Exploring how polyploidy enhances a plant-bacterial mutualism

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Nicole J. Forrester

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This dissertation was presented

by

Nicole J. Forrester

It was defended on

March 21, 2019

and approved by

Dr. Walter Carson, Associate Professor, Department of Biological Sciences, University of Pittsburgh

Dr. Jeffrey Lawrence, Professor, Department of Biological Sciences, University of Pittsburgh

Dr. Corinne Richards-Zawacki, Associate Professor, Department of Biological Sciences, University of Pittsburgh

Dr. Joel Sachs, Associate Professor, Department of Biology, University of California, Riverside

Thesis Advisor/Dissertation Director: Dr. Tia-Lynn Ashman, Distinguished Professor, Department of Biological Sciences, University of Pittsburgh

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Nicole J. Forrester, PhD

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Polyploidy (i.e., the possession of more than two complete sets of chromosomes) is a major driver of ecological and evolutionary processes in plants. Previous work has illuminated how polyploidy affects genotypes, phenotypes, and abiotic interactions, yet little is known about how it alters plant-biotic interactions. The legume-rhizobia symbiosis is a model interaction between plants and mutualistic microbes, in which rhizobia bacteria fix nitrogen in exchange for carbon provided by plant hosts. This mutualism regulates global nutrient cycles and plays a prominent role in the distribution and diversification of legume taxa. Despite the widespread importance of this mutualism, it remains unclear how polyploidy affects mutualism traits and host benefits from it. To address this fundamental gap in knowledge, I developed a framework of mechanistic hypotheses for how plant polyploidy might directly enhance the quantity and quality of rhizobial symbionts hosted, subsequently improving plant growth benefits. I tested mechanisms within this framework using stabilized polyploids of *Medicago sativa* by asking whether polyploids exhibited greater niche breadth, increased host benefits, and reduced fitness plasticity across a broad range of *Sinorhizobium* symbionts relative to diploids. Finally, to isolate the direct effects of plant polyploidy on the legume-rhizobia mutualism, I created synthetic neotetraploid *M. sativa* plants and compared them to their diploid progenitors. Using confocal microscopy, I quantified the direct effects of plant polyploidy on the internal structure of mature root nodules. These studies reveal that polyploid plants obtain greater benefits from rhizobial symbionts and maintain them across a broad range of rhizobial symbionts relative to diploid plants, which may be due to direct changes

in internal nodule structure. Overall, this dissertation uncovers novel patterns and underlying mechanisms for how plant polyploidy alters a model species interaction, and in doing so, contributes to ecological and evolutionary theories concerning the widespread success of polyploid plants.

Table of contents

List of tables

List of figures

Preface

I am deeply grateful for all the individuals that contributed to my scientific, intellectual, and career development as a doctoral student. First and foremost, I want to thank Dr. Tia-Lynn Ashman for her exceptional mentorship and endless curiosity. It's truly impossible to capture the impact she has had on me, but I can say that without her brilliance, dedication, and support, I would never have become the scientist or person I am today. I would also like to thank my outstanding committee members who contributed substantially to the development of this dissertation. A monumental thanks to J. Sachs, K. Gano-Cohen, K. Quides, and C. Wendlandt for welcoming me into their lab and showing me all there is to know about the world of rhizobia. I am forever grateful to the members of the Ashman lab for their insightful comments and critical feedback on my work. M. H. Koski, M. Rebolleda-Gomez, and C. Wood played particularly pivotal roles in shaping my approach to science. Finally, I want to thank my amazing community of family and friends that always keep me going.

1.0 The direct effects of plant polyploidy on the legume-rhizobium mutualism

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1.1 Introduction

Polyploidy (the condition of having more than two complete sets of chromosomes) is a major driver of evolutionary novelty and speciation in flowering plants (Levin, 2002; Soltis et al., 2014; PS Soltis and DE Soltis, 2016; Zhan et al., 2016). Although we have made significant advances in understanding how plant polyploidy affects genotypes, phenotypes, and interactions with the abiotic environment (Balao et al., 2011; Husband et al., 2013; Soltis et al. 2014; Alix et al., 2017), much less is known about how it influences biotic interactions (Thompson et al., 2004; Segraves and Anneberg, 2016; Segraves, 2017). Although recent work has found that plant polyploidy can significantly alter plant-pollinator and plant-herbivore interactions (Segraves and Thompson, 1999; Nuismer and Cunningham, 2005; Arvanitis et al., 2008; Halverson et al., 2008), only a handful of published studies have explored the effects of plant polyploidy on their interactions with mutualistic soil microbes (Segraves and Anneberg, 2016; Segraves, 2017). Furthermore, most of these studies focus on mutualistic fungi (Tesitelova, et al., 2013; Sudova et al., 2014), with relatively few testing the effects of polyploidy on mutualisms with rhizobia (but see Table 1).

The legume-rhizobia mutualism has significant impacts on global ecosystems as it is a key regulator of nitrogen (N) cycles in natural and agricultural environments (Herridge et al., 2008;

Vitousek et al., 2013). Moreover, N is an essential and limiting resource for plants (Vitousek et al., 2002), and legumes associated with rhizobia have greater plant biomass and reproductive success (Daehler, 1998; Ndlovu et al., 2013). Although the effects of ancient whole genome duplication (WGD) on the legume-rhizobia mutualism have been well studied (Cannon et al., 2010; Doyle et al., 2011; Li et al., 2013; Cannon et al., 2014), we do not fully understand the direct effects of plant polyploidy on key features of this interaction or the potential ecological and evolutionary consequences. In the following paragraphs, we briefly summarize the salient features of the mutualism and then consider novel ways in which polyploidy could directly alter it.

1.1.1 The legume-rhizobia mutualism

As a model system for studying mutualisms, rhizobia fix atmospheric N into ammonia, a compound usable by the plant hosts, in exchange for carbon and other photosynthetic resources from their host plant (Heath and Tiffin, 2007; Jones et al., 2007). Legume taxa exhibit variation in rhizobial infection method, nodulation type, products of N_2 fixation, as well as other mutualism traits (Sprent, 2009). Root hair infection and differentiation of rhizobia within symbiosomes are two of the most common features among nodulating legume taxa (Sprent, 2009; Ferguson et al., 2010; Sprent 2013) and will therefore be the focus of this review. For these legume taxa, the mutualism is initiated when legumes release flavonoids into the soil, triggering free-living rhizobia to produce signaling molecules 'Nod factors' (Wang et al., 2012). Nod factors are perceived by Nod factor receptors of the plant host, stimulating root hair deformation and the development of nodules (Wang et al., 2012). Following successful initiation of the symbiosis, rhizobia enter the developing root nodules and differentiate into 'bacteroids' that fix atmospheric N (Wang et al., 2012). In legume taxa that produce indeterminate nodules, rhizobia terminally differentiate into

bacteroids and lose the ability to reproduce, whereas rhizobia retain the ability to reproduce in legume taxa that produce determinate nodules (Kiers et al., 2003). Root nodules provide protective environments for N fixation to occur (Gage, 2004; Heath and Tiffin, 2007), and the amount of oxygen (O_2) within nodules is strictly regulated because O_2 is required for rhizobial respiration, yet also irreversibly inhibits nitrogenase and the amount of N fixed (Hunt and Layzell, 1993). Several factors regulate O_2 concentration within nodules, primarily nodule permeability and leghemoglobin (Hunt and Layzell, 1993).

Because the process of symbiotic N fixation can be costly to plants and rhizobia, the interaction is finely regulated to ensure cooperation among partners (Kiers and Denison, 2008; Sachs et al., 2010). Although regulation can occur via multiple mechanisms, two primary ways in which legume hosts can stabilize cooperation with their rhizobial symbionts are partner choice, the establishment of the symbiosis with beneficial rhizobial partners based on recognition signals (e.g., flavonoids, Nod factors), and host sanctions, the ability of a plant to assess nodule efficiency and invest more in efficient nodules than inefficient ones (Kiers and Denison, 2008). Despite our extensive understanding of the establishment and maintenance of the legume-rhizobia mutualism, little is known about how plant polyploidy directly affects mutualism traits, whether it immediately increases plant host access to fixed N, and if so, by what mechanism(s).

1.1.2 Ancient polyploidy and the legume-rhizobia mutualism

Although the direct effects of plant polyploidy on the legume-rhizobia mutualism remain unresolved, studies evaluating the effects of ancient WGD on the evolution of nodulation suggest that polyploidy may have enhanced key aspects of the mutualism (Cannon et al., 2010; Doyle, 2011; Young et al., 2011; Li et al., 2013). Ancient WGD was not required for the evolution of

nodulation, but the genetic material acquired and retained from a WGD event in the Papilionoideae is hypothesized to have led to enhanced and more complex interactions with rhizobia (Cannon et al., 2010; Young et al., 2011; Li et al., 2013). Notably, the Papilionoideae is the largest and most geographically widespread subfamily within the legumes and 90% of taxa exhibit nodulation (Sprent, 2007; Sprent, 2009).

Hypotheses about whether WGD led to enhancements of the legume-rhizobia mutualism focus primarily on gene copies retained during the papilionoid WGD event (~58 mya) that function in mutualism establishment and maintenance (Young et al., 2011; Li et al., 2013). Young et al. (2011) determined that several nodulation genes retained from the WGD event have undergone sub- or neofunctionalization in *Medicago truncatula*, thereby increasing the complexity of genes involved in rhizobial signaling (e.g., flavonoids, Nod factor receptors) and mutualism function (e.g., nodule-specific cysteine-rich peptides, leghemoglobins). These patterns are also found across the Papilionoideae subfamily; Li et al., (2013) determined that a portion of duplicated genes retained from the papilionoid WGD event diverge in expression patterns and function in mutualism establishment (e.g., rhizobial signaling, nodule organogenesis, rhizobial infection) and maintenance (e.g., nutrient exchange). Furthermore, Werner et al. (2015) suggest that genome duplications may reduce the rate of symbiotic loss and increase symbiotic persistence over evolutionary time.

The relationship between ancient WGD and the evolution of nodulation in legumes is complex and warrants further investigation (Cannon et al., 2014; Werner et al., 2015); however, these studies support an overall role of polyploidy in enhancing key aspects of the legume-rhizobia mutualism. Evaluating the immediate and direct effects of plant polyploidy on the mutualism, in

addition to the effects of ancient WGD, will clarify the relationship between polyploidy and improvements in nodulation as well as uncover underlying mechanisms.

1.1.3 The direct effects of polyploidy on the legume-rhizobia mutualism

Polyploidy could directly enhance the legume-rhizobia mutualism by increasing the quantity and/or quality of rhizobial symbionts hosted, which may occur by altering plant traits that function in mutualism establishment and maintenance. We organize the ways polyploidy can directly affect the legume-rhizobia mutualism into an effects hierarchy (Fig. 1) and then evaluate the weight of current evidence for each node within this framework.

To do this, we conducted an exhaustive review of studies of the effects of plant ploidal level on one or more components of our framework. Specifically, we searched ISI Web of Science using the key words "polyploid* AND nodul*" and "tetraploid* AND nodul*" for studies published between 1900 and 2016 and obtained seven studies. We then evaluated the references that cited these seven studies and found nine additional published studies to include, as well as data from Forrester et al. (University of Pittsburgh, USA, unpubl. res.), for a total of 17 studies in this dataset (Table 1). Three approaches have been used to test the effects of plant polyploidy on the mutualism (Table 1 A): (1) Natural Comparisons, in which traits are compared among natural diploids and polyploids within or among species (N = 13 studies), (2) Phylogenetically Informed Comparisons, in which polyploids are compared to their isogenic diploid progenitors with known time of WGD events (N = 1 study), and (3) Experimental Manipulations, in which polyploid plants are synthesized and compared to their diploids progenitors (N = 3 studies). Experimental Manipulation studies allow for separating the effects of polyploidy from the effects of hybridization and other evolutionary changes since the WGD event, but generating neopolyploids

is challenging (Shi et al., 2015) and studies using this approach are limited (Table 1 A3). Although studies using Natural Comparisons and Phylogenetically Informed Comparisons approaches do not test the direct and immediate effects of polyploidy on the mutualism, they can inform whether polyploid plants have altered and/or enhanced relationships with rhizobia over evolutionary time.

Studies in the dataset tested ploidy effects in 24 species across six genera; however, over half (nine of 17 studies) used *Trifolium* species (Table 1 B). Furthermore, 13 of the 17 plant taxa are autopolyploids with only four using allopolyploid taxa (Table 1 C). The majority of studies (11 of 17) compared diploids and tetraploids, but several included other ploidy levels (triploids, hexaploids, and octoploids; Table 1 D). All studies were conducted in pots, test tubes, or jars in either glasshouses or growth chambers (Appendix A Table 2). These studies reveal long-standing interests in the effects of polyploidy on the legume-rhizobia mutualism, as 11 of the 17 studies were conducted between 1954 – 1980. They also reveal a striking gap in experimental studies addressing this question, especially given recent advancements in genetic and genomic techniques (Dufresne et al., 2013).

Although the relatively small number of studies limits quantitative analyses, results from these studies can be synthesized using several approaches to gain insight into the direct effects of plant polyploidy on the legume-rhizobia mutualism. First, a qualitative synthesis of general outcomes across studies allows for identifying broad patterns of the effects of plant polyploidy on the mutualism (Table 1 E). Variation in approach, origin of polyploid plants, nodulation traits, and experimental methods among studies may lead to idiosyncratic or species specific outcomes when synthesizing results across the dataset; thus, in-depth details about each study are provided in Appendix A, Table 2. Second, evaluating specific outcomes of polyploidy on key mutualism traits (e.g., nodule number, plant N content; Table 1 F), aids in determining specific mechanisms by

which polyploidy alters the mutualism. To assess the weight of current evidence for specific traits in the hierarchy, we organized results from all studies in the dataset by each trait in Appendix A, Table 3. Third, considering case studies using the Experimental Manipulation approach provides insight into the direct and immediate effects of polyploidy on mutualism traits. In addition to these perspectives, we discuss data from studies of the effects of ancient WGD on the mutualism as well as studies of synthetic neopolyploid plants alone to test the immediate effects of WGD on plant traits (e.g., flavonoid composition, photosynthetic rate) to predict their effects on the legume-rhizobia mutualism.

1.1.4 Overview of framework

Fundamental features of polyploidy such as increased cell size and alterations to genetic content and activity (Song et al., 1995; Levin, 2002; Beaulieu et al., 2008; Shi et al., 2015) could directly and immediately enhance the legume-rhizobia mutualism thereby allowing plants to access more fixed N, ultimately increasing plant growth and reproductive success (Fig. 1 VI; Parker, 1995; Heath and Tiffin, 2007; Munoz et al., 2016). Such enhancements could result from increases in the quantity or the quality of rhizobial symbionts hosted by legume plants.

First, enhancements in the quantity of rhizobial symbionts could be achieved if polyploid plants host more bacteroids than diploids. Direct changes in root architecture resulting from polyploidy (e.g., increase in root length and volume; Kulkarni and Borse, 2010) could enhance infection rate by rhizobia, thereby increasing the total number of nodules produced and bacteroids hosted (Fig. 1 I; Nutman, 1967; Kabi and Bhaduri, 1978). Enlarged cell size immediately resulting from polyploidy may increase nodule size and subsequently the number of bacteroids contained within nodules (Fig. 1 II; Kondorosi et al., 2000; Beaulieu et al., 2008; Maroti and Kondorosi,

2014). Additionally, WGD may directly alter plant host factors that control terminal bacteroid differentiation, thereby increasing the number and symbiotic efficiency of bacteroids hosted by polyploid plants relative to diploids (Fig. 1 III; Mergaert et al., 2006; Oono & Denison, 2010; Van de Velde et al., 2010; Kondorosi et al., 2013)).

Second, enhancements in the quality of rhizobial symbionts hosted by legume plants could be achieved through two additional pathways: improving the nodule environment for rhizobia or by altering the identity of rhizobial symbionts engaged in the mutualism. In terms of the nodule environment, polyploidy might immediately change O_2 and nutrient diffusion rates into nodules and leghemoglobin quantity and functions relative to diploids, thereby providing a more efficient environment for N fixation to occur (Fig. 1 IV; Robson and Postgate, 1980; Denison and Layzell, 1991; Hunt and Layzell, 1993; Warner and Edwards, 1993; Levin, 2002). Moreover, polyploid plants may have more photosynthetic resources to allocate to nodules than diploid plants (Warner and Edwards, 1993; Levin, 2002; Ramsey and Schemske, 2013). In addition, changes in plant chemistry resulting from polyploidy could affect the identity of rhizobial symbionts via partner choice and host sanctioning mechanisms (Fig. 1 V; Levy, 1976; Levin, 2002; Powell and Doyle, 2015).

Despite the numerous pathways by which polyploidy could enhance the legume-rhizobia symbiosis, few studies have tested any specific mechanisms. The thickness of boxes within the hierarchy reflects the number of published studies that have explicitly tested each hypothesis (Fig. 1). Hypotheses that have been never been tested are outlined in thin boxes, hypotheses that have been tested in one to five published studies are outlined in medium boxes, and hypotheses that have been tested in six or more published studies are outlined in thick boxes. In the following paragraphs, we formalize hypotheses for how polyploidy might directly enhance the quantity and

quality of mutualists hosted by legumes, confront these with current evidence, and in doing so highlight areas in great need of empirical work.

1.2 Quantity of symbionts: nodule number

Increased cell size and genomic changes resulting from polyploidy may alter root and nodule traits, leading to the production of more nodules that can accommodate more rhizobial symbionts than diploids (Fig. 1 I; Kondorosi et al., 2000; Levin, 2002; Beaulieu et al., 2008; Melino et al., 2012; Shi et al., 2015).

Nodule number is partially influenced by timing of nodulation, root size and architecture, and autoregulation of nodulation (Nutman, 1967; Diatloff and Ferguson, 1970; Kabi and Bhaduri, 1978; Reid et al., 2011; Thilakarathna et al., 2012). Reduced time to nodulation might occur if polyploidy alters plant signaling molecules that function in mutualism establishment (e.g., flavonoids, Nod factor receptors; Powell and Doyle, 2015). Plants that nodulate earlier have more time to develop root nodules, which could increase nodule production and result in a greater quantity of bacteroids hosted by the plant (Hely, 1957; Evans and Jones, 1966; Diatloff and Ferguson, 1970). Early effective nodulation is thought to be particularly important for plant survival and fitness in N limited environments (Diatloff and Ferguson, 1970).

Across all studies, there were no consistent effects of polyploidy on time to nodulation: five studies found that polyploid plants nodulated earlier and four studies found the opposite (Table 1 FI). However, synthetic neotetraploids of *Phaseolus aureus* produced nodules significantly earlier than its diploid progenitors, suggesting that plant polyploidy immediately reduces time to nodulation. In contrast, synthetic neotetraploid and neooctoploid *M. sativa* plants did not differ

from their diploid progenitors in time to nodulation (Table 1 A3 FI). While plant polyploidy might not directly and consistently reduce time to nodulation, these results might also reflect variation in experimental approach across the studies (Appendix A Table 2). Moreover, five of the nine studies either did not conduct statistical analyses or only report anecdotally that diploids and tetraploids differ in time to nodulation (Nilsson and Rydin, 1954; Hely, 1957; Weir, 1961b; Evans and Jones, 1966; Diatloff and Ferguson, 1970).

Enhancements in root length and lateral root production due to polyploidy can enhance rhizobial infection rate and subsequently increase nodule production per plant (Nutman, 1948; Nutman, 1967; Kabi and Bhaduri, 1978). Across all studies, four of four found that polyploid plants produced roots with greater size or biomass than diploid plants (Table 1 FI). Although not a legume, in *Capsicum annuum*, synthetic neotetraploid plants produced longer primary roots and more lateral roots than diploids, suggesting an immediate effect of polyploidy on root size and morphology (Kulkarni and Borse, 2010). In *P. aureus*, synthetic neotetraploid plants had significantly greater volumes of tap and lateral roots, and a higher infection rate by rhizobia (Table 1 A3 FI; Kabi & Bhaduri, 1978). Consistent with this, Powell and Doyle (2016) found a higher rate of root hair deformation in allopolyploid, *Glycine dolichocarpa*, relative to its diploid progenitors (Table 1 A2 FI). Combined, these results show positive, direct effects of plant polyploidy on root size and architecture, and rhizobial infection rate.

However, increases in root morphology and rhizobial infection rate of polyploid plants did not lead to increases in nodule production. Across all studies, polyploid plants did not consistently produce more nodules than diploids: seven studies found that polyploids produced more nodules than diploids and eight found the opposite (Table 1 FI). Allopolyploid *G. dolichocarpa* did not differ in nodule production compared to its diploid progenitors (Table 1 A2 FI; Powell and Doyle,

2016). Of the three studies that used synthetic polyploids to evaluate the direct effects of polyploidy on nodule production, one found the neotetraploids produced fewer nodules than diploids (Kabi and Bhaduri, 1978), while the other two found no significant differences among diploids and polyploids (Table 1 A3 FI; Leps et al., 1980; Pfieffer et al., 1980).

