019). These effects might also be at play in the extant polyploids and would explain the differences in nodule size and color between ploidies observed here.

The effects of polyploidy on generalization are likely to extend to other nutrient acquisition (e.g. plant–mycorrhizal) and reproductive (e.g. plant–pollinator, plant–seed disperser) mutualisms, as well as other plant–biotic interactions (e.g. herbivores, parasites) that vary in niche breadth and effects on host fitness (Segraves & Anneberg, 2016; Wood *et al.*, 2018). Although previous studies have demonstrated that most organisms interact with multiple mutualistic partners, the benefits of generalization and underlying mechanisms remained largely unresolved (Douglas, 1998; Heath & Stinchcombe, 2014). This study reveals that polyploidy is a key genetic driver of generalization in a bacterial mutualism, uncovers a potential mechanism underlying the widespread success of polyploid legumes, and provides a general framework for understating how variation in biotic interactions can be affected by polyploidy.

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Author contributions

NJF, MR-G, JLS and T-LA planned and designed the experiment. NJF and MR-G performed the experiment and analyzed the data. NJF, MR-G and T-LA wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phylogeny of the 21 *Sinorhizobium* strains used in the experiment.

Fig. S2 Mean total biomass of diploid and autotetraploid lineages of *Medicago sativa* associated with 21 *Sinorhizobium* strains.

Fig. S3 Average host growth response of diploid and autotetraploid *Medicago sativa* associated with 21 *Sinorhizobium* strains.

Fig. S4 Nodule traits of diploid and autotetraploid lineages of *Medicago sativa* associated with 17 *Sinorhizobium* strains.

Table S1 Diploid and autotetraploid accessions of the *Medicago sativa* species complex used in the study.

Table S2 Estimated marginal means for plant growth and nodule traits of diploid and autotetraploid *Medicago sativa* lineages associated with *Sinorhizobium* strains.

Table S3 ANCOVAs for nodule number and total nodule biomass of diploid and autotetraploid *Medicago sativa* when grown with 17 single strains of *Sinorhizobium* and including root biomass as a covariate.

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