Lack of ploidy effects on nodule production may be due to variation in methods among experimental studies (Appendix A Table 2) or any number of factors that are known to affect nodule number (e.g., plant biomass, rhizobium genotype, environmental conditions; Heath and Tiffin, 2007; Regus et al. 2015). Because production and investment in nodules can be energetically costly to plant hosts, nodule production is regulated via autoregulation of nodulation (Caetano-Anolles and Gresshoff, 1991; Reid et al., 2011). Autoregulation of nodulation may function similarly in diploid and polyploid plants and explain the lack of ploidy effects on nodule production. This process occurs in response to host infection condition and soil N availability and is characterized by a nodulation phenotype in which nodules form near the crown of the roots and decrease along the root surface (Reid et al., 2011). Autoregulation of nodulation occurs systemically and involves a signaling circuit between root and shoot tissue, ultimately restricting the production of additional nodules (Reid et al., 2011). Although the molecular basis of this process is relatively well understood (Ferguson et al., 2010; Reid et al., 2011), it remains unclear whether and how plant polyploidy directly alters it and how it may constrain differences in nodule production in diploid and polyploid plants (Fig. 1 I, thin boxes).

1.3 Quantity of symbionts: nodule size and biomass

Even if polyploidy does not directly alter nodule number, it may increase nodule size, resulting in a greater quantity of symbionts hosted by polyploid plants relative to diploids (Fig. 1 II; Heath and Tiffin, 2007; Regus et al., 2015). Genome size is strongly correlated with cell size across 101 angiosperm species (Beaulieu et al., 2008) and polyploidy directly increases cell size in synthetically produced *Capiscum annuum*, *Chamerion angustifolium*, *Vicia cracca*, as well as other plant taxa (Maherali et al., 2009; Kulkarni and Borse, 2010; Munzbergova, 2017). Therefore, polyploids may be predisposed to hosting large numbers of bacteroids because larger cells are needed to accommodate N-fixing bacteroids; indeed many legumes undergo endoreduplication in nodule tissue to achieve greater cell sizes (Mergaert et al., 2006; Kondorosi et al., 2013; Maroti and Kondorosi, 2014). Endoreduplication of nodule tissue has been detected in legume species with indeterminate, determinate, and lupinoid nodules (Gonzalez-Sama et al., 2006; Kondorosi et al., 2013). However, the content and distribution of polyploid nuclei vary across legume hosts, and some legume taxa (e.g., *Glycine*) do not undergo endoreduplication of nodule tissue at all (Schwent, 1983; Gonzalez-Sama et al., 2006).

Of the taxa that do undergo endoreduplication of nodule tissue, polyploid plants may produce nodule cells with higher ploidal levels than diploids, and thereby accommodate a greater quantity of bacteroids. Consistent with this, in nodules of isogenic diploid, tetraploid, and octoploid *M. sativa* plants, diploid plants produced nodules with mostly tetraploid nuclei, tetraploid plants produced nodules with tetraploid and octoploid nuclei, and octoploid plants produced nodules with mostly octoploid nuclei (Shanklin and Schrader, 1986). Alternatively, even if ploidal level of nodule cells is the same for diploid and polyploid plants, nodule cells of polyploids may reach their max ploidal level (e.g., 32C or 64C) faster than nodule cells of diploids

because they experience fewer cycles of endoreduplication (Kondorosi et al., 2000; Gonzalez-Sama et al., 2006).

Across all studies, seven of ten found that polyploids produced larger nodules or nodules with greater biomass than diploids (Table 1 FII). Allopolyploid *G. dolichocarpa* produced nodules with greater biomass than both of its diploid progenitors (Table 1 A2 FII; Powell and Doyle, 2016). Synthetic neotetraploid plants of *P. aureus* also produced larger nodules than its diploid progenitor, supporting an immediate and direct effect of polyploidy on nodule size (Table 1 A3 FII; Kabi and Bhaduri, 1978). However, nodule size did not differ between diploid and synthetic neotetraploid *M. sativa* plants (Pfieffer et al., 1980). These studies suggest that polyploidy directly increases nodule size and biomass, although these effects may depend on host taxa, symbiont taxa, or both.

While these results suggest polyploid plants ought to host more rhizobial symbionts per plant than diploids, additional work is needed to evaluate this hypothesis as well as the potential underlying mechanisms (Fig. 1 I-II, thin boxes). Although the hypotheses regarding endoreduplication and ploidal level of nodule cells in diploid and polyploid plants are theoretically possible, empirical tests are lacking and therefore we can only speculate about potential effects of plant polyploidy on these traits. Experimental Manipulation studies evaluating the direct effects of polyploidy on timing of nodulation, root architecture, and nodule number will be imperative to tease apart the direct effects of polyploidy from other evolutionary changes that have occurred since the WGD event. Moreover, to our knowledge, no published studies have measured bacteroid quantity within nodules of diploid and polyploid plants. Such experiments are essential for determining whether polyploid plants host more bacteroids than diploids, thereby increasing access to fixed N and host benefit from the mutualism.

1.4 Quantity and quality of symbionts: terminal bacteroid differentation

In addition to changes in the quantity of the mutualism via nodule number and size, plant polyploidy might directly alter plant host factors regulating terminal bacteroid differentiation, thereby increasing the number and symbiotic efficiency of bacteroids hosted by polyploid plants relative to diploids (Fig. 1 III; Oono & Denison, 2010). Terminal bacteroid differentiation occurs when rhizobia enter the plant host cell and undergo cell expansion, genome endoreduplication, and membrane permeabilization (Van de Velde et al., 2010; Kereszt et al., 2011; Maroti et al., 2011; Alunni and Gourion, 2016). Terminal bacteroid differentiation is regulated by plant antimicrobial peptides 'nodule-specific cysteine-rich peptides' (NCRs, Van de Velde et al., 2010). Nodule-specific cysteine-rich peptides were identified in legumes of the inverted repeat-lacking clade and functional homologues of NCRs were recently found in the genus, *Aeschynomene*, but terminal bacteroid differentiation does not occur in all legume taxa (Van de Velde et al., 2010; Maroti et al., 2011; Alunni and Gourion, 2016). Notably, plants that impose terminal bacteroid differentiation on rhizobia have more symbiotically efficient bacteroids and benefit more from the mutualism than plants that do not (Oono & Denison, 2010).

Nodule-specific cysteine-rich peptides exhibit extensive diversity (e.g., *M. truncatula* contains over 600 NCRs) and are hypothesized to differ in function, modes of action, and bacterial targets; yet many specific functions remain unresolved (Farkas et al., 2014; Maroti and Kondorosi, 2014; Horvath et al., 2015). While no studies have explicitly tested the direct effects of plant polyploidy on the composition and function of NCRs, genome duplication is known to alter expression patterns of polypeptides and peptide transporters in *Brassica* and *Utricularia*, respectively (Albertin et al., 2006; Lan et al., 2017). Moreover, in *M. truncatula*, ancient WGD is hypothesized to have enhanced the legume-rhizobia mutualism because many amplified gene

families, including the NCR gene family, have nodule specific functions (Young et al., 2011). If polyploidy directly enhances the diversity and functions of NCRs, then polyploid plants may have a greater ability to regulate terminal bacteroid differentiation, thereby increasing bacteroid quantity and symbiotic efficiency. However, no published studies have tested whether plant polyploidy directly alters the composition and functions of NCRs or the process of terminal bacteroid differentiation (Fig. 1 III, thin boxes); thus, we do not have sufficient data to decisively conclude whether plant polyploidy affects these mutualism traits.

1.5 Quality of symbionts: nodule environment

The other primary pathway by which polyploidy could enhance the legume-rhizobia mutualism is by improving the quality of the symbiosis, and this could be achieved by enhancing the nodule environment (Fig. 1 IV). Improvements in the nodule environment may allow for finer regulation of O_2 content within nodules, which is critical for rhizobial growth and nitrogenase function (Robson and Postgate, 1980; Sheehy et al., 1983; Kiers et al., 2003). Nodule permeability and leghemoglobin are two key factors that regulate O_2 concentration within nodules (Robson and Postgate, 1980; Denison and Layzell, 1991; Hunt and Layzell, 1993), both of which could be altered by polyploidy (Warner and Edwards, 1993; Kondorosi et al., 2000; Levin, 2002).

Nodule permeability is primarily limited by one or more layers of densely packed cells that comprise the nodule inner cortex (Denison and Layzell, 1991; Hunt and Layzell, 1993; Denison, 2015). Enlargements in cortex cell size due to polyploidy might increase cortex thickness or adjust the size and distribution of intercellular spaces in the inner cortex layer, which could either decrease or increase nodule permeability relative to nodules produced by diploid plants.

Leghemoglobin is an O_2-binding protein that facilitates O_2 diffusion to respiring bacteroids (Appleby, 1984; Hunt and Layzell, 1993), and its content within nodules is correlated with N-fixing ability (Appleby, 1984). Polyploidy can have drastic effects on plant genomes by altering gene expression patterns and sub- and neo-functionalization of duplicated gene copies (Levin, 2002; Doyle et al., 2008; Li et al., 2013; Shi et al., 2015); thus, polyploid plants may produce more leghemoglobin or have modified functions of leghemoglobin gene copies compared to diploids. Consistent with this, Young et al. (2011) found that the leghemoglobin gene family was amplified in the *M. truncatula* genome following WGD and contains nine symbiotic leghemoglobins (double those present in *Lotus japonicas* and *G. max*), supporting the hypothesis that ancient WGD provided the genetic material to increase the complexity of rhizobial symbioses.

Another consequence of increased cell size due to polyploidy is a reduction in the surface area to volume ratio of the cell, which can influence the rate of nutrient exchange (Kondorosi et al., 2000). Reduced surface area to volume ratios of polyploid cells may result in a greater barrier to O_2 diffusion into nodules of polyploid plants, thereby providing a more efficient environment for nitrogenase function (Appleby, 1984). There are no published studies of the direct effects of polyploidy on the surface area to volume ratio of nodules and subsequent impacts on nutrient exchange, but a study on the effects of ancient WGD hypothesizes a positive effect of polyploidy on nutrient exchange in the legume-rhizobia symbiosis. In the Papilionoideae, paralogues derived from the WGD event that function in nutrient exchange (e.g., ammonium assimilation) have been retained across many papilionoid taxa (Li et al., 2013). Moreover, many gene families that function in nutrient exchange have been amplified following this WGD event, suggesting that polyploidy may have provided genes to enhance the symbiosis (Li et al., 2013).

In addition to potential changes in O_2 concentration and nutrient exchange rates, polyploid cells often have greater metabolic and transcriptional activity than diploid cells (Levin, 2002; Doyle et al., 2008; Shi et al., 2015). Therefore, nodules that grow via endoreduplication may have an increased ability to provide energy and nutrients to rhizobia for the metabolically costly process of nitrogen fixation (Kondorosi et al., 2000; E Kondorosi and A Kondorosi, 2004; Mergaert et al., 2006). Since photosynthate supply and N fixation rate are positively correlated (Lawrie and Wheeler, 1973; Singleton and van Kessel, 1987; Walsh et al., 1987), if polyploid plants provide more photosynthetic resources to rhizobia within nodules, then they may acquire more fixed N via the symbiosis. Studies experimentally manipulating ploidy level have found that polyploidy directly increases photosynthetic rate and chloroplast number per cell, although these changes do not always scale to the entire plant (Warner and Edwards, 1993; Levin, 2002; Maherali et al., 2009). Although these data suggest that polyploidy can directly alter photosynthetic processes, which may result in polyploid plants having more photosynthates to allocate to nodules, empirical tests are lacking and therefore we can only speculate about potential effects of plant polyploidy on resource allocation to nodules.

Despite the numerous ways in which polyploidy may directly improve the nodule environment and increase access to fixed N, limited work is available to evaluate these hypotheses. While specific mechanisms for how polyploidy may enhance the nodule environment have not been explicitly tested, two of three studies found that polyploid plants fix N at a higher rate than diploids (Table 1 FIV). This result suggests that polyploid plants have an increased ability to fix N relative to diploids, but it is not clear whether this is due to direct modifications of the nodule environment via polyploidy. To our knowledge, no studies have explicitly tested whether polyploidy directly alters nodule structure and permeability, leghemoglobin production and

function, nutrient exchange, or photosynthetic supply to nodules (Fig. 1 IV, thin boxes). Research addressing these hypotheses will be particularly insightful for understanding whether polyploidy directly improves the nodule environment allowing polyploid plants to access more fixed N.

1.6 Quality of symbionts: identity of rhizobial symbionts

The final pathway by which polyploidy might enhance the quality of the legume-rhizobia mutualism is via the identity of rhizobia engaged in the symbiosis relative to diploids (Fig. 1 V). Although ensuring cooperation in the legume-rhizobia mutualism is complex, legume hosts use two primary mechanisms, partner choice and host sanctions, to influence the identity and efficiency of their rhizobial partners (Sachs et al., 2004; Kiers and Denison, 2008; Sachs et al., 2010).

Partner choice is the establishment of the symbiosis with rhizobial partners based on recognition signals (e.g., flavonoids, Nod factors, Nod factor receptors; Sachs et al., 2004; Kiers and Denison, 2008), which are genetically determined and may be altered by polyploidy (Stacey et al., 2006; Young et al., 2011; Li et al., 2013; Powell and Doyle, 2015). Polyploidy can directly increase the composition, concentration, and diversity of flavonoids produced by the host plant (Levy, 1976; Levin, 2002), thereby broadening the suite of symbionts solicited for the symbiosis (i.e., host promiscuity; Li et al., 2013; Powell and Doyle, 2015).

Although not a legume, synthetically created autotetraploids of *Phlox drummondii* produced 14 novel flavonoids that were not present in their diploid progenitors, supporting a direct effect of plant polyploidy on flavonoid composition (Levy, 1976). Additionally, the flavonoid biosynthetic pathway in *M. truncatula* expanded considerably post WGD (Young et al., 2011) and

Li et al. (2013) found at least eight enzymes in the flavonoid biosynthetic pathway were retained following the WGD event in the Papilionoideae.

Similar expansions have been observed for Nod factor receptors of *M. truncatula* (Young et al., 2011) as well as other Papilionoideae taxa (Li et al., 2013). Specifically, the Nod factor receptor (*NFP*) and transcription factor (*ERN1*) retained from the papilionoid WGD event exhibit nodule-enhanced expression patterns in *M. truncatula*, potentially reflecting sub-functionalization of ancestral genes following WGD (Young et al., 2011). Consistent with this, Li et al. (2013) found duplicated genes retained from the papilionoid WGD event that amplified the *LysM* receptors gene family, which are key components to Nod factor receptors. Together, these studies suggest that polyploidy can increase the abundance and diversity of flavonoids and Nod factor receptors, which can lead to enhanced and more complex signaling to rhizobial partners (Young et al., 2011; Li et al., 2013; Powell and Doyle, 2015).

Across four studies, all found that polyploids could form effective symbioses with a broader range of rhizobial symbionts than diploids (i.e., greater host promiscuity; Table 1 FV). Moreover, in synthetic autotetraploid *P. aureaus*, diploids and tetraploids differed in nodule occupancy when co-inoculated with two rhizobial strains, suggesting an immediate effect of polyploidy on the identity of rhizobial symbionts hosted within nodules (Table 1 A3 FV; Kabi and Bhaduri, 1978).

Although limited, these data support the hypothesis that polyploid plants have the potential for increased host promiscuity and altered communities of rhizobial symbionts relative to diploids, and this has been confirmed in five cases. Additional studies testing nodulation capabilities of diploid and polyploid plants across diverse rhizobial strains will provide insight into whether polyploidy increases host niche breadth and thereby access to more beneficial symbionts. Studies

evaluating whether polyploidy directly enhances the abundance, composition, and/or diversity of flavonoids and Nod factor receptors will be critical for understanding the mechanistic basis of partner choice and host promiscuity apart from subsequent evolution that occurred after WGD (Fig. 1 V, thin boxes).

The second key mechanism for regulating the identity of rhizobia occupying root nodules is host sanctioning, the ability of the plant to assess nodule efficiency and invest more in efficient nodules than inefficient ones (Kiers and Denison, 2008; Sachs et al., 2010; Regus et al; 2014). The process of N fixation imposes a high metabolic cost for rhizobia; thus, ineffective rhizobia may have increased fitness relative to effective rhizobia (Kiers et al., 2003; Kiers and Denison, 2008; Sachs et al., 2010). In the context of the legume-rhizobia mutualism, where a host species often interacts with multiple symbionts, host sanctions can ensure cooperation among partners (Kiers and Denison, 2008; Sachs 2010). Hosts are hypothesized to impose sanctions via several mechanisms, but primarily by limiting the supply of carbon or O_2 to inefficient nodules and allocating more photosynthetic resources to highly efficient nodules (Singleton and van Kessel, 1987; Kiers et al., 2003). If polyploidy alters O_2 permeability and concentration within nodules, as described in regards to the nodule environment, then polyploids may also exhibit differences in host sanctioning abilities relative to diploids. Moreover, if polyploid plants have increased photosynthetic resources relative to diploids, then allocating photosynthates to highly effective nodules may result in an even greater amount of fixed N acquired via the symbiosis. Although it is theoretically possible for plant polyploidy to alter host sanctions, no studies have explicitly addressed whether diploid and polyploid plants differ in host sanctioning abilities nor tested any of the underlying mechanisms proposed here (Fig. 1 V, thin boxes).

1.7 Access to fixed nitrogen via enhanced symbioses

Direct alterations in the quantity and quality of rhizobial symbionts due to polyploidy could allow polyploid plants greater access to fixed N (Fig. 1 VI), thereby increasing their biomass and reproductive success relative to diploids (Parker, 1995; Heath and Tiffin, 2007; Munoz et al., 2016). Host benefit from the mutualism can be tested by comparing N content of plant tissue or overall plant size (Munoz et al., 2016), although fitness estimates (e.g., seed production) would be best, it is difficult to assay nodule traits and plant reproductive success simultaneously (Regus et al., 2015).

Across all studies, most (11 of 15) found that polyploid plants had greater N content, size, and/or biomass than diploids (Table 1 F VI). Most tested the effects of polyploidy on plant biomass when plants were inoculated with single rhizobial strains. Importantly, only two cases directly compared plant biomass to uninoculated controls, the most salient metric of host benefit from the mutualism, within ploidy level, both of which found polyploids produced more biomass when inoculated with rhizobia than diploids (Evans and Jones, 1966; Leps et al., 1980). However, the work of Weir (1961 *b*) can shed additional light on this issue, as our post hoc comparison between inoculated and uninoculated plants within ploidy level in his study revealed that polyploids had greater increases in plant biomass when inoculated with rhizobia than diploids.

Taken together these data suggest that polyploid plants benefit more from the mutualism than diploids, but it is important to note that many do not include uninoculated controls and/or rely on indirect measures of host benefit. Comparisons of growth of inoculated to uninoculated plants within ploidy levels (i.e., host growth response) for diploids and polyploids are essential to separate the effects of the mutualism from the effects of ploidy alone. If possible for the plant taxa, assaying nodule traits and plant reproductive success (e.g., seed set) within an experiment will aid in

determining the effects of the mutualism on host fitness. In addition, experiments measuring N content derived from the mutualism rather than the environment among diploid and polyploid plants can be conducted using ^{15}N methodologies, acetylene reduction assays, or other techniques (Anglade et al., 2015; Chalk et al., 2016). Such studies are imperative to determine whether polyploid plants have increased access to N relative to diploids and whether polyploid plants benefit more when engaged in the symbiosis as opposed to obtaining N solely from the environment.

1.8 Recommendations for future work

Our synthetic framework makes clear the myriad ways in which plant polyploidy can directly and immediately affect how legumes interact with their rhizobial symbionts as well as the magnitude of the benefits they derive from the interaction. However, our literature review also illustrates that we do not yet have a consensus on whether polyploidy directly enhances host benefits from the mutualism and we have very limited understanding of the mechanisms that underlie the variation in results achieved thus far (Fig. 1, thin and medium boxes). Previous work using Natural Comparisons, Phylogenetically Informed Comparisons, and Experimental Manipulation approaches as well as studies evaluating the effects of ancient WGD on the mutualism provide strong support for the role of polyploidy in enhancing key aspects of the symbiosis, yet rigorous experimental tests are lacking. Here, we highlight three key areas in need of attention to clarify the direct effects of plant polyploidy on the legume-rhizobia mutualism: improved experimental tests, untested mechanistic hypotheses, and studies in natural environments.

Several limitations in the work conducted thus far are the small number of studies that have experimentally tested the effects of plant polyploidy on the mutualism and the lack of variation in plant taxa, rhizobial taxa, nodulation type, and polyploid type within these studies. Only three studies have used the Experimental Manipulation approach and only one study has used the Phylogenetically Informed Comparisons approach to compare isogenic diploid and polyploid plants (Table 1 A2,3). Additional studies using the Experimental Manipulation approach (Table 1 A3) by synthesizing auto- and allopolyploids will be particularly informative for assessing the immediate and direct effects of polyploidy on the mutualism, and allow separation of these from subsequent adaptation of the host or rhizobia post WGD (Segraves, 2017). Another limitation from the previous work is lack of taxonomic diversity, as most studies used *Trifolium* species (Table 1 B). Studies across a broader range of legume hosts are needed to test aspects of this framework and gain a generality. Moreover, these studies exhibit limited diversity in nodulation type as 12 of the 17 used legume taxa that produce indeterminate nodules. It would be interesting to determine how hypotheses within this framework vary for legumes with different nodulation types (e.g., desmodioid, aeschynomenoid, lupinoid) as well as additional mechanisms that can be included in the framework relevant to these different nodulation types (e.g., hormones, flavonoids that affect auxin fluxes; Grunewald, 2009; Ferguson, 2010; Sprent 2013). Lastly, the majority of legume taxa used in these studies are autopolyploids (Table 1 C); thus, additional tests of allopolyploid legumes and their known diploid progenitors are essential for evaluating how the effects of polyploidy as well as hybridization impact the mutualism.

Another major gap is that many mechanisms in this framework have never been tested (Fig. 1, thin boxes). Although several studies have quantified ploidy effects on mutualism traits that alter the quantity of rhizobial symbionts (e.g., nodule number, nodule size and biomass), we still

lack an understanding of how plant polyploidy influences symbiont quantity inside the nodule. Studies comparing bacteroid quantity and function as well as the process of terminal bacteroid differentiation in isogenic diploids and polyploids will provide critical insight into whether and how polyploidy affects the quantity and quality of bacteroids. Moreover, we have limited understanding of the mechanisms that may underlie variation among diploid and polyploid plants in terms of symbiont quantity. Studies testing whether and how plant polyploidy alters autoregulation of nodulation and endoreduplication of nodule tissue may reveal pathways by which polyploidy has directly enhanced mutualisms with rhizobia.

The current data also highlights key gaps in our understanding of how plant polyploidy affects the quality of rhizobial symbionts hosted by legumes (Fig. 1 III – V, thin boxes). To our knowledge, no studies have tested whether polyploidy alters the process of terminal bacteroid differentiation, thereby increasing the quality of bacteroids hosted by polyploid plants. Studies assessing how polyploidy directly alters the internal structure of nodules of diploids and polyploids are urgently needed to clarify how differences in cell size can impact nodule cortex structure, O2 concentration, nutrient exchange, and N fixation capacity. Tests of photosynthetic rates and resource allocation to nodules will also contribute to a mechanistic understanding of whether and how plant polyploidy enhances the nodule environment, potentially increasing host access to fixed N. Lastly, studies testing whether isogenic diploids and polyploids differ in signaling to rhizobia (e.g., flavonoids, Nod factor receptors) and subsequent effects on the identity of rhizobial symbionts will inform how polyploidy might directly alter host promiscuity and sanctioning mechanisms..

Finally, all aspects of the framework should be evaluated in ecologically relevant contexts (Segraves, 2017). Because all experimental studies were conducted in either a glasshouse or

growth chamber (Appendix A Table 2) it remains unclear how plant polyploidy affects the mutualism in natural environments. Moreover, mutualism traits and host benefit are often context dependent and influenced by environmental conditions (Heath and Tiffin, 2007; Kiers et al., 2010). For instance, varying parameters such as light limitation and nutrient availability in the soil, are likely to affect the relative importance of specific mechanisms (e.g., efficiency of N fixation, allocation of O2 and photosynthates), and subsequent differences between diploid and polyploid plants. Studies conducted in natural environments and evaluating how specific environmental parameters alter mutualism traits are essential for addressing the ecological and evolutionary consequences of plant polyploidy on the symbiosis. Interestingly, polyploidy and the bacterial mutualism are hypothesized to enhance plant invasion success (Daehler, 1998; te Beest et al., 2012; Pandit et al., 2014), and it would be particularly informative to evaluate the proposed mechanisms among diploid and polyploid taxa in native and non-native habitats.

1.9 Conclusions

The conceptual framework reveals tantalizing support for the role of plant polyploidy in directly enhancing the legume-rhizobia mutualism and provides novel mechanistic hypotheses that may underlie this pattern, but it also highlights many unexplored avenues that warrant further investigation. Thus, it makes clear where future work on the effects of plant polyploidy on the legume-rhizobia mutualism will be most beneficial. Such work is even more pressing in light of current global concerns such as food security and climate change, yet we cannot address these challenges without a thorough understanding of the direct effects of polyploidy on the mutualism as well as the underlying mechanisms.

Table 1. Summary of published studies testing the effects of plant polyploidy on the legume-rhizobia mutualism. Studies are organized by A, Approach; B, Plant Taxa, then C, Polyploid Type. E, General Outcome for each study summarizes whether polyploids (P) have enhanced (>), reduced (<), or no difference (=) in nodulation traits and/or host benefit relative to diploids (D). F, Specific Outcomes for each study always compare polyploid plants to diploids and are organized by traits (I-VI) within the hierarchy.

A. Approach	B. Plant Taxa	C. Polyploid Type	D. Ploidy Levels Tested	E. General Outcome	F. Specific Outcomes					Reference
					I	II	IV	V	VI	
1. Natural Comparisons										
	Arachis	Allopolyploid	2X-4X	P > D	Polyploids produce more nodules	Polyploids produce larger nodules	Polyploids fix N at a higher rate		Polyploids produce more biomass	Stalker et al., 1994
	Glycine wightii (*Neonotonia wightii*)	Autopolyploid	2X-4X	P > D	Polyploids nodulate earlier and produce more nodules				Polyploids produce more biomass and have higher N content	Diatloff and Ferguson, 1970
	Medicago sativa	Autopolyploid	2X-4X	P > D	Polyploids produce more root biomass and nodules	Polyploids produce nodules with greater average biomass			Polyploids do not differ in shoot biomass	Forrester et al., PA, USA unpubl. res.
	Stylosanthes hamata, S. seabrana	Allopolyploid	2X-4X	P > D				Polyploids form effective symbioses with unique and more strains		Date, 2010
	Trifolium ambiguum	Autopolyploid	2X-6X	P > D	Polyploids nodulate earlier and produce more nodules	Polyploids produce smaller nodules		Polyploids form effective symbioses with more strains	Polyploids produce more biomass	Hely, 1957
	Trifolium ambiguum	Autopolyploid	2X-4X-6X	P > D	Polyploids nodulate earlier and produce more nodules	Polyploids produce larger nodules			Polyploids produce more biomass	Evans and Jones, 1966
	Trifolium ambiguum, T. pratense, T. repens	Autopolyploid	2X-4X-6X	P > D				Polyploids form effective symbioses with more strains		Beauregard et al., 2004
	Trifolium pratense	Autopolyploid	2X-4X	P ≤ D	Polyploids do not differ in timing of nodulation and produce fewer nodules				Polyploids do not differ in N content	Nilsson and Rydin, 1954

Trifolium pratense	Autopolyploid	2X-4X	P > < D	Polyploids nodulate later and produce fewer nodules	Polyploids produce larger nodules			Polyploids produce more biomass	Weir, 1961b
Trifolium pratense	Autopolyploid	2X-4X	P > D	Polyploids nodulate earlier, produce larger roots and more nodules				Polyploids produce more biomass	Thilakarathna et al., 2012
Trifolium pratens, T. repens	Autopolyploid	2X-4X	P > < D	Polyploids more nodules (*T. pratense*) or less nodules (*T. repens*)	Polyploids produce larger nodules			Polyploids produce more (*T. pratense*) or less (T. repens) biomass	Weir, 1961a
Trifolium repens	Allopolyploid	2X-4X	P ≤ D	Polyploids produce fewer nodules	Polyploids do not differ in nodule size			Polyploids produce less biomass	Weir, 1964
Trifolium subterraneum	Autopolyploid	2X-4X	P ≤ D	Polyploids do not differ in timing of nodulation and produce fewer nodules				Polyploids do not differ in biomass	Nutman, 1967
2. Phylogenetically Informed Comparisons									
Glycine dolichocarpa, G. syndetica, G. tomentella	Allopolyploid	2X-4X	P ≥ D	Polyploids have higher rates of root hair deformation, produce more root biomass, do not differ in nodule number	Polyploids produce larger nodules		Polyploids form effective symbioses with more strains	Polyploids produce more biomass	Powell and Doyle, 2016
3. Experimental Manipulations									
Medicago sativa	Autopolyploid	2X-4X-8X	P ≥ D	Polyploids do not differ in timing of nodulation or nodule number		Polyploids fix N at a higher rate		Polyploids have greater N content	Leps et al., 1980
Medicago sativa	Autopolyploid	2X-4X-8X	P ≥ D	Polyploids do not differ in nodule number	Polyploids do not differ in nodule size	Polyploids do not differ in N fixation rate		Polyploids produce more biomass	Pfeiffer et al., 1980
Phaseolus aureus	Autopolyploid	2X-3X-4X	P ≥ D	Polyploids nodulate earlier, produce larger tap roots, have higher rates of rhizobial infection, produce fewer nodules	Polyploids produce larger nodules		Polyploids differ in nodule occupancy of rhizobial symbionts	Polyploids produce more biomass	Kabi and Bhaduri, 1978

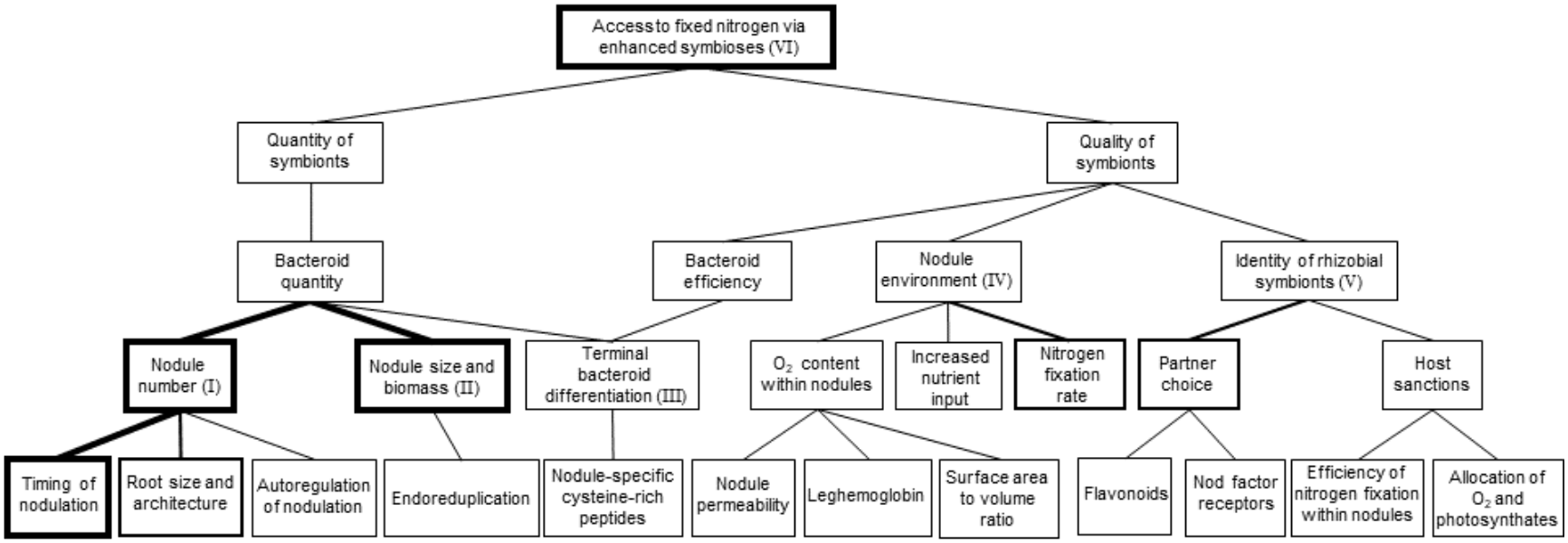

Figure 1. Framework of hypotheses for how plant polyploidy might directly enhance the legume-rhizobia mutualism. The framework is structured into a hierarchy with the predicted outcome that polyploid plants have greater access to fixed nitrogen via enhanced symbioses with rhizobia. Enhanced symbioses can broadly be categorized by improvements in the quantity and/or quality of rhizobial symbionts hosted. Specific mechanisms for how polyploidy can directly alter plant traits that affect the symbiosis are proposed. Hypotheses that have been never been tested are outlined in thin boxes, hypotheses that have been tested in one to five published studies are outlined in medium boxes, and hypotheses that have been tested in six or more published studies are outlined in thick boxes.

2.0 Polyploid plants benefit more from a nutrient acquisition mutualism than diploids by maintaining fitness across diverse partners

2.1 Introduction

Nearly all organisms engage in mutualisms, in which two species interact and benefit one another (Bronstein, 1994; Kiers et al., 2010; Afkhami and Stinchcombe, 2016). Generalists interact with and obtain benefits from a broad range of mutualistic partners, whereas specialists establish mutualisms with fewer but potentially more beneficial partners (Douglas, 1998; Ehinger et al., 2014). Organisms engaged in specialized mutualisms may outcompete generalists if they are locally adapted to symbionts in their environment, use resources more efficiently, or avoid interactions with ineffective partners or cheaters (Futuyma and Moreno, 1988; Ehinger et al., 2014; Batstone et al., 2018). However, generalization of mutualistic interactions may be favored in temporally and spatially heterogenous environments where partner availability and quality vary (reviewed in Batstone et al., 2018). Variation in the degree of generalization of mutualisms can be attributed in part to an organism's ability to interact with a broad taxonomic range of partners (i.e., niche breadth), obtain and maintain fitness benefits across a wide range of interactions (i.e., low plasticity in fitness), and/or reduce costs of associating with lower quality partners (Futuyma and Moreno, 1988; Douglas, 1998; Batstone et al., 2018). While numerous studies have characterized the degree of generalization in mutualisms across a wide range of plant and animal taxa, the mechanisms driving generalization in niche breadth and host benefits remain relatively unclear (Bascompte et al., 2003; Poisot et al., 2011; Afkhami and Stinchcombe, 2016).

A major genetic polymorphism that has the potential to shape generalization in mutualisms is polyploidy, or the condition in which an organism contains more than two complete sets of chromosomes from one or more donors (Levin, 1983; Husband et al., 2013; Soltis and Soltis, 2016). Polyploidy occurs in every major eukaryotic lineage, but is particularly common in plants, where all angiosperms are derived from a polyploid ancestor and 24% of extant plant species are polyploids (Husband et al., 2013; Barker et al., 2016; Soltis and Soltis, 2016). Because plants engage in mutualisms that serve a variety of reproductive (e.g., pollinators, seed dispersers; Segraves and Anneberg, 2016) and nutrient acquisition functions (e.g., mycorrhizae, nitrogen-fixing bacteria; Shantz et al., 2016), polyploidy could have profound effects on generalization in diverse types of species interactions. Specifically, increases in cell size, enhancements in genetic diversity, and physiological changes that occur after polyploidy events may permit plants to establish mutualisms with broader range of partners or obtain greater benefits from them (Segraves and Anneberg, 2016; Forrester and Ashman, 2018a). The few studies testing this hypothesis largely focus on reproductive mutualisms and have produced variable results (Thompson and Merg, 2008; reviewed in Segraves and Anneberg, 2016). Thus, it remains unclear whether polyploidy alters generalization in 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 plant (legume) - bacterial (rhizobia) symbiosis, in which rhizobia fix atmospheric nitrogen (N) into a plant-usable form in exchange for photosynthetic resources 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 across these partners, akin to 'biotic environments' (Forrester and Ashman, 2018a). Plants showing more generalized rhizobial interactions may have the ability to establish mutualisms with more diverse rhizobial partners, maintain high fitness across rhizobial strains (i.e., exhibit reduced plasticity in fitness), or reduce costs of associating with lower quality partners, resulting in greater and more consistent benefits obtained from the mutualism (Rodriguez-Echeverria et al., 2008).

Generalization may be enhanced by plant polyploidy if it increases the amount and diversity of resources available to invest in rhizobial symbionts (Powell and Doyle, 2015; Forrester and Ashman, 2018a). Polyploid plants often have faster photosynthetic rates 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, leghaemoglobins; Young et al., 2013; Li et al., 2013). In addition, polyploid plants have larger cells, which may allow them to host a greater quantity of rhizobia, thereby increasing the amount of N obtained (Forrester and Ashman, 2018a). These changes may 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 in Forrester and Ashman, 2018a). Although previous studies have characterized differences in nodule traits of diploid and polyploid legumes, it remains unclear whether these differences translate to greater generalization in taxonomic niche breadth and host benefits obtained by polyploids across diverse rhizobial environments (Segraves and Anneberg, 2016; Forrester and Ashman, 2018a).

We conducted a controlled inoculation experiment using geographically widespread sampling of diploid (2X) and autotetraploid (4X) lineages of the plant species complex, *Medicago sativa*, and a diverse panel of *Sinorhizobium* symbionts. We sought to determine whether

autotetraploid plants: (*i*) establish mutualisms with a broader range of rhizobial symbionts, (*ii*) obtain greater fitness benefits from rhizobial mutualisms, (*iii*) exhibit reduced plasticity in fitness across rhizobial environments, and (*iv*) show reduced costs of specialization in rhizobial interactions relative to diploids. This study demonstrates that autotetraploid plants exhibit greater generalization in bacterial mutualisms than diploids not due to increased niche breadth, but by obtaining greater fitness and maintaining it across a broad range of bacterial symbionts. We uncover traits driving these differences, and in doing so, provide insight into the increased ability of polyploid legumes to establish and spread across diverse biotic environments.

2.2 Materials and methods

2.2.1 Plant host selection and rhizobial strains

Medicago sativa is a species complex that consists of diploid and autotetraploid plant lineages. *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). Seeds from ten wild accessions were obtained from the USDA National Genetic Resources Program and $2x$ and $4x$ were matched by geographic origin when possible (Appendix B Table 4; http://www.ars-grin.gov/). Previous work has revealed significant genetic variation within diploid (Sakiroglu et al., 2010) and tetraploid (Ilhan et al., 2016) accessions of *M.* sativa, therefore we used accessions as a proxy for genetic lineages. Twenty-one strains of *Sinorizobium* were used to evaluate nodulation propensity, host growth response, and nodulation traits of diploid and autotetraploid *M. sativa* (Sugawara et al., 2013;

Appendix B Fig. 9). *Sinorhizobium meliloti* USDA1002 was obtained from Patrick Elia (National Rhizobium Germplasm Resource Collection) and all other strains were obtained from Michael Sadowsky (University of Minnesota).

2.2.2 Seed scarification and growth conditions

Scarified and surface-sterilized seeds were planted in sterilized growth pouches (CYG, Mega International) containing 20 mL of sterile, nitrogen-free Fahraeus solution, as described in the *Medicago truncatula* Handbook (https://www.noble.org/medicago-handbook/). For each *M. sativa* lineage, eight seeds were planted for each rhizobial strain or control treatment (four seeds/pouch, two pouch replicates/lineage/treatment). Pouches were sorted by treatment and replicate, then placed into sterilized plastic containers that held ten pouches each (one pouch/lineage/treatment). Each treatment had two replicate containers. Containers were transferred to a growth room set to 25°C, 60% humidity, and with supplemental lighting to achieve 16-hour days.

2.2.3 Experimental design, inoculations, and harvesting

The experiment was divided into four temporal blocks with each block using four to six unique rhizobial strains and a water-inoculated control treatment. Each block lasted six weeks and occurred between May and October 2017. Size-matched plants were randomly assigned to strains and pouches. Each plant was inoculated with 1.0 x 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 strains. Plants were given nine ml of nitrogen-

free Fahraeus solution once per week. All surviving plants (n = 1138) were harvested by removing them from pouches, counting number of leaves and nodules produced, and recording nodule color. Plants were then dissected into shoot, root, and nodule tissue, and dried in an oven at 55°C for at least four days. To quantity plant and nodule biomass for each plant, shoot and root tissue was weighed in grams using a Mettler Toledo AE-200 Analytical Balance, and total nodule biomass was measured in milligrams using a Cahn C-31 Microbalance. Of the control plants (n = 180), only one plant produced a single nodule and was excluded from analyses.

2.2.4 Statistical methods

Trait values of plants within pouches were averaged and the resulting data were used for subsequent analyses. Nodule color was quantified on a scale from zero (white, ineffective nodules) to one (pink, effective nodules). To evaluate potential bias in this scale, we re-ran nodule color analyses with a different scale, and the results did not change. Host growth response was quantified as the mean percentage difference in plant shoot biomass between inoculated and uninoculated controls within each lineage ((biomass inoculated plant – average biomass uninoculated plants)/average biomass uninoculated plants)*100; Regus et al., 2015) for the 17 nodulating strains. Linear mixed effects models were used to test for effects of ploidy, strain, and their interaction (fixed) and lineage nested within subspecies (random) on nodule traits and host benefit using the lme4 (v1.1-and lmerTest (v3.0-1) packages in R (v1.1.453). Relative distance plasticity index (RDPI; Valladares et al., 2006) and relative distance from max host growth response were calculated across all 21 rhizobial environments in R, and t-tests were used to test for significant differences between ploidy levels (stats package v3.3.3). Data was visualized using ggplot2 (v3.0.0). Additional details are given in Appendix B.

2.3 Results

Diploid and autotetraploid *M. sativa* exhibited similar niche breadth in the taxonomic range of rhizobial partners with which they could establish mutualisms. Specifically, all diploid and autotetraploid lineages of *M. sativa* from a broad geographic range (Appendix B Table 4) were nodulated by the same 17 of 21 possible rhizobial strains that span the *Sinorhizobium* phylogeny (Appendix B Fig. 9).

Even though diploid and autotetraploid *M. sativa* established mutualisms with the same range of *Sinorhizobium* symbionts, autotetraploids benefited more from these interactions, as demonstrated by host growth response, the percentage increase (or decrease) in shoot biomass relative to water-inoculated control plants within lineage (Regus et al., 2015). This bias-free metric distinguishes host benefits obtained from the mutualism from the effects of polyploidy on plant size. When associated with rhizobia, autotetraploid *M. sativa* plants exhibited a greater positive growth response of shoot biomass on average compared to diploids across the 17 nodulating strains (>2-fold increase in shoot biomass vs. 1.5-fold increase; $F_{1,156} = 5.32$; $P = 0.05$; Fig. 2, Appendix B Table 5). These patterns held across rhizobial environments even though strains significantly differed in their effects on host growth response ($F_{16,126} = 9.81$; $P < 0.001$), ranging from costly to highly beneficial (-19 to 574% for autotetraploid plants and -67 to 733% for diploids). Given this extensive variation in strain partner quality, we explored whether autotetraploids were better able to maintain high fitness benefits across diverse rhizobial environments (i.e., low plasticity in fitness) and reduce fitness costs of associating with lower quality strains relative to diploids.

To capture variation in benefits obtained across all 21 rhizobial environments, we estimated plasticity in fitness (relative distance plasticity index, or RDPI, of host growth response; Valladares et al., 2006; see Fig. 3) for diploid and autotetraploid *M. sativa* lineages. Autotetraploid *M. sativa*

had a significantly lower RDPI of host growth response compared to diploids (0.63 vs 0.72; t = 3.55, df = 7.18, P = 0.008; Fig. 3A). These results reveal that autotetraploid *M. sativa* plants maintained high fitness benefits (i.e., low plasticity) across a broad range of rhizobial partners, reflecting greater generalization in host benefits obtained from mutualistic interactions relative to more specialized diploids.

Although diploids exhibited greater variation in fitness benefits obtained across rhizobial environments, it was unclear whether specialization came at a fitness cost. To explore potential costs of specialization in the legume-rhizobial interactions, we calculated the relative distance from the maximum host growth response for diploid and autotetraploid lineages across all rhizobial strains. While this metric can be correlated with RDPI (and is in this case; r^2 = 0.76, P = 0.01), the point of comparison differs and, as a result, provides additional insight into the factors driving variation in benefits obtained. 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 host growth response across a broad range of symbionts (0.65 vs 0.82; t = 5.02, df = 7.64, P = 0.001; Fig. 3B,C). Taken together, these results indicate that autotetraploid *M. sativa* plants not only exhibit less plasticity in fitness, but also reduced costs of specialization in rhizobial interactions. 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.

Nodulation traits that reflect the quantity (nodule number and biomass) and quality (nodule color as a proxy for N fixation) of rhizobial symbionts hosted may underlie the increased fitness benefits obtained by autotetraploid *M. sativa* relative to diploids. There was a strong effect of

polyploidy on nodule traits (MANOVA, $F_{3,48} = 33.88$, $P < 0.001$; Appendix B Table 6). We found autotetraploid *M. sativa* produced significantly more nodules ($F_{1,132} = 14.69$, $P < 0.001$) and more total nodule biomass than diploids across rhizobial strains ($F_{1,132} = 102.39$, $P < 0.001$; Fig. 4; Appendix B Fig. 10, Table 7), indicating they host a greater quantity of symbionts as rhizobial abundance is correlated with nodule biomass (Kiers et al., 2003; Heath and Tiffin, 2007; Regus et al., 2015). These patterns were not solely due to the larger size of polyploidy plants, as ploidy remained significant for nodule number ($F_{1,132} = 21.43$, $P < 0.001$) and nodule biomass ($F_{1,132} = 168.48$, $P < 0.001$) when root biomass was included as a covariate (Appendix B Table 8). Moreover, the effects of polyploidy on nodule traits were evident even though rhizobial strain influenced nodule number ($F_{16,132} = 1.78$, $P = 0.04$) and total nodule biomass ($F_{16,132} = 3.70$, $P < 0.001$). Although nodule color also varied across rhizobial strains ($F_{16,132} = 25.82$, $P = 0.001$), autotetraploid *M. sativa* consistently produced significantly darker nodules than diploids ($F_{1,132} = 8.07$, $P < 0.01$; Figs. 2, 4), suggesting effective N fixation by rhizobial symbionts (Imaizumi-Anraku et al., 1997; Burghardt et al., 2018). As expected if color is related to N fixation, host growth response was positively correlated with average nodule color for both diploid ($r^2 = 0.55$; $P < 0.001$) and autotetraploid plants ($r^2 = 0.61$; $P < 0.001$). These results imply that autotetraploid *M. sativa* obtain more N than diploids from the same rhizobial strains, thereby increasing benefits acquired from bacterial mutualisms.

2.4 Discussion

By focusing on a model belowground mutualism, we demonstrate that autotetraploid *M. sativa* obtained greater benefits from rhizobial partners not due to increased niche breadth (i.e.,

ability to interact with a broad taxonomic range of patterns), but because they maintained high benefits from a wide range of interactions (i.e., reduced plasticity in fitness). These results uncover a potential mechanism underlying the invasive success of polyploid legumes and provide a general framework for understating how variation in biotic interactions may be affected by polyploidy.

The similar taxonomic niche breadth of diploids and polyploids we observed is consistent with previous studies exploring the effects of plant polyploidy on the range of potential mutualistic partners. For example, diploid and polyploid plants often share similar pollinator communities (Castro et al., 2011; Nghiem et al., 2011; Borges et al., 2012; but see Thompson and Merg, 2008) and mycorrhizal fungal associations (Tesitelova et al., 2013; Sudova et al., 2018). Furthermore, numerous studies have addressed whether plant polyploidy is associated with increases in abiotic niche breadth, yet no clear patterns have emerged (Husband et al., 2013; Brittingham et al., 2018). Although some polyploid plant taxa occupy larger abiotic niches than their diploid progenitors (Lowry and 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 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 polyploid plants may not be attributed to expansion in the range of mutualistic partners (or habitats), but rather their ability to obtain greater benefits from interactions and/or maintain fitness across biotic (or abiotic) environments once established.

Here, we demonstrate that autotetraploid *M. sativa* not only obtain greater benefits from rhizobial symbionts, but also maintain high 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 and supports the

characterization of polyploid plants as “jacks-of-all-trades” and “masters-of-some” (Richards et al., 2006), here extended to engagement in bacterial symbioses. These patterns 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 and Strauss, 2017; Wei et al., 2018). Autotetraploid *M. sativa* plants may 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 number and biomass, and darker nodule color) relative to diploids. Additional studies are needed to uncover specific mechanisms permitting polyploid plants to invest more in bacterial mutualisms; however, this work suggests that generalization is beneficial and may 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 and Thompson, 2001; Leitch and Leitch, 2008; Shimizu-Inatsugi et al., 2017), and may 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 fitness benefits of mutualistic interactions contribute to polyploid success.

Polyploid plants that benefit more from nutrient acquisition mutualisms and maintain high fitness benefits across biotic environments may be better able to establish and spread in novel habitats. Legumes are overrepresented among invasive taxa and increased generalization of rhizobial mutualisms is thought to facilitate legume invasion success (Daehler, 1998; Rodriguez-Echeverria et al., 2008). Furthermore, polyploidy is associated with plant invasive success (te Beest et al., 2012, Pandit et al., 2014). Our study highlights a potential link between these two patterns by suggesting that increased generalization in fitness benefits obtained from bacterial

mutualisms may underlie the increased invasive success of polyploid legume plants (Harrison et al., 2018).

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 may facilitate the occurrence of diverse bacterial symbionts within or across environments (Heath and Stinchcombe, 2014). In contrast, more specialized diploids may enrich the environment with a few highly beneficial strains and, in doing so, 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 may maintain high diversity of symbiotic partners (Batstone et al., 2018). Specifically, intraspecific diploid and polyploid plants in mixed-ploidy populations may increase population-level breadth of mutualistic partners or species-level partner breadth across the geographic range (Batstone et al., 2018). These processes may occur in autopolyploid species, including *Medicago sativa* used here; however, it is possible that allopolyploids exhibit even greater generalization in species interactions, which could lead to greater variation in mutualistic partners. Future studies testing these hypotheses would be particularly informative.

The effects of polyploidy on generalization likely 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 and Anneberg, 2016, Wood et al., 2018). Although previous studies have demonstrated that highly specialized mutualisms are rare in nature and most organisms interact with multiple mutualistic partners, the benefits of generalization and underlying

mechanisms remained largely unresolved (Douglas, 1998; Heath and Stinchcombe, 2014). This study reveals that polyploidy is a key genetic driver of generalization in bacterial mutualisms through reducing plasticity in fitness rather than increasing taxonomic niche breadth, and establishes novel connections between biotic interactions and the widespread success of polyploid plants.

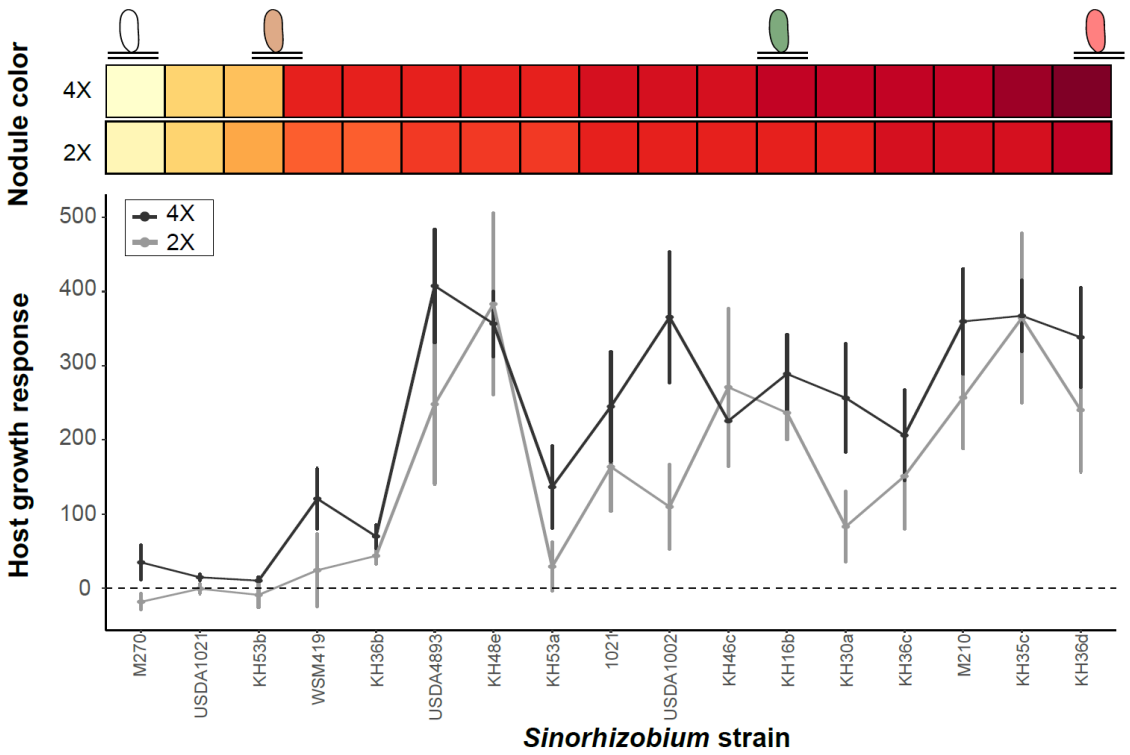

Figure 2. Mean host growth response of diploid (2X, gray) and autotetraploid (4X, black) lineages (*n* = 10) of *Medicago sativa* plants associated with diverse rhizobial strains spanning the *Sinorhizobium* phylogeny (*n* = 17). Host growth response is a bias-free fitness metric that quantifies the percentage change in dry shoot biomass of inoculated plants (*n* = 765) relative to water inoculated control plants (dashed line, *n* = 179) within lineage. Error bars show SEM. *Sinorhizobium* strains are ordered by average nodule color, a common metric of nitrogen fixation function that ranges from white (ineffective) to dark red (highly effective), produced by 2X (upper bar) and 4X (lower bar) plants. Four additional strains used in this study nodulated five or fewer plants and were not included in these analyses. These were *Sinorhizoibum fredii* USDA205, *S. fredii* USDA207, and *S. meliloti* M30, which did not nodulate any plants, and *S. terangae* USDA4894, which only produced nodules on five plants (one diploid and four tetraploids).

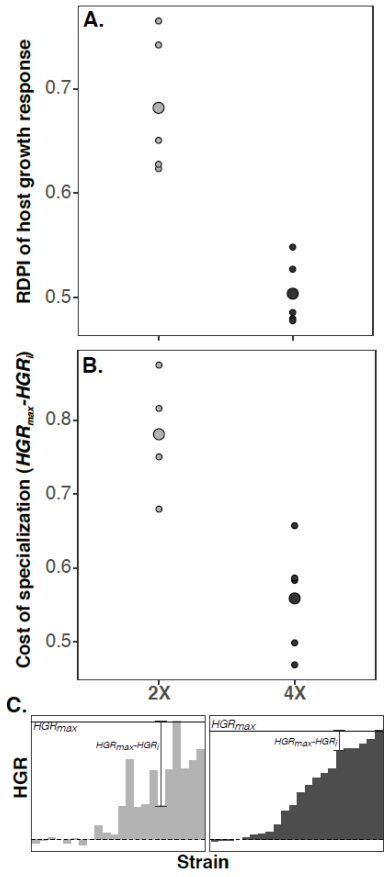

Figure 3. Plasticity of fitness and cost of specialization of diploid (2X, gray) and autotetraploid (4X, black) lineages of *Medicago sativa* inoculated with 21 strains of *Sinorhizobium* bacteria. (A) Relative distance plasticity index (RDPI) for host growth response of shoot biomass of 2X and 4X lineages. RDPI captures the variation in benefits obtained by diploid and autotetraploid *M. sativa* lineages across rhizobial environments. RDPI values range from 0 to 1, with values closer to 0 reflecting more generalized rhizobial interactions as the quality of interactions is maintained across biotic environments. (B) Cost of specialization as estimated by the average of the individual distances (HGRi) from the maximum growth response (HGRmax) of shoot biomass for 2X and 4X plants. Similar to RDPI, these values range from 0 to 1, with values closer to 0 indicating greater generalization in the quality of the mutualism as plants obtained benefits closer to the maximum across rhizobial strains. Average RDPI or cost of specialization is shown for each plant lineages as small circles and for each ploidy level as large circles. (C) Histogram of host growth response of 2X and 4X plants associated with 21 strains of *Sinorhizobium*.

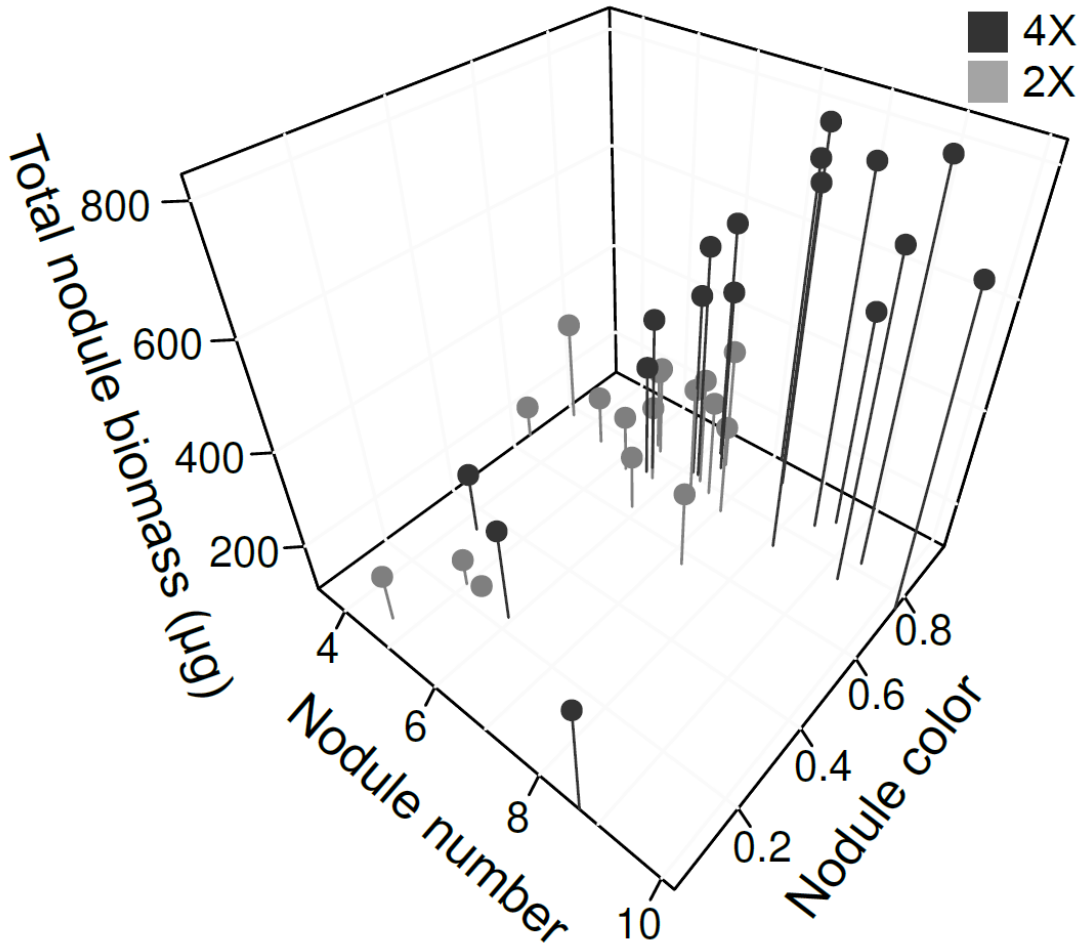

Figure 4. Nodule traits (biomass, number, and color) of diploid (2X) and autotetraploid (4X) *Medicago sativa* plants associated with 17 strains of *Sinorhizobium* bacteria. Each data point represents the average nodule trait value produced by diploid (gray, *n* = 316 plants) or autotetraploid (black, *n* = 449 plants) plants associated with a *Sinorhizobium* strain.

3.0 Synthetic autotetraploids show that polyploidy alters the mutualism interface of legume-rhizobia interactions in *Medicago sativa* subsp. *caerulea*

3.1 Introduction

The legume-rhizobia interaction is a model nutrient acquisition mutualism that regulates global nutrient cycles, supplies nitrogen (N) to natural and agricultural environments, and contributes to the widespread distribution of legume taxa (Fabaceae; Daehler et al., 1988; Herridge et al., 2008; Sprent, 2009; Vitousek et al., 2013). In this mutualism, rhizobia bacteria fix atmospheric N into a plant-usable form in exchange for photosynthetic resources provided by legume hosts inside root nodules (Grillo et al., 2016). Previous work has revealed extensive variation in the fitness benefits legume plants obtain from rhizobial mutualisms (Burdon et al., 1999; Heath and Stinchcombe, 2014; Wendlandt et al., 2019; Forrester et al., in prep). This variation is frequently attributed to complex genotype x genotype x environment interactions between legume hosts, rhizobial symbionts, and their environmental context (Heath, 2010; Heath et al., 2010; Forrester and Ashman, 2018b); however, the mechanisms driving this variation remain poorly understood.

From a plant perspective, polyploidy (i.e., the possession of more than two complete sets of chromosomes from one or more genetic donors) is a key driver of genetic variation (Levin, 2002), which may have profound effects on interactions between legume plants and rhizobial symbionts. Ancient polyploidy is hypothesized to have enhanced the legume-rhizobia mutualism (Canon et al., 2010; Doyle, 2011) by increasing the diversity of signaling factors that function in mutualism establishment (e.g., flavonoids, Nod factor receptors) and maintenance (e.g., nodule-

specific cysteine-rich peptides, leghaemoglobins; Young et al., 2011; Li et al., 2013; Powell and Doyle, 2015). Consistent with this, empirical studies have demonstrated that established polyploid plants host a greater quantity of symbionts and obtain greater growth benefits from the mutualism relative to diploids (Stalker et al., 1994; Powell and Doyle, 2016; Forrester et al., in prep). Although these studies support a role of plant polyploidy in altering the legume-rhizobia mutualism, it is unclear whether these effects are the immediate and direct result of increased ploidy or evolutionary changes that occurs after a polyploidy event.

Fundamental changes that directly result from an increase in ploidy, such as larger cell size, faster photosynthetic rate, and composition changes in genes that function in mutualism establishment and maintenance (Levin, 2002; Beaulieu et al., 2008; Maherali et al., 2009; Young et al., 2011; Martin and Husband, 2012; Li et al., 2013; Doyle and Coate, 2019), could alter legume interactions with rhizobia. Specifically, polyploid plants that have larger cells or more resources to allocate to the mutualism might host a greater quantity of or higher quality rhizobial symbionts relative to diploids, resulting in increased host benefits obtained (reviewed in Forrester and Ashman, 2018a; Forrester et al., in prep). Only a few studies have isolated the direct effects of increased ploidy on the legume-rhizobia mutualism, which found that synthetic neopolyploids produced larger nodules (Kabi and Bhaduri, 1978) and fixed N at a higher rate (Leps et al., 1980) than their diploids progenitors. However, no studies have tested whether polyploidy directly alters the internal structure of nodules, which represent the interface of legume-rhizobial interactions.

Root nodules are plant-derived structures that house rhizobia and provide protective environments for N-fixation to occur. Legume plants initiate nodule development in response to Nod factor signals released by free-living rhizobial cells (Jones et al., 2007; Wang et al., 2012). As the nodule develops, rhizobial cells that are trapped inside the plant tissue differentiate into N-

fixing bacteroids within plant-derived symbiosomes (Wang et al., 2012; Fig. 5A). Although legume taxa differ in nodule morphology (Sprent, 2007), nodule development can be broadly categorized as determinate or indeterminate. Determinate nodules grow to a certain stage of development and then senesce, whereas indeterminate nodules are defined by continuous growth (Sprent 2007; Regus et al. 2017). The continual growth of indeterminate nodules results in distinct regions within nodules, including a persistent meristem, interzone, N-fixation zone, and senescence zone. Within the N-fixation zone of indeterminate nodules, each symbiosome contains a single, terminally-differentiated bacteroid (Haynes et al., 2004; Jones et al., 2007).

Polyploidy may directly alter the internal structure of nodules due to increased cell size or genomic changes that result from polyploidy events (Forrester and Ashman, 2018a. Specifically, increased cell size of polyploid plants may lead to the production of larger nodules with larger N-fixation zones. Polyploidy may also directly increase the size of symbiosomes and the N-fixing bacteroids hosted within them (Forrester and Ashman, 2018a). Because larger bacteroids fix more N (Oono and Denison, 2010), direct changes in internal nodule structure resulting from polyploidy may permit polyploid legumes to access more N than diploids, thereby obtaining greater benefits from rhizobial mutualisms relative to their diploid progenitors (Forrester et al., in prep).

We used the *Medicago* (legume) - *Sinorhizobium* (bacteria) system to evaluate the direct effects of plant polyploidy on the internal structure of indeterminate root nodules. To achieve this, we created synthetic neotetraploids of *M. sativa* subsp. *caerulea* and inoculated seeds derived from them and their diploid progenitors with two strains of *Sinorhizobium*. We assessed nodule number and quantify traits related to the internal structure of nodules using confocal microscopy. This approach allowed us to reveal how tetraploidy immediately and directly alters the legume-rhizobia mutualism.

3.2 Materials and methods

3.2.1 Plant host selection and neotetraploid creation

The species complex *Medicago sativa* consists of diploid and autotetraploid plant lineages with *M. s.* subsp. *caerulea* ($2n = 2x = 16$) being the diploid progenitor of autotetraploid *M. s.* subsp. *sativa* ($2n = 4x = 32$; Havanada et al. 2011). Seeds from wild diploid *M. sativa* subsp. *caerulea* GRIN lineages (referred to by their accession numbers) were obtained from the USDA National Genetic Resources Program (http://www.ars-grin.gov/; Sakiroglu et al. 2010).

Neotetraploid *M. sativa* subsp. *caerulea* were created using colchicine on Nov 15 and 30 2014, following established methods (Joshi and Verma, 2004). Seeds from PI 464714 (40 seeds in 0.0075% colchicine), and PI 440500, 440501, and 440507 (500 seeds each in 0.01% colchicine) were placed in Petri dishes containing filter paper and soaked for eight hours. For both treatments, seeds were immediately rinsed after the colchicine soak with deionized water for one hour, planted into plug trays filled with about 2" of Sunshine Germination Mix (Sun Gro Horticulture Inc., Agawam, Massachusetts, USA) and grown in the greenhouse under 14-hour days.

Colchicine treatments resulted in 127 germinated seedlings. Once seedlings produced mature leaves, a fresh leaf (50-100 mg) was sampled from each plant and analyzed to determine ploidy level using flow cytometry. Leaf samples from confirmed diploid and tetraploid *M. sativa* plants were included as controls. Leaf tissue was finely chopped using a double-edged razor blade in Petri dish containing 1 mL Galbraith buffer (Galbraith et al., 1983). The homogenate was filtered using a 40 µm pore size (600 mesh) nylon membrane (WUJI White Bridal Crinoline, Jo-Ann Stores Inc., Hudson, Ohio, USA) and the obtained suspension was treated with 10 µL/mL RNaseA for 10 minutes. Each sample was then stained with 200 µL/mL of propidium iodide for 30 minutes.

Ploidy level was determined by analyzing at least 1000 nuclei per sample on a BD Accuri C6 flow cytometer (BD Accuri C6; BD Biosciences, Inc., San Jose, California, USA), which resulted in five confirmed neotetraploid plants.

These neotetraploid plants were transplanted into 3" square pots in a 2:1:1 mixture of Fafard #4 (Sun Gro Horticulture, Inc., Agawam, Massachusetts, USA), 1020 Course Sand (Browns Hill Sand, Homestead, Pennsylvania, USA), and Course Perlite (PVP Industries, Inc., Orwell, Ohio, USA), and grown in the greenhouse under 16-hour days. Diploid seeds from the same lineages were grown in 3" square pots in a 2:1:1 mixture of Fafard #4, 1020 Course Sand, and Course Perlite, and grown in the greenhouse under 16-hour days. Flowering diploid (n = 5) and neopolyploid plants (n = 5) were hand-pollinated at random and repeatedly within ploidy level from July 2017 through January 2018 to produce stocks of diploid and neopolyploid seeds under the same greenhouse conditions.

3.2.2 Rhizobial strains

Two strains of *Sinorizobium* were used to evaluate the direct effects of autotetrploidy on the internal structure of nodules. *Sinorhizobium meliloti* M210 and *S. medicae* KH36d were obtained from Michael Sadowsky (University of Minnesota), and determined to be effective symbionts of natural diploid and tetraploid *M. sativa* lineages (Forrester et al. in prep.).

3.2.3 Seed preparation and treatments

Scarified and surface sterilized seeds produced by diploid (n = 130) and neotetraploid (n = 192) plants in the greenhouse were planted in sterilized growth pouches (1 seed/pouch, CYG seed

germination pouch, Mega International, Newport, Minnesota, USA) containing 20 mL of sterile, N-free Fahraeus solution, as described in the *Medicago truncatula* Handbook (https://www.noble.org/medicago-handbook/). Size-matched plants were randomly assigned to one of three treatments: *S. meliloti* M210, *S. medicae* KH36d, or controls. For diploid seeds, 45 were assigned to each rhizobial treatment and 40 were assigned to the control treatment. Because we could not evaluate the ploidy levels of neotetraploid seeds before planting, we planted extra seeds for each treatment: 72 seeds for *S. meliloti*, 60 seeds for *S. medicae*, and 60 seeds for controls. Pouches were sorted by treatment and replicate, then placed into sterilized plastic containers. Each treatment had five replicate containers, which were transferred to a growth room set to 25°C, 60% humidity, and with supplemental lighting to achieve 16-hour days.

3.2.4 Inoculation and growth conditions

Rhizobial strains were grown on tryptone-yeast media with 0.3 µg ml^{-1} biotin (Watson et al. 2001) and inocula were prepared by scraping plates into sterile ddH_2O. Five days after planting seedlings into pouches, each seedling was inoculated with 1.0 x 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 grown for six weeks post-inoculation. Plants were fertilized with nine ml of N-free Fahraeus solution. None of the control plants produced nodules.

3.2.5 Confirmation of plant ploidy level

To confirm ploidy level of plants used in the experiment, flow cytometry of fresh leaf tissue was conducted five weeks post-inoculation of all putative neotetraploids (n = 49) that produced nodules, following the methods described previously. Leaf samples from two diploid plants and confirmed diploid and tetraploid *M. sativa* stocks not used in the study were included as controls. Flow cytometry analyses were conducted in a single day and identified 25 neotetraploid plants and 24 revertant diploid plants (i.e., diploids produced by neotetraploid mothers).

3.2.6 Confocal microscopy

Flow cytometry data were used to select diploid, revertant diploid, and neotetraploid plants for confocal microscopy. Mature nodules were from harvested plants 34 days post-infection into 80 mm PIPES buffer and sectioned longitudinally into thin slices using a double-edged razor blade (Haynes et al. 2004; Regus et al. 2017). Nodule sections were stained in 1 μL ml^{-1} SYTO 13 for 15 minutes and then mounted onto slides and sealed with coverslips for confocal processing (Haynes et al. 2004).

Confocal images were acquired on a Leica SP5 laser scanning confocal microscope (Leica Microsystems, Inc., Buffalo Grove, Illinois, United States) using a x10 lens (Leica Microsystems, Inc., Buffalo Grove, Illinois, United States) and 488 nm (Argon) and 514 nm (DPSS) excitation beams. An emission range of 531 – 638 nm was used to detect SYTO 13. Images were captured as single optical sections (2D) with SYTO 13 fluorescence depicted in green and plant autofluorescence in blue.

3.2.7 Data collection and analysis

Confocal images of mature nodules were obtained from ten (nine) diploids, six (seven) revertant diploids, and eight (eight) neotetraploid plants for *S. meliloti* M210 (and *S. medicae* KH36d). For each image, nodule histology traits including total nodule area, the area of the N-fixation zone, and average area of five symbiosomes per nodule were measured using Fiji (Fig. 5A; Schindelin et al., 2012).

A MANOVA was conducted to test for effects of plant ploidy, bacterial strain, and their interaction on nodule histology traits, and an ANOVA was conducted for nodule number using the stats package (v3.5.2) in R (v3.5.2). Significant differences among ploidy levels were determined using Tukey's honest significant difference tests, and data was visualized using ggpubr (v0.2).

3.3 Results

Medicago sativa subsp. *caerulea* natural diploids, synthetic neotetraploids, and revertant diploids that were inoculated with *S. medicae* or *S. meliloti* produced numerous, dark-pink, putatively N-fixing nodules that grew throughout the experiment (Imaizumi-Anraku et al. 1997). Confocal analysis revealed fully infected nodules with distinct regions including the total nodule area and N-fixation zone, as well as symbiosomes containing enlarged bacteroids for all ploidies (Fig. 5).

There was a strong direct effect of increased ploidy on nodule histologic traits (MANOVA, $P < 0.001$), but not nodule number ($F_{2,47} = 0.797$, $P = 0.45$). These patterns were evident across

rhizobial strains, as strain identity nor its interaction with ploidy were significant in the MANOVA ($P = 0.16$ and $P = 0.41$, respectively) or any subsequent ANOVA models ($P > 0.15$ and $P > 13$).

Neotetraploid *M. sativa* subsp. *caerulea* produced the largest nodules, followed by natural diploids and then revertant diploids ($F_{2,47} = 2.93$, $P = 0.06$; Fig. 6). Neotetraploids produced nodules with 29% more area than diploids and 62% more area than revertant diploids, the latter difference was statistically significant ($P = 0.05$) while the former was not ($P = 0.27$).

In addition to larger nodule size, neotetraploid *M. sativa* subsp. *caerulea* produced nodules with significantly larger N-fixation zones ($F_{2,47} = 3.78$, $P = 0.03$; Fig. 7). This effect was also driven by pronounced elevation of the neotetraploids relative to the revertant diploids (88% $P = 0.03$), followed by the natural diploids (47%, $P = 0.12$). Natural and revertant diploids were not different from each other ($P = 0.66$).

Within the N-fixation zone, ploidy significantly affected the average area of symbiosomes that host bacteroids ($F_{2,47} = 1.29$, $P > 0.001$; Fig. 8), with neotetraploid *M. sativa* subsp. *caerulea* producing significantly larger symbiosomes than those of both diploid types (natural $P < 0.001$; revertant $P < 0.001$). Specifically, symbiosomes in nodules produced by neotetraploids had 83% more area on average than those within nodules produced by diploids.

3.4 Discussion

Overall, this study demonstrates that polyploidy has direct effects on the plant-rhizobial interface. We reveal that autotetraploidy directly alters the internal structure of root nodules, and in doing so, uncover an important genetic mechanism shaping plant-microbial mutualisms. More broadly, the direct increase in plant-microbe interaction metrics suggests a mechanism by which

newly formed polyploid plants overcome challenges of establishing and spreading in natural environments.

One of the most well-established direct impacts of polyploidy is an increase in cell size (Beaulieu et al., 2008, Doyle and Coate, 2019), which likely underlies the increases in nodule area and N-fixation zone area in nodules produced by neotetraploid *M. sativa* subsp. *caerulea* plants. These patterns are consistent with previous work demonstrating that synthetically-induced polyploidy directly increased externally measured nodule size in *Phaseolus aureus* (Kabi and Bhaduri, 1978), as well as those finding that established natural polyploid plants produced larger nodules than diploids (e.g., Evans and Jones, 1966; Stalker et al., 1994; reviewed in Forrester and Ashman, 2018a). Enlargements in nodule size and the N-fixation zone directly resulting from increases in ploidy may have important consequences for legume-rhizobial interactions, as nodule size is positively correlated with rhizobial abundance (Kiers et al., 2003; Heath and Tiffin, 2007; Regus et al., 2015). Specifically, polyploidy may directly increase the quantity of rhizobial symbionts hosted relative to their diploid progenitors, thereby allowing them to obtain more fixed N from bacterial mutualisms relative to diploids (Forrester and Ashman, 2018a). While we were not able to rigorously assess the direct effects of polyploidy on the number of symbiosomes within nodules, there is evidence that nodule area is positively correlated with symbiosome number ($r = 0.63$, $P < 0.001$). Thus, if polyploidy directly increases nodule area, then neopolyploid plants may also host more symbiosomes than diploids. Additional studies quantifying the direct effects of polyploidy on the number of symbiosomes that contain bacteroids within nodules (e.g., using electron microscopy) are essential for evaluating this hypothesis.

In addition to potential increases in the quantity of rhizobial symbionts hosted, polyploidy may directly alter the quality of rhizobia housed within nodules (Forrester and Ashman, 2018a).

In indeterminate nodules, such as those produced by *Medicago* plants, each symbiosome hosts a single bacteroid (Haynes et al., 2004), thus symbiosome size can be used as a proxy for bacteroid size. In this experiment, we found that neopolyploid *M. sativa* subsp. *caerulea* produced symbiosomes approximately twice the size of those produced by diploids, which suggests complementary increases in bacteroid size. Previous work has revealed that enlarged, swollen bacteroids fix N more effectively than smaller, non-swollen bacteroids, resulting in greater host benefits obtained from rhizobial mutualisms (Oono and Denison, 2010). Therefore, increases in symbiosome and bacteroid size directly resulting from polyploidy may enhance the efficiency of N-fixation by rhizobial symbionts, and thus the amount of N provided to plant hosts (Oono and Denison, 2010; Forrester and Ashman, 2018a). Consistent with this, polyploidy directly enhanced N-fixation rate in neotetraploid and neooctoploid *M. sativa* (Leps et al., 1980). Together, these results suggest that neopolyploids may host more effective rhizobial symbionts relative to diploids, and future work explicitly testing this hypothesis will provide critical insights into how polyploidy alters the quality of legume-rhizobial interactions.

Revertant diploid *M. sativa* subsp. *caerulea* consistently exhibited the smallest nodule traits relative to diploids and neotetraploids. These patterns may reflect detrimental effects of colchicine (Trojak-Goluch and Skomra, 2013; Husband et al., 2016; Van Drunen and Husband, 2018) or other genetic, developmental, or physiological alterations present in revertant diploids (Munzbergova, 2017). Negative effects of colchicine on converted and unconverted plants are well established (Joshi and Verma, 2004; Husband et al., 2016; Munzbergova, 2017), therefore we used seeds produced by colchicine-treated neotetraploids to avoid any direct effects of colchicine on nodule traits. Yet, we still uncovered distinctions between natural and revertant diploid plants, which complement the one other study comparing diploid and autotetraploid progeny of

neotetraploid mothers (Munzbergova, 2017). Although we cannot isolate the causal mechanism driving reduced nodule traits of revertant diploids, if they are artifacts of colchicine, then the effects of increased ploidy far outweighed any trait reductions due to colchicine treatments. This was evident as neotetraploids produced consistently larger nodule traits than revertant diploids.

Within each ploidy level, plants exhibited extensive variation in internal nodule traits, which may be due to interactions between legume host genotypes and rhizobial genotypes (Heath and Tiffin, 2007; Heath, 2010; Wendlandt et al., 2019). The greatest variation we observed was in total nodule area, followed by the N-fixation zone, which are regulated by interactions between the plant host and rhizobial symbiont (Oldroyd et al., 2011; Wang et al., 2012; Maroti and Kondorosi, 2014). Host genotype x rhizobial genotype interactions may increase variation in these traits, which may explain the lack of significant differences detected between natural diploids and neotetraploid *M. sativa* subsp. *caerulea*. Future studies using a broad range of diploid and neotetraploid host genotypes as well as diverse rhizobial symbionts are needed to empirically evaluate this hypothesis. Symbiosome size was far less variable, which may be due to greater host control over these structures (Mergaert et al., 2006; Oldroyd et al., 2011; Alunni and Guorion, 2016; de la Pena et al., 2018). Although not explicitly tested here, if these traits are not shaped by interactions between host and symbiont genotypes to the same extent as nodule area and N-fixation zone area, then this may explain the drastic differences observed between neotetraploids and both diploid types. Yet even in the context of this variation, direct increases in ploidy had similar outcomes for all internal nodule traits measured.

On a broader scale, uncovering a mechanistic basis for how polyploidy can directly affect plant interactions with mutualistic microbes can shed light on how newly formed polyploids establish and spread in natural environments. An outstanding question in ecological and

evolutionary biology is how neopolyploid plants overcome challenges related to survival, growth and reproduction, such as competition with their diploid progenitors and minority cytotype exclusion (Ramsey and Schemske, 1998; Maherali et al., 2009; Ramsey, 2011). If polyploidy directly increases the size of plant traits that function in nutrient acquisition mutualisms, as we have demonstrated with the internal structure of nodules, then neopolyploids may host a greater quantity of or more effective microbial symbionts than diploids (Forrester and Ashman, 2018a). These changes in mutualism traits may allow neopolyploid plants to acquire more resources than their diploid progenitors, permitting them to establish and spread across diverse environments.

3.5 Conclusions

Polyploidy is a major genetic driver of ecological and evolutionary processes in plants, yet little is known about its effects on biotic interactions (Segraves and Anneberg, 2016). In this study, we explore how the legume-rhizobia mutualism is directly impacted by plant polyploidy, thereby isolating the effects of polyploidy on species interactions apart from other evolutionary changes that occurred after the polyploidy event. Using synthetic neotetraploid *Medicago sativa* plants, their diploid progenitors, and revertant diploids, we reveal that polyploidy directly modifies the internal structure of root nodules. These changes may lead to increases in the quantity or quality of rhizobial symbionts hosted by legume plants, potentially enhancing nutrients acquired from the mutualism, and permitting newly formed polyploids to establish and spread in natural environments.

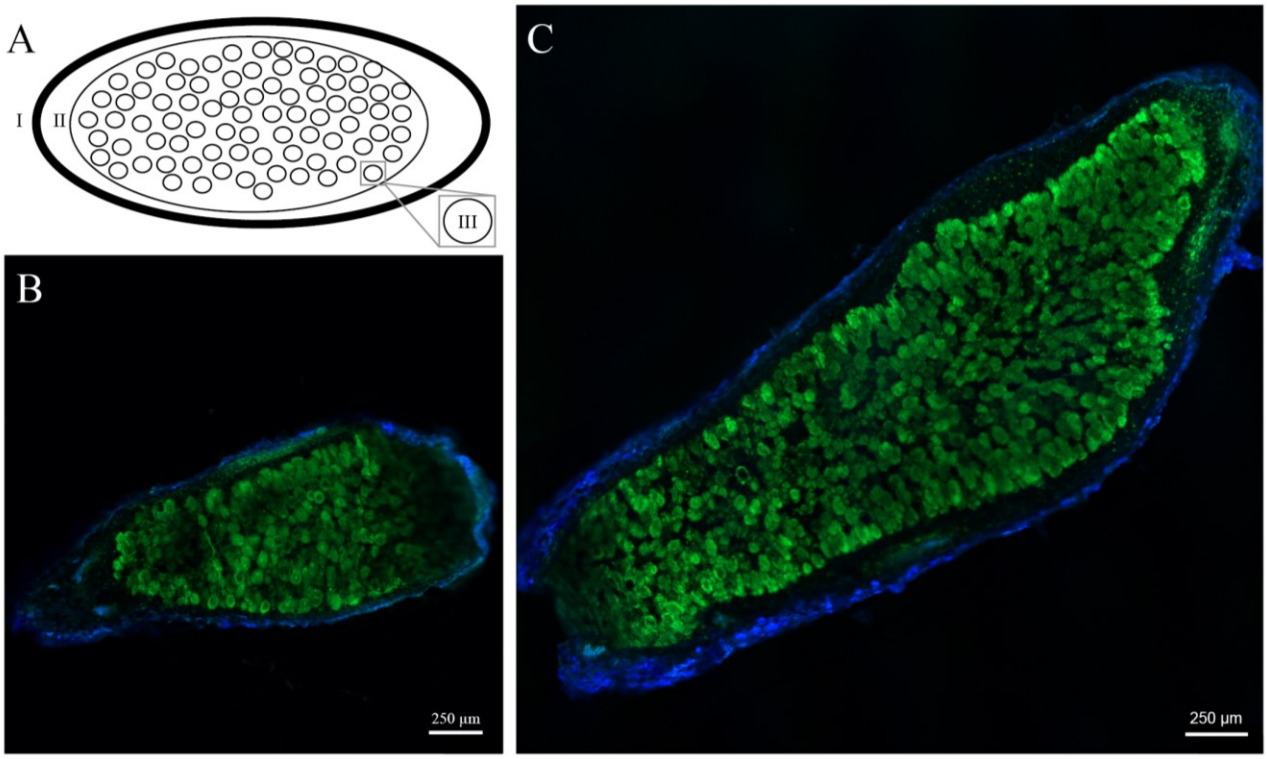

Figure 5. Exemplar longitudinal sections of mature root nodules from *Medicago sativa* subsp. *caerulea* at 34 days post-inoculation with *Sinorhizobium medicae* KH36d visualized using confocal microscopy. (A) Diagram of an indeterminate root nodule with distinct regions: (I) total nodule area, (II) nitrogen-fixation zone, and (III) a symbiosome within the nitrogen-fixation zone. Nodules from diploid (B) and neotetraploid (C) plants. Bacteroids (green) and plant cells (blue).

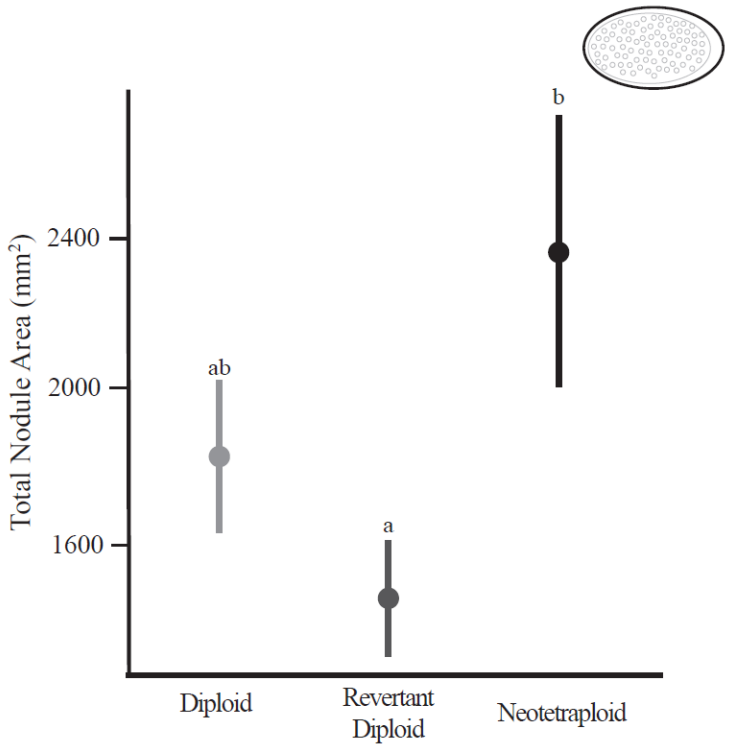

Figure 6. Total area of root nodules of diploid, revertant diploid, and neotetraploid *Medicago sativa* subsp. *caerulea* associated with two strains of *Sinorhizobium*.

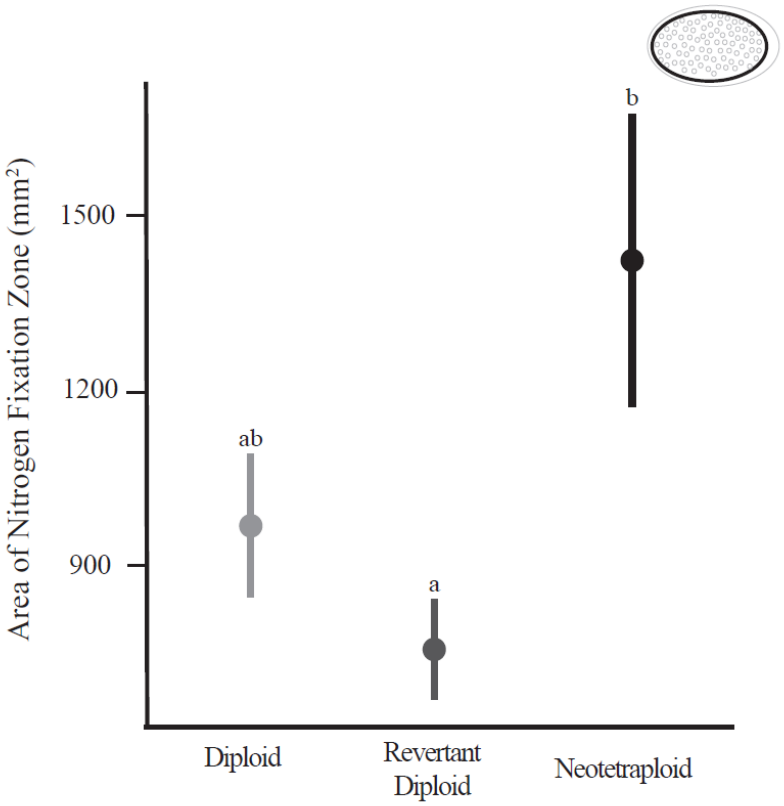

Figure 7. Area of the nitrogen-fixation zone in root nodules from diploid, revertant diploid, and neotetraploid *Medicago sativa* subsp. *caerulea* associated with two strains of *Sinorhizobium*.

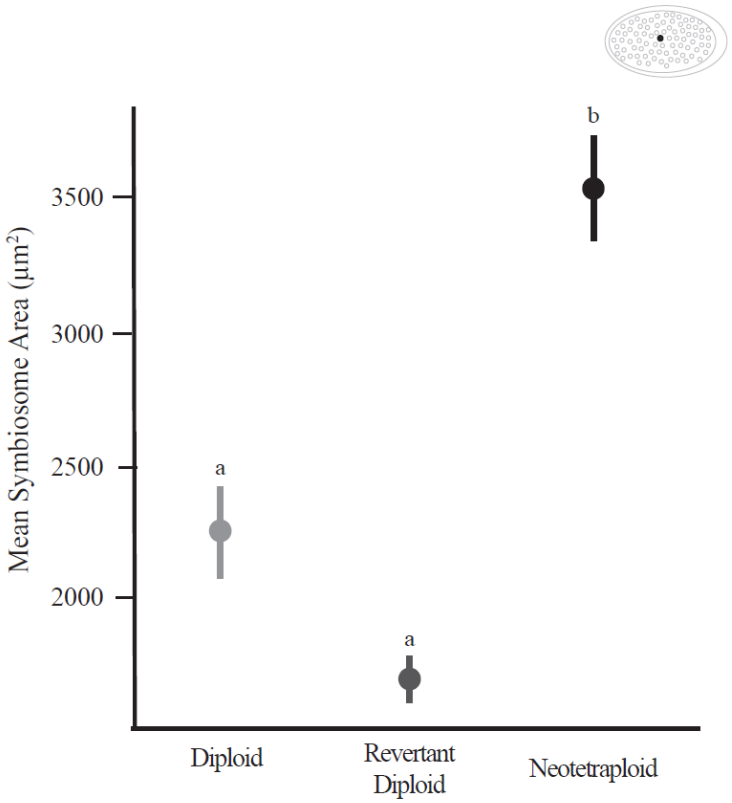

Figure 8. Mean area of symbiosomes in the nitrogen-fixation zone in root nodules from diploid, revertant diploid, and neotetraploid *Medicago sativa* subsp. *caerulea* associated with two strains of *Sinorhizobium*. In indeterminate nodules of *M. sativa* subsp. *caerulea*, each symbiosome contains a single, enlarged bacteroid.

Appendix A Additional tables (chapter 1)

Table 2. Summary of plant taxa, polyploid information, and experimental methods for each study included in the review. Blank cells indicate variables that were not explicitly described in the publication. Studies are organized alphabetically by first author. Nod Type, nodulation type; I, indeterminate; D, determinate; A, aeschynomenoid. Infect Method, method of rhizobial infection. Geo Distrib, geographic distribution of legume taxa; Temp, temperate; Trop, tropical. Polyploid Type, Auto, autopolyploid; Allo, allopolyploid. Supp N, use of supplemental nitrogen in the experiment. Inoc, inoculation; Uninoc, uninoculated. Inoc Method, quantity of cells and method used to inoculate plants. N+, uninoculated control plants given nitrogen.

Reference	Plant Taxa	Nod Type	Infect Method	Geo Distrib	Ploidy Levels Tested	Polyploid Type	Polyploid Origin	Growth Conditions	Supp N	Plant Size at Inoc	Inoc Method	Rhizobia Taxa	No Strains Used	Controls	Growth Post Inoc	Data Analysis
Beauregard et al., 2004	*Trifolium ambiguum, T. repens, T. pratense*	I	Root hair	Temp	2X-4X-6X	Auto	Natural	Tubes, turface	None	Seeds	1ml of 10-1 rhizobia dilution in ddh20		13	Uninoc	40 days	None
Date, 2010	*Stylosanthes hamata, S. seabrana*	A	Crack	Trop	2X-4X	Allo	Natural	Jars, sand	None	5 - 7 days post sowing		*Bradyrhizobium*	20	Uninoc and N+	6 - 8 weeks	Pattern analysis (UPGMA)
Diatloff and Ferguson, 1970	*Glycine wightii* (*Neonotonia wightii*)	D	Root hair	Trop	2X-4X	Auto	Natural	Tubes, agar	None	Seeds	100,000 nodule bacteria	*Rhizobium*	3	None	40 days	LSD (5%) of accession effects
Diatloff and Ferguson, 1970	*Glycine wightii* (*Neonotonia wightii*)	D	Root hair	Trop	2X-4X	Auto	Natural	Glasshouse: jars, sand	None	7 day old seedlings	1 mL 10 day old bacterial suspension	*Rhizobium*	3	None	9 weeks	LSD (5%) of accession effects

Evans and Jones, 1966	*Trifolium ambiguum*	I	Root hair	Temp	2X-4X-6X	Auto	Natural	Glasshouse: boxes	None	Seeds		*Rhizobium*	1	Uninoc	4 months	T-tests (inoc vs. uninoc w/in ploidy)
Forrester et al., unpubl. res.	*Medicago sativa*	I	Root hair	Temp	2X-4X	Auto	Natural	Greenhouse: pots, turface	None	7 day old seedlings	5 mL of 10^8 cells in sterile ddH2O	*Sinorhizobium meliloti*	3	Uninoc	6.5 weeks	Linear mixed effects models
Hely, 1957	*Trifolium ambiguum*	I	Root hair	Temp	2X-6X	Auto	Natural	Glasshouse: tubes, agar	None			*Rhizobium trifolii*	2 - 4	None	80 days	None
Kabi and Bhaduri, 1978	*Phaseolus aureus*	D	Root hair	Trop	2X-3X-4X	Auto	Synthetic	Lab: jars, sand	None	Seeds	Coinoculation (1:1), 10^8 cells/culture	*Rhizobium* 'cowpea miscellani'	2	None	30 or 45 days	Report significance at 1 and 5% level but no details
Leps et al., 1980	*Medicago sativa*	I	Root hair	Temp	2X-4X-8X	Auto	Synthetic	Growth room: vial, vermiculite	2 trts	Seedlings	0.5 ml of 10^10 - 10^11 cells	*Rhizobium meliloti*	1	Uninoc and N+	3 weeks	T- tests to compare 2X-4X and 4X-8X
Nilsson and Rydin, 1954	*Trifolium pratense*	I	Root hair	Temp	2X-4X	Auto	Natural	Greenhouse: tubes, agar	None				30	Uninoc	9 - 45 days	None
Nutman, 1967	*Trifolium subterraneum*	I	Root hair	Temp	2X-4X	Auto	Natural	Lab: tubes, agar	None				3			
Pfeiffer et al., 1980	*Medicago sativa*	I	Root hair	Temp	2X-4X-8X	Auto	Synthetic	Growth chamber: pots, vermiculite	7.5 mM KNO3	Plants cut back to 5 cm height		*Rhizobium meliloti*		None	Variable across ploidies	F-test, t-test
Powell and Doyle, 2016	*Glycine dolichocarpa, G. syndetica, G. tomentella*	D	Root hair	Trop	2X-4X	Allo	Natural	Lab: microfuge tubes	None	5 day old seedlings	200 ul of 10^9 cells	*Ensifer fredii, Bradyrhizobium japonicum*	5	Uninoc	60 hours	Mixed effects model
Powell and Doyle, 2016	*Glycine dolichocarpa, G. syndetica, G. tomentella*	D	Root hair	Trop	2X-4X	Allo	Natural	Growth room: tubes, Jensens	Some trts, 0.1% KNO3	7 day old seedlings	10^9 cells	*Ensifer fredii, Bradyrhizobium japonicum*	5	Uninoc and N+	10 weeks	Logistic regression model, mixed effects models, Hurdle models

Stalker et al., 1994	*Arachis*	A	Crack	Trop	2X-4X	Allo	Natural	Greenhouse: jars, sand	None		10 ml of 10^9 cells in YAM	*Bradyrhizobium*	3	Uninoc	60 days	General linear model
Stalker et al., 1994	*Arachis villosa*	A	Root hair	Temp	2X-4X	Auto	Natural	Greenhouse: jars, sand,	None		10 ml of 10^9 cells in YAM	*Bradyrhizobium*	3	Uninoc	60 days	General linear model
Thilakarathna et al., 2012	*Trifolium pratense*	I	Root hair	Temp	2X-4X	Auto	Natural	Lab: slides	None	7 day old seedlings	200 ul of OD600 0.1 cells	*Rhizobium leguminosarum* biovar *trifolii*	1	Uninoc	4, 12, 24 hrs	Split plot ANOVA
Thilakarathna et al., 2012	*Trifolium pratense*	I	Root hair	Temp	2X-4X	Auto	Natural	Growth room: pouches	Some trts	7 day old seedlings	1 ml of 10^8 cells	*Rhizobium leguminosarum* biovar *trifolii*	1	None	8 weeks	Split plot ANOVA, PCA
Weir, 1961a	*Trifolium pratense*	I	Root hair	Temp	2X-4X	Auto	Natural	Lab: pots, unsterilized soil	None	2 week old seedlings	5 ml of 5 day old inoculum in YM	*Rhizobium trifolii*	1	None	16 weeks	None
Weir, 1961a	*Trifolium repens*	I	Root hair	Temp	2X-4X	Allo	Natural	Lab: pots, unsterilized soil	None	2 week old seedlings	5 ml of 5 day old inoculum in YM	*Rhizobium trifolii*	1	None	16 weeks	None
Weir, 1961a	*Trifolium repens*	I	Root hair	Temp	2X-4X	Allo	Natural	Lab: jars, agar	None				1	None	8 weeks	None
Weir, 1961b	*Trifolium pratense*	I	Root hair	Temp	2X-4X	Auto	Natural	Greenhouse: jars, agar	None	2 day old seedlings	2 day old inoculum in YM water	*Rhizobium trifolii*	3	Uninoc	7 weeks	None
Weir, 1964	*Trifolium repens*	I	Root hair	Temp	2X-4X	Allo	Natural	Greenhouse: pots, soil	None	2 week old seedlings		*Rhizobium trifolii*	1	None	17 weeks	ANOVA

Table 3. Summary of studies organized by five subsections (I, II, IV, V, VI) within the effects hierarchy. Within each subsection, the table is organized by specific mutualism traits within the hierarchy and a predication for how polyploid and diploid plants will differ. All studies that have tested a specific trait are included with a general outcome and information about each study.

Trait	Prediction	General Outcome	Plant Taxa	Ploidy Levels Tested	Polyploid Type	Polyploid Origin	Reference
I. Quantity of symbionts: Timing of nodulation, root size and architecture, nodule number							
Timing of nodulation	Polyploid plants nodulate earlier than diploid plants (P < D)	P < D	*Glycine wightii* (*Neonotonia wightii*)	2X - 4X	Autopolyploid	Natural	Diatloff and Ferguson, 1970*
		P < D	*Trifolium ambiguum*	2X - 6X	Autopolyploid	Natural	Hely, 1957*
		P < D	*Trifolium ambiguum*	2X - 4X - 6X	Autopolyploid	Natural	Evans and Jones, 1966‡
		P < D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Thilakarathna et al., 2012
		P < D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
		P = D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Nilsson and Rydin, 1954*
		P = D	*Trifolium subterraneum*	2X - 4X	Autopolyploid	Natural	Nutman, 1967
		P = D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synthetic	Leps et al., 1980
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Weir, 1961b*
Root size and biomass	Polyploid plants produce larger roots and/or more root biomass than diploids (P > D)	P > D	*Medicago sativa*	2X - 4X	Autopolyploid	Natural	Forrester et al., unpubl. res.
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Thilakarathna et al., 2012
		P > D	*Glycine dolichocarpa, G. syndetica, G. tomentella*	2X - 4X	Allopolyploid	Natural	Powell and Doyle, 2016
		P > D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
Lateral root production	Polyploid plants produce more lateral roots than diploids (P > D)	P = D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
Root hair deformation	Polyploid plants have a higher percentage of deformed roots per plant than diploids (P > D)	P > D	*Glycine dolichocarpa, G. syndetica, G. tomentella*	2X - 4X	Allopolyploid	Natural	Powell and Doyle, 2016†
Root hair infection	Polyploid plants have a higher percentage of root hair infection than diploids (P > D)	P > D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
Nodule number	Polyploid plants produce more nodules than diploid plants (P > D)	P > D	*Glycine wightii* (*Neonotonia wightii*)	2X - 4X	Autopolyploid	Natural	Diatloff and Ferguson, 1970*
		P > D	*Medicago sativa*	2X - 4X	Autopolyploid	Natural	Forrester et al., unpubl. res.
		P > D	*Trifolium ambiguum*	2X - 4X - 6X	Autopolyploid	Natural	Evans and Jones, 1966
		P > D	*Trifolium ambiguum*	2X - 6X	Autopolyploid	Natural	Hely, 1957*
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Thilakarathna et al., 2012
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Weir, 1961a*

		P > D	*Arachis*	2X - 4X	Allopolyploid	Natural	Stalker et al., 1994
		P = D	*Arachis villosa*	2X - 4X	Autopolyploid	Natural	Stalker et al., 1994
		P = D	*Glycine dolichocarpa, G. syndetica, G. tomentella*	2X - 4X	Allopolyploid	Natural	Powell and Doyle, 2016
		P = D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synethic	Leps et al., 1980
		P = D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synethic	Pfieffer et al., 1980
		P < D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Nilsson and Rydin, 1954*
		P < D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Weir, 1961b*
		P < D	*Trifolium subterraneum*	2X - 4X	Autopolyploid	Natural	Nutman, 1967
		P < D	*Trifolium repens*	2X - 4X	Allopolyploid	Natural	Weir, 1961a*
		P < D	*Trifolium repens*	2X - 4X	Allopolyploid	Natural	Weir, 1964
		P < D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
II. Quantity of symbionts: nodule size and biomass							
Nodule size and biomass	Polyploid plants produce larger nodules and/or nodules with greater biomass than diploid plants (P > D)	P > D	*Medicago sativa*	2X - 4X	Autopolyploid	Natural	Forrester et al., unpubl. res.
		P > D	*Trifolium ambiguum*	2X - 4X - 6X	Autopolyploid	Natural	Evans and Jones, 1966‡
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Weir, 1961a*‡
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Weir, 1961b*‡
		P > D	*Arachis*	2X - 4X	Allopolyploid	Natural	Stalker et al., 1994
		P > D	*Glycine dolichocarpa, G. syndetica, G. tomentella*	2X - 4X	Allopolyploid	Natural	Powell and Doyle, 2016
		P > D	*Trifolium repens*	2X - 4X	Allopolyploid	Natural	Weir, 1961a*‡
		P > D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
		P = D	*Trifolium repens*	2X - 4X	Allopolyploid	Natural	Weir, 1964
		P = D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synthetic	Pfieffer et al., 1980
		P < D	*Trifolium ambiguum*	2X - 6X	Autopolyploid	Natural	Hely, 1957*
IV. Quality of symbionts: nodule environment							
Nitrogen fixation rate	Polyploid plants fix nitrogen at a higher rate than diploid plants (P > D)	P > D	*Arachis villosa*	2X - 4X	Autopolyploid	Natural	Stalker et al., 1994
		P > D	*Arachis*	2X - 4X	Allopolyploid	Natural	Stalker et al., 1994
		P > D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synthetic	Leps et al., 1980
		P = D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synthetic	Pfieffer et al., 1980
V. Quality of symbionts: identity of rhizobial symbionts							
Partner choice	Polyploid plants associate with distinct rhizobial partners than diploid plants (P ≠ D)	P ≠ D	*Stylosanthes hamata, S. seabrana*	2X - 4X	Allopolyploid	Natural	Date, 2010
		P ≠ D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
Host promiscuity	Polyploid plants can form effective symbioses with more rhizobial symbionts than diploid plants (P > D)	P > D	*Trifolium ambiguum*	2X - 4X - 6X	Autopolyploid	Natural	Beauregard et al., 2004*
		P > D	*Trifolium ambiguum*	2X - 4X - 6X	Autopolyploid	Natural	Hely, 1957*
		P > D	*Glycine dolichocarpa, G. syndetica, G. tomentella*	2X - 4X	Allopolyploid	Natural	Powell and Doyle, 2016
		P > D	*Stylosanthes hamata, S. seabrana*	2X - 4X	Allopolyploid	Natural	Date, 2010
VI. Access to fixed nitrogen via enhanced symbioses							

Plant nitrogen content	Polyploid plants have higher N content in vegetative tissue than diploid plants (P > D)	P > D	*Glycine wightii* (*Neonotonia wightii*)	2X - 4X	Autopolyploid	Natural	Diatloff and Ferguson, 1970*
		P > D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synthetic	Leps et al., 1980
		P = D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Nilsson and Rydin, 1954*
Plant size and biomass	Polyploid plants produce more biomass when associating with rhizobia than diploid plants (P > D)	P > D	*Glycine wightii* (*Neonotonia wightii*)	2X - 4X	Autopolyploid	Natural	Diatloff and Ferguson, 1970*
		P > D	*Trifolium ambiguum*	2X - 6X	Autopolyploid	Natural	Hely, 1957*
		P > D	*Trifolium ambiguum*	2X - 4X - 6X	Autopolyploid	Natural	Evans and Jones, 1966†
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Weir, 1961a*
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Weir, 1961b*
		P > D	*Trifolium pratense*	2X - 4X	Autopolyploid	Natural	Thilakarathna et al., 2012
		P > D	*Arachis*	2X - 4X	Allopolyploid	Natural	Stalker et al., 1994
		P > D	*Glycine dolichocarpa, G. syndetica, G. tomentella*	2X - 4X	Allopolyploid	Natural	Powell and Doyle, 2016
		P > D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synthetic	Leps et al., 1980†
		P > D	*Medicago sativa*	2X - 4X - 8X	Autopolyploid	Synthetic	Pfieffer et al., 1980
		P > D	*Phaseolus aureus*	2X - 3X - 4X	Autopolyploid	Synthetic	Kabi and Bhaduri, 1978
		P = D	*Medicago sativa*	2X - 4X	Autopolyploid	Natural	Forrester et al., unpubl. res.
		P = D	*Trifolium subterraneum*	2X - 4X	Autopolyploid	Natural	Nutman, 1967
		P < D	*Trifolium repens*	2X - 4X	Allopolyploid	Natural	Weir, 1961a*
		P < D	*Trifolium repens*	2X - 4X	Allopolyploid	Natural	Weir, 1964

* No statistical analyses conducted

† Compared inoculated plants to uninoculated controls within a ploidy level

‡ Anecdotal report, no measurements taken

Appendix B Additional information, figures, and tables (chapter 2)

Supplemental 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., 2006, Havananda et al., 2011). Plant lineages from two independent autopolyploidy events within the *Medicago sativa* complex 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 et al., 2011) and seeds from ten accessions were obtained from the USDA National Genetic Resources Program. Accessions were used as a proxy for genetic variation within taxa, as previous studies found significant variation among accessions in *M. sativa* (Sakiroglu et al., 2010; Ilhan et al., 2016). Diploid and autotetraploid lineages within ploidy pairs were matched by geographic origin when possible (http://www.ars-grin.gov/; Table 4). For clarity, accessions will be referred to as lineages throughout the remainder of the text.

Seed scarification and planting

Seeds were scarified with 72% (w/w) sulfuric acid for ten minutes, rinsed with sterile ddH_2O, and sterilized with 10% bleach for ten minutes following standard protocols (Heath and

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℃ refrigerator for two-four days to synchronize germination. Seeds were then transferred to a dark cabinet at room temperature for one-three days to induce germination. Once seeds developed radicles they were planted into sterilized growth pouches (CYG, Mega International) 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/pouch, two pouch replicates/lineage/treatment). Pouches were sorted by treatment and replicate and then placed into plastic containers to prevent cross contamination (Sterilite 18058606 Large Flip Top, Clear). Containers were sterilized prior to housing pouches by soaking them in a 10% commercial bleach solution for five minutes. Each container held ten pouches (one pouch/lineage/treatment) and each treatment had two replicate containers. Containers were transferred to a growth room set to 25℃, 60% humidity, and with supplemental lighting to achieve 16-hour days.

Rhizobial strains

Twenty-one strains of *Sinorizobium* 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* (Appendix B Fig. 9). These strains span the *Sinorhizobium* phylogeny, have genetic resources available, and exhibit diverse symbiotic phenotypes with *M. truncatula* (Sugawara et al., 2013). Twenty strains were obtained from Michael Sadowsky at the University of Minnesota and one

strain (*S. meliloti* USDA1002) was obtained from Patrick Elia at the National Rhizobium Germplasm Resource Collection.

Experimental design and treatments

The experiment was divided into four temporal blocks with each block using four to six unique rhizobial strains and a water inoculated control treatment. The first temporal block used four rhizobial strains, the second block used five strains, and the third and fourth blocks used six strains each. Prior to planting, seedlings were sorted into size groups to avoid effects of initial plant size at time of inoculation and each seedling within a size group was assigned to a rhizobial treatment or the water inoculated control. Each round lasted six weeks and occurred between May and October 2017.

Inoculation and plant growth

For the first round, 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 (based on OD_{600}). For all other rounds, rhizobial strains were cultured on TY plates, scraped, and resuspended in 10 ml sterile ddH_2O to achieve 10^9 cells/ml.

Seven or eight days after planting (depending on the round), each plant was inoculated with 1.0 x 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 nine ml of nitrogen-free Fahraeus solution eight days after inoculation. Three weeks post-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 within a round were harvested three-weeks post inoculation and within two – four days.

Plant harvest and data collection

Pouches were cut open to expose plant tissue and scanned (CanoScan LiDE 220, Canon Inc., United States). Plants were removed from pouches, number of leaves and nodules were counted, and nodule color was recorded by a single observer as pink, white, brown, and/or green to estimate the presence of leghaemoglobin and nitrogen fixation function (Imaizumi-Anraku et al., 1997). Plants were then dissected into shoot, root, and nodule tissue, dried in an oven at 55°C for at least four days, and weighed. Of the 180 control plants, only one plant produced a single nodule and was excluded from analyses.

Nodule traits and host benefit analyses

Nodule and plant biomass traits were collected for all plants that survived in the experiment. For pouches that contained more than one plant, an average value was calculated for all plants within each pouch. Only strains that nodulated 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 used in the following analyses (Appendix B Fig. 9).

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 zero as they are likely not fixing nitrogen and pink nodules received a score of one as they are likely fixing nitrogen and providing it to their plant hosts (Imaizumi-Anraku et al., 1997). Green nodules received a score of 0.75, as they likely fixed nitrogen but are in the early stages of senescence. Brown nodules received a score of 0.5 as they completely senesced but may have fixed nitrogen during the experiment. 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 re-ran 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.

To test whether autotetraploid plants benefitted more from rhizobial symbioses than diploids, we calculated host growth response (HGR) for plants in each lineage and rhizobial treatment combination. HGR was quantified as the mean percentage difference in dried shoot biomass between inoculated and uninoculated controls within each lineage ((shoot biomass inoculated plant – average shoot biomass uninoculated plants)/average shoot biomass uninoculated plants)*100; Regus et al., 2015). Therefore, this metric allowed us to separate the benefits plants obtain from rhizobial mutualism from the effects of polyploidy on plant growth. Nodule traits and HGR metrics were approximately normally distributed, and model assumptions were checked. Linear mixed effects models were used to test for effects of ploidy, strain, and their interaction (fixed) and lineage nested within subspecies (random) on nodule traits and host benefit using the lme4 (v1.1-and lmerTest (v3.0-1) packages in R (v1.1.453).

Plasticity analyses

We used plasticity indices to test whether autotetraploid *M. sativa* lineages exhibited reduced plasticity in fitness across rhizobial environments relative to diploids. Specifically, we calculated the relative distance plasticity index (RDPI; Valladares et al., 2006) of host growth response of shoot biomass across all 21 rhizobial strains for diploid and autotetraploid lineages. Within each lineage, we calculated the average pairwise distance in HGR for all combinations of rhizobial environments using the Canberra method. RDPI values range from 0 to 1, with values closer to 0 reflecting more generalized rhizobial interactions because the quality of interactions is maintained across biotic environments. To evaluate potential costs associated with specialization in rhizobial interactions, we calculated the average distance from the maximum host growth response of shoot biomass obtained for diploid and tetraploid lineages across all rhizobial environments. RDPI and cost of specialization were calculated in R using the vegan package (v2.5-4), and t-tests were used to test for significant differences between ploidy levels (stats package v3.3.3). We also re-ran these analyses for the 17 nodulating strains, but the results did not change. Data were visualized using ggplot2 (v3.0.0).

Table 4. Diploid (2X) and autotetraploid (4X) accessions of the *Medicago sativa* species complex used in the study. *Medicago* sativa subsp. caeruluea (2X) gave rise to *M. s.* subsp. *sativa* (4X) and *M. s.* subspecies *falcata* contains both 2X and 4X populations. Seeds from ten wild accessions (or lineages) were from the USDA National Genetic Resources Program that spanned a broad geographic range and potential genetic diversity. When possible, 2X and 4X accessions within subspecies pairs were matched by origin. Elevation and GPS locations for each accession provided when available.

Plant Species	Subspecies	Ploidy	Accession	Origin	Collection Type	Elevation (m)	GPS
Medicago sativa	caerulea	2X	PI 315466	Russia	Wild		
Medicago sativa	caerulea	2X	PI 440502	Kazakhstan	Wild	493	43.1236 N, 71.2248 E
Medicago sativa	caerulea	2X	PI 464715	Turkey	Wild	780	
Medicago sativa	sativa	4X	PI 253451	Slovenia	Wild	518	46.3833 N, 16.4000 E
Medicago sativa	sativa	4X	PI 314713	Kazakhstan	Wild		
Medicago sativa	sativa	4X	PI 577574	Tajikistan	Wild		
Medicago sativa	falcata	2X	PI 325387	Russia	Wild		44.0666 N, 41.6000 E
Medicago sativa	falcata	2X	PI 631707	China	Wild	1960	43.4905 N, 81.1233 E
Medicago sativa	falcata	4X	PI 502451	Russia	Wild		
Medicago sativa	falcata	4X	PI 631704	China	Wild	1320	43.1644 N, 81.6200 E

Table 5. ANOVA for host growth response of dried shoot biomass of *Medicago sativa* diploid and autotetraploid plants when grown with 17 single strains of *Sinorhizobium*. *P = 0.05; ***P < 0.001. Numerator d.f. and F values are shown for each effect. Denominator d.f. = 126.

	d.f.	host growth response of shoot biomass
Ploidy	1	5.32*
Strain	16	9.81***
Ploidy:Strain	16	0.78

Table 6. MANOVA for nodule number, total nodule biomass, and nodule color for Medicago sativa diploid and autotetraploid plants when grown with 17 single strains of *Sinorhizobium*. ***P < 0.001.

	d.f.	Pillai	approx F	num df	den df
Ploidy	1	0.44	33.88***	3	132
Strain	16	1.00	4.24***	48	402
Ploidy:Strain	16	0.27	0.82	48	402
Residuals	134				

Table 7. ANOVAs for nodule traits of *Medicago sativa* diploid and autotetraploid plants when grown with 17 single strains of Sinorhizobium. *P < 0.05; **P < 0.01; ***P < 0.001. Numerator d.f. and F values are shown for each effect. Denominator d.f. = 132 for all traits.

	d.f.	nodule number	total nodule biomass	nodule color
Ploidy	1	14.69***	102.39***	8.07**
Strain	16	1.78*	3.70***	25.82***
Ploidy:Strain	16	0.43	1.49	0.74

Table 8. ANCOVAs for nodule number and total nodule biomass of *Medicago sativa* diploid and autotetraploid plants when grown with 17 single strains of *Sinorhizobium* and including root biomass as a covariate. **P < 0.01; ***P < 0.001. Numerator d.f. and F values shown for each effect. Denominator d.f. = 132 for all traits.

	d.f.	nodule number	total nodule biomass
Ploidy	1	21.43***	168.48***
Strain	16	2.60**	6.09***
Root Biomass	1	58.27***	100.77***
Ploidy:Strain	16	0.89	1.62

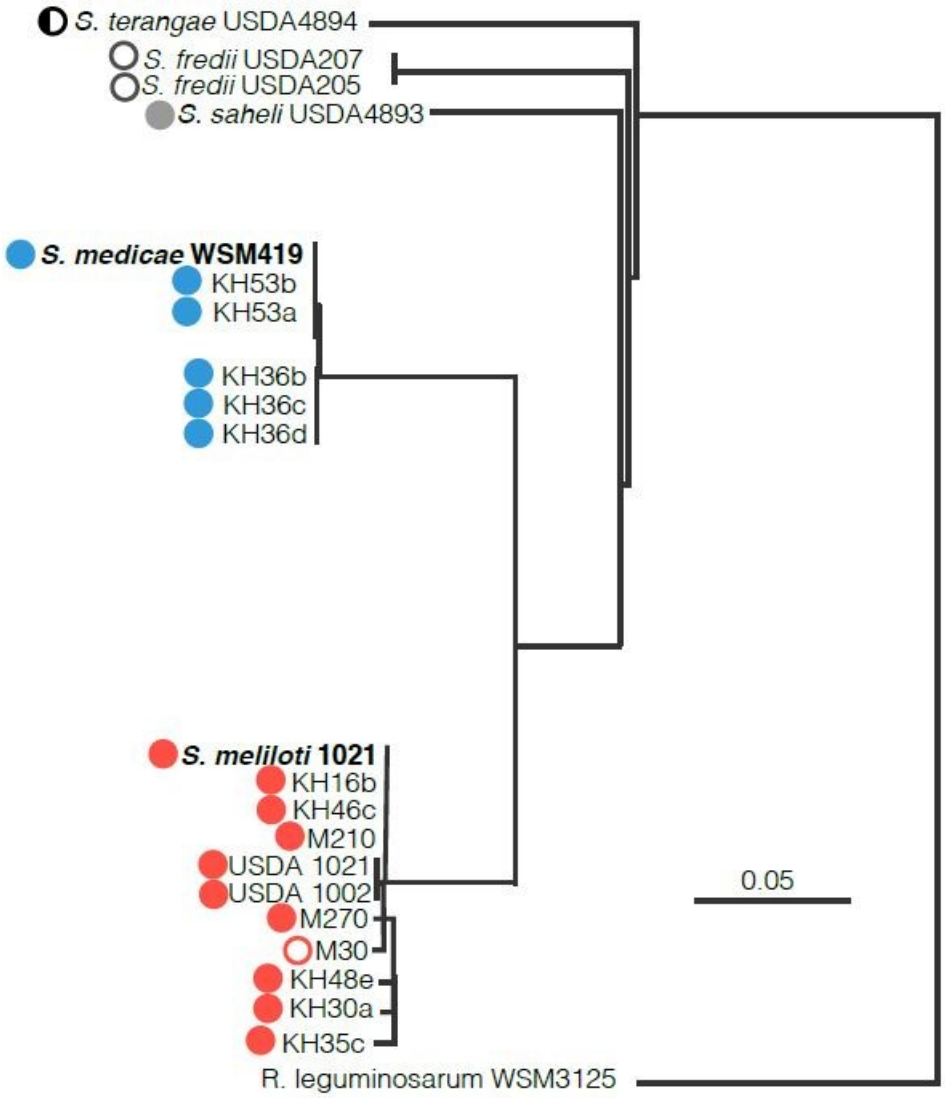

Figure 9. Phylogeny of the 21 *Sinorhizobium* strains used in the experiment. *Sinorhizobium medicae* strains are shown in blue, *S. meliloti* strains are shown in red, and all other strains shown in black or gray circles. Filled circles indicate the strain produced nodules with all diploid and autotetraploid *Medicago sativa* lineages, empty circles indicate the strain did not nodulate any *M. sativa* lineages (*S. meliloti* M30, *S. fredii* USDA207 and UDSA205), and the partially filled circle indicates the strain that only nodulated a portion of the *M. sativa* lineages used.

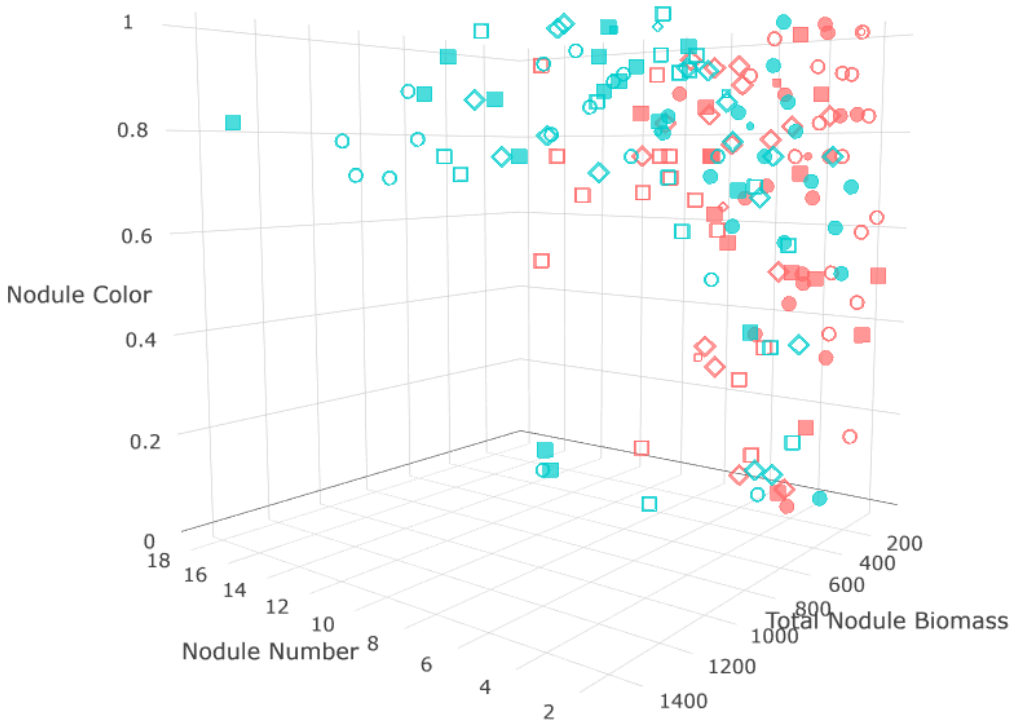

Figure 10. Nodule traits of diploid (2X) and autotetraploid (4X) lineages of *Medicago sativa* associated with 17 strains of *Sinorhizobium* bacteria. Each shape represents a different lineage. Each data point represents the average nodule trait value produced by diploid (red, *n* = 316 plants) or tetraploid (blue, *n* = 449 plants) lineages associated with a *Sinorhizobium* strain. Total nodule biomass shown in micrograms (µg).

Bibliography

Alunni B, B Gourion. 2016. Terminal bacteroid differentiation in the legume-rhizobium symbiosis: nodule-specific cysteine-rich peptides and beyond. *New Phytol* 211(2): 411-417.

Anglade J, G Billen, J Garnier. 2015. Relationships for estimating N^2 fixation in legumes: incidence for N balance of legume-based cropping systems in Europe. *Ecosphere* 6(3): 37.

Appleby C. 1984. Leghemoglobin and *Rhizobium r*espiration. *Ann Rev Plant Physiol* 35: 443-478.

Arvanitis L, C Wiklund, J Ehrlen. 2008. Plant ploidy level influences selection by butterfly seed predators. *Oikos* 117: 1020-1025.

Balao F, J Herrera, S Talavera. 2011. Phenotypic consequences of polyploidy and genome size at the microevolutionary scale: a multivariate morphological approach. *New Phytol* 192(1): 256-265.

Barker MS, N Arrigo, AE Baniaga, Z Li, DA Levin. 2016. On the relative abundance of autopolyploids and allopolyploids. *New Phytol* 212: 391-398.

Bascompte J, P Jordano, CJ Melian, JM Olesen. 2003. The nested assembly of plant-animal mutualistic networks. *Proc Natl Acad Sci USA* 100(16): 9383-9387.

Batstone RT, KA Carscadden, ME Afkhami, ME Frederickson. 2018. Using niche breadth theory to explain generalization in mutualisms. *Ecology* 99(5): 1039-1050.

Beaulieu JM, IJ Leitch, S Patel, A Pendharkar, CA Knight. 2008. Genome size is a strong predictor of cell size and stomatal density in angiosperms. *New Phytol* 179: 975-986.

Borges LA, LGR Souza, M Guerra, IC Machado, GP Lewis, AV Lopes, A. 2012. Reproductive isolation between diploid and tetraploid cytotypes of *Libidibia ferrea* (= *Caesalpinia ferrea*) (Leguminosae): Ecological and taxonomic implications. *Plant Systematics and Evolution* 298(7): 1371-1381.

Bretagnolle F, JD Thompson. 2001. Phenotypic plasticity in sympatric diploid and autotetraploid *Dactylis glomerata*. *Int J Plant Sci* 162(2): 309-316.

Brittingham HA, MH Koski, TL Ashman. 2018. Higher ploidy is associated with reduced range breadth in the Potentilleae tribe. *Am J Bot* 105(4): 700-710.

Bronstein JL. 1994. Our current understanding of mutualism. *Q Rev Biol* 69: 31-51.

Burdon J, A Gibson, S Searle, M Woods, J Brockwell. 1999. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian *Acacia*: within-species interactions. *Journal of Applied Ecology* 36: 398-408.

Burghardt LT, B Epstein, J Guhlin, MS Nelson, MR Taylor, ND Young, P Tiffin. 2018. Select and resequence reveals relative fitness of bacteria in symbiotic and free-living environments. *Proc Natl Acad Sci U S A* 115(10): 2425-2430.

Caetano-Anolles G, PM Gresshoff. 1991. Plant genetic control of nodulation. *Annu Rev Microbiol* 45: 345-382.

Cannon SB, D Ilut, AD Farmer, SL Maki, GD May, SR Singer, JJ Doyle. 2010. Polyploidy did not predate the evolution of nodulation in all legumes. *PLoS One* 5(7): e11630.

Cannon SB, MR McKain, A Harkess, MN Nelson, S Dash, MK Deyholos, J Leebens-Mack. 2015. Multiple polyploidy events in the early radiation of nodulating and nonnodulating legumes. *Mol Biol Evol* 32(1): 193-210.

Castro S, Z Münzbergová, J Raabová, J Loureiro. 2010. Breeding barriers at a diploid–hexaploid contact zone in *Aster amellus*. *Evolutionary Ecology* 25(4): 795-814.

Chalk PM, SK Lam, D Chen. 2016. (15)N methodologies for quantifying the response of N2-fixing associations to elevated [CO2]: A review. *Sci Total Environ* 571: 624-632.

Coba de la Pena T, E Fedorova, JJ Pueyo, MM Lucas. 2017. The symbiosome: Legume and rhizobia co-evolution toward a nitrogen-fixing organelle? *Front Plant Sci* 8: 2229.

Coughlan JM, S Han, S Stefanovic, TA Dickinson. 2017. Widespread generalist clones are associated with range and niche expansion in allopolyploids of Pacific Northwest Hawthorns (Crataegus L.). *Mol Ecol* 26(20): 5484-5499.

Daehler CC. 1998. The taxonomic distribution of invasive angiosperm plants: ecological insights and comparison to agricultural weeds. *Biological Conservation* 84: 167-180.

Date RA. 2010. *Bradyrhizobium* effectiveness responses in *Stylosanthes hamata* and *S. seabrana*. *Tropical Grasslands* 44: 141-157.

Denison RF, DB Layzell. 1991. Measurement of legume nodule respiration and O_2 permeability by noninvasive spectrophotometry of leghemoglobin. *Plant Physiology* 96: 137-143.

Denison RF. 2015. A Darwinian perspective on improving nitrogen-fixation efficiency of legume crops and forages. In *Crop Physiology* (pp. 207-222).

Diatloff A, JE Ferguson. 1970. Nodule number, time to nodulation and its effectiveness in eleven accessions of *Glycine wightii*. *Tropical Grasslands* 4: 223-228.

Douglas AE. 1998. Host benefit and the evolution of specialization in symbiosis. *Heredity* 81: 599-603.

Doyle JJ, LE Flagel, AH Paterson, RA Rapp, DE Soltis, PS Soltis, JF Wendel. 2008. Evolutionary genetics of genome merger and doubling in plants. *Annu Rev Genet* 42: 443-461.

Doyle JJ. 2011. Phylogenetic perspectives on origins of nodulation. *Molecular Plant-Microbe Interactions* 24: 1289-1295.

Doyle JJ, JE Coate. 2019. Polyploidy, the nucleotype, and novelty: The impact of genome doubling on the biology of the cell. *International Journal of Plant Sciences* 180(1): 1-52.

Dufresne F, M Stift, R Vergilino, BK Mable. 2014. Recent progress and challenges in population genetics of polyploid organisms: An overview of current state-of-the-art molecular and statistical tools. *Mol Ecol* 23(1): 40-69.

Ehinger M, TJ Mohr, JB Starcevich, JL Sachs, SS Porter, EL Simms. 2014. Specialization-generalization trade-off in a *Bradyrhizobium* symbiosis with wild legume hosts. *BMC Ecol* 14: 8.

Evans AM, DG Jones. 1966. The response to inoculation of the three chromosome races of *Trifolium ambiguum* sown with and without a companion grass. *The Journal of Agricultural Science* 66: 315-319.

Ferguson BJ, A Indrasumunar, S Hayashi, MH Lin, YH Lin, DE Reid, PM Gresshoff. 2010. Molecular analysis of legume nodule development and autoregulation. *J Integr Plant Biol* 52(1): 61-76.

Forrester NJ, TL Ashman. 2018a. The direct effects of plant polyploidy on the legume-rhizobia mutualism. *Ann Bot* 121(2): 209-220.

Forrester NJ, TL Ashman. 2018b. Nitrogen fertilization differentially enhances nodulation and host growth of two invasive legume species in an urban environment. *Journal of Urban Ecology* 4(1).

Futuyma DJ, G Moreno. 1988. The evolution of ecological specialization. *Ann Rev Ecol Syst* 19: 207-233.

Galbraith D, K Harkins, J Maddox, N Ayres, D Sharma, E Firoozabady. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220: 1049-1051.

Gage DJ. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev* 68(2): 280-300.

Gonzalez-Sama A, TC de la Pena, Z Kevei, et al. 2006. Nuclear DNA endoreduplication and expression of the mitotic inhibitor Ccs52 associated to determinate and lupinoid nodule organogenesis. *Molecular Plant-Microbe Interactions* 19: 173-180.

Grillo MA, JR Stinchcombe, KD Heath. 2016. Nitrogen addition does not influence pre-infection partner choice in the legume-rhizobium symbiosis. *Am J Bot* 103(10): 1763-1770.

Halverson K, SB Heard, JD Nason, JO Stireman 3rd. 2008. Differential attack on diploid, tetraploid, and hexaploid *Solidago altissima* L. by five insect gallmakers. *Oecologia* 154(4): 755-761.

Harrison TL, AK Simonsen, JR Stinchcombe, ME Frederickson. 2018. More partners, more ranges: generalist legumes spread more easily around the globe. *Biol Lett* 14(11). doi:10.1098/rsbl.2018.0616

Havananda T, EC Brummer, JJ Doyle. 2011. Complex patterns of autopolyploid evolution in alfalfa and allies (*Medicago sativa*; Leguminosae). *Am J Bot* 98(10): 1633-1646.

Haynes JG, KJ Czymmek, CA Carlson, H Veereshlingam, R Dickstein, DJ Sherrier. 2004. Rapid analysis of legume root nodule development using confocal microscopy. *New Phytol* 163(3): 661-668.

Heath KD, P Tiffin. 2007. Context dependence in the coevolution of plant and rhizobial mutualists. *Proc Biol Sci* 274(1620): 1905-1912.

Heath KD. 2010. Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. *Evolution* 64(5): 1446-1458.

Heath KD, AJ Stock, JR Stinchcombe. 2010. Mutualism variation in the nodulation response to nitrate. *J Evol Biol* 23(11): 2494-2500.

Heath KD, JR Stinchcombe. 2014. Explaining mutualism variation: A new evolutionary paradox? *Evolution* 68(2): 309-317.

Herridge DF, MB Peoples, RM Boddey. 2008. Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* 311(1-2): 1-18.

Hely FW. 1957. Symbiotic variation in *Trifolium ambiguum* M. Bieb. with special reference to the nature of resistance. *Australian Journal of Biological Sciences* 10: 1-16.

Hunt S, DB Layzell. 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. *Annual Review of Plant Physiology and Plant Molecular Biology* 44: 483-511.

Husband BC, SJ Baldwin, J Suda. 2013. The incidence of polyploidy in natural plant populations: Major patterns and evolutionary processes. In *Plant Genome Diversity Volume 2* (pp. 255-276).

Husband BC, SJ Baldwin, HA Sabara. 2016. Direct vs. indirect effects of whole-genome duplication on prezygotic isolation in *Chamerion angustifolium*: Implications for rapid speciation. *Am J Bot* 103(7): 1259-1271.

İlhan D, X Li, EC Brummer, M Şakiroğlu. 2016. Genetic diversity and population structure of tetraploid accessions of the *Medicago sativa* species complex. *Crop Science* 56(3). doi:10.2135/cropsci2015.12.0750

Imaizumi-Anraku H, M Kawaguchi, H Koiwa, S Akao, K Syono. 1997. Two ineffective-nodulating mutants of *Lotus japonicus*—Different phenotypes caused by the blockage of endocytotic bacterial release and nodule maturation. *Plant and Cell Physiology* 38: 871-881.

Jones KM, H Kobayashi, BW Davies, ME Taga, GC Walker. 2007. How rhizobial symbionts invade plants: The *Sinorhizobium-Medicago* model. *Nat Rev Microbiol* 5(8): 619-633.

Joshi P, R Verma. 2004. High frequency production of colchicine induced autotetraploids in faba bean (*Vicia faba* L.). *Cytologia* 69: 141-147.

Kabi MC, PN Bhaduri. 1978. Nodulating behavior of colchicine induced polyploids of *Phaseolus aureus* Roxb. *Cytologia* 43: 467-475.

Kiers ET, RA Rousseau, SA West, RF Denison. 2003. Host sanctions and the legume-rhizobium mutualism. *Nature* 425: 78-81.

Kiers ET, RF Denison, R. F. 2008. Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annual Review of Ecology, Evolution, and Systematics* 39(1): 215-236.

Kiers ET, TM Palmer, AR Ives, JF Bruno, JL Bronstein. 2010. Mutualisms in a changing world: An evolutionary perspective. *Ecol Lett* 13(12): 1459-1474.

Kondorosi E, F Roudier, E Gendreau. 2000. Plant cell-size control: growing by ploidy? *Current Opinion in Plant Biology* 3: 488-492.

Kondorosi E, A Kondorosi. 2004. Endoreduplication and activation of the anaphase-promoting complex during symbiotic cell development. *FEBS Lett* 567(1): 152-157.

Kondorosi E, P Mergaert, A Kereszt. 2013. A paradigm for endosymbiotic life: Cell differentiation of *Rhizobium* bacteria provoked by host plant factors. *Annu Rev Microbiol* 67: 611-628.

Kulkarni M, T Borse. 2009. Induced polyploidy with gigas expression for root traits in *Capsicum annuum*(L.). *Plant Breeding* 129(4): 461-464.

Lawrie AC, CT Wheeler. 1973. The supply of photosynthetic assimilates to nodules of *Pisum sativum* L. in relation to the fixation of nitrogen. *New Phytol* 72: 1341-1348.

Leps WT, WJ Brill, ET Bingham. 1980. Effect of alfalfa ploidy on nitrogen fixation. *Crop Science* 20: 427-230.

Levin DA. 1983. Polyploidy and novelty in flowering plants. *The American Naturalist* 122:1-25.

Levy M. 1976. Altered glycoflavone expression in induced autotetraploids of *Phlox drummondii*. *Biochemical Systematics and Ecology* 4: 229-254.

Li QG, L Zhang, C Li, JM Dunwell, YM Zhang. 2013. Comparative genomics suggests that an ancestral polyploidy event leads to enhanced root nodule symbiosis in the Papilionoideae. *Mol Biol Evol* 30(12): 2602-2611.

Lowry E, SE Lester. 2006. The biogeography of plant reproduction: potential determinants of species' range sizes. *Journal of Biogeography* 33(11): 1975-1982.

Maherali H, AE Walden, BC Husband. 2009. Genome duplication and the evolution of physiological responses to water stress. *New Phytol* 184(3): 721-731.

Maroti G, A Kereszt, E Kondorosi, P Mergaert. 2011. Natural roles of antimicrobial peptides in microbes, plants and animals. *Res Microbiol* 162(4): 363-374.

Maroti G, E Kondorosi. 2014. Nitrogen-fixing *Rhizobium*-legume symbiosis: Are polyploidy and host peptide-governed symbiont differentiation general principles of endosymbiosis? *Front Microbiol* 5: 326.

Martin SL, BC Husband. 2012. Whole genome duplication affects evolvability of flowering time in an autotetraploid plant. *PLoS One* 7(9): e44784.

McIntyre PJ, SS Strauss. 2017. An experimental test of local adaptation among cytotypes within a polyploid complex. *Evolution* 71(8): 1960-1969.

Melino VJ, EA Drew, RA Ballard, WG Reeve, G Thomson, RG White, GW O'Hara. 2012. Identifying abnormalities in symbiotic development between *Trifolium* spp. and *Rhizobium leguminosarum* bv. *trifolii* leading to sub-optimal and ineffective nodule phenotypes. *Ann Bot* 110(8): 1559-1572.

Mergaert P, T Uchiumi, B Alunni, G Evanno, A Cheron, O Catrice, E Kondorosi. 2006. Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis. *Proc Natl Acad Sci U S A* 103(13): 5230-5235.

Munoz N, X Qi, MW Li, M Xie, Y Gao, MY Cheung, et al. 2016. Improvement in nitrogen fixation capacity could be part of the domestication process in soybean. *Heredity* 117(2): 84-93.

Munzbergova Z. 2017. Colchicine application significantly affects plant performance in the second generation of synthetic polyploids and its effects vary between populations. *Ann Bot* 120(2): 329-339.

Ndlovu J, DM Richardson, JRU Wilson, JJ Le Roux, P Ladiges. 2013. Co-invasion of South African ecosystems by an Australian legume and its rhizobial symbionts. *Journal of Biogeography* 40(7): 1240-1251.

Nghiem CQ, CE Harwood, JL Harbard, AR Griffin, TH Ha, A Koutoulis. 2011. Floral phenology and morphology of colchicine-induced tetraploid *Acacia mangium* compared with diploid *A. mangium* and *A. auriculiformis*: Implications for interploidy pollination. *Australian Journal of Botany* 59(6): 582-592.

Nilsson PE, C Rydin. 1954. Studies on symbiotic nitrogen fixation by a new strain of tetraploid red clover (UO36). *Archives of Microbiology* 29: 398-403.

Nuismer SL, BM Cunningham. 2005. Selection for phenotypic divergence between diploid and autotetraploid *Heuchera grossulariifolia*. *Evolution* 59(9): 1928-1935.

Nutman PS. 1967. Varietal differences in the nodulation of subterranean clover. *Australian Journal of Agricultural Research* 18: 381-425.

Oldroyd GE, JD Murray, PS Poole, JA Downie. 2011. The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* 45: 119-144.

Oono R, RF Denison. 2010. Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant Physiol* 154(3): 1541-1548.

Pandit MK, SM White, MJ Pocock. 2014. The contrasting effects of genome size, chromosome number and ploidy level on plant invasiveness: a global analysis. *New Phytol* 203(2): 697-703.

Parker MP. 1995. Plant fitness variation caused by different mutualist genotypes. *Ecology* 76: 1525-1535.

Petit C, JD Thompson, F Bretagnolle. 1996. Phenotypic plasticity in relation to ploidy level and corm production in the perennial grass *Arrhenatherum elatius*. *Can J Bot* 74: 1964-1973.

Pfeiffer T, LE Schrader, ET Bingham. 1980. Physiological comparisons of isogenic diploid-tetraploid, tetraploid-octoploid alfalfa populations. *Crop Science* 20: 299-303.

Poisot T, JD Bever, A Nemri, PH Thrall, ME Hochberg. 2011. A conceptual framework for the evolution of ecological specialisation. *Ecol Lett* 14(9): 841-851.

Powell AF, JJ Doyle. 2015. The implications of polyploidy for the evolution of signalling in rhizobial nodulation symbiosis. In *Plant Microbe Interactions* (pp. 149-190).

Powell AF, JJ Doyle. 2016. Enhanced rhizobial symbiotic capacity in an allopolyploid species of Glycine (Leguminosae). *Am J Bot* 103(10): 1771-1782.

Ramsey J, DW Schemske. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology, Evolution, and Systematics* 29: 467-501.

Ramsey J, DW Schemske. 2002. Neopolyploidy in flowering plants. *Annual Review of Ecology and Systematics* 33(1): 589-639.

Ramsey J. 2011. Polyploidy and ecological adaptation in wild yarrow. *Proc Natl Acad Sci U S A,* 108(17): 7096-7101.

Regus JU, KA Gano, AC Hollowell, JL Sachs. 2014. Efficiency of partner choice and sanctions in *Lotus* is not altered by nitrogen fertilization. *Proc Biol Sci* 281(1781): 20132587.

Regus JU, KA Gano, AC Hollowell, V Sofish, JL Sachs. 2015. *Lotus* hosts delimit the mutualism-parasitism continuum of *Bradyrhizobium*. *J Evol Biol* 28(2): 447-456.

Regus JU, KW Quides, MR O'Neill, R Suzuki, EA Savory, JH Chang, JL Sachs. 2017. Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *Am J Bot* 104(9): 1299-1312.

Reid DE, BJ Ferguson, S Hayashi, YH Lin, PM Gresshoff. 2011. Molecular mechanisms controlling legume autoregulation of nodulation. *Ann Bot* 108(5): 789-795.

Richards CL, O Bossdorf, NZ Muth, J Gurevitch, M Pigliucci. 2006. Jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. *Ecol Lett* 9(8): 981-993.

Robson RL, JR Postgate. 1980. Oxygen and hydrogen in biological nitrogen fixation. *Annual Review of Microbiology* 34: 183-207.

Rodríguez-Echeverría S, JA Crisóstomo, C Nabais, H Freitas. 2008. Belowground mutualists and the invasive ability of *Acacia longifolia* in coastal dunes of Portugal. *Biological Invasions* 11(3): 651-661.

Sachs JL, JE Russell, YE Lii, KC Black, G Lopez, AS Patil. 2010. Host control over infection and proliferation of a cheater symbiont. *J Evol Biol* 23(9): 1919-1927.

Sakiroglu M, JJ Doyle, EC Brummer. 2010. Inferring population structure and genetic diversity of broad range of wild diploid alfalfa (*Medicago sativa* L.) accessions using SSR markers. *Theor Appl Genet* 121(3): 403-415.

Schwent RM, M Abe, S Higashi. 1983. Cytological study of the root nodule of *Vigna mungo* (Fabaceae). *Journal of the Arizona-Nevada Academy of Science* 18: 22-25.

Segraves KA, JN Thompson. 1999. Plant polyploidy and pollination: Floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. *Evolution* 53: 1114-1127.

Segraves KA, TJ Anneberg. 2016. Species interactions and plant polyploidy. *Am J Bot* 103(7): 1326-1335.

Segraves KA. 2017. The effects of genome duplications in a community context. *New Phytol* 215(1): 57-69.

Shanklin J, LE Schrader. 1986. Is there a relationship between infection by rhizobia and occurrence of disomatic nuclei in nodules of alfalfa (*Medicago sativa* L.)? *Plant Physiol* 80: 280-282.

Shantz AA, NP Lemoine, DE Burkepile. 2016. Nutrient loading alters the performance of key nutrient exchange mutualisms. *Ecol Lett* 19(1): 20-28.

Sheehy JE, FR Minchin, JF Witty. 1983. Biological control of the resistance to oxygen flux in nodules. *Annals of Botany* 52: 565-571.

Shimizu-Inatsugi R, A Terada, K Hirose, H Kudoh, J Sese, KK Shimizu. 2017. Plant adaptive radiation mediated by polyploid plasticity in transcriptomes. *Mol Ecol* 26(1): 193-207.

Singleton PW, C van Kessel. 1987. Effect of localized nitrogen availability to soybean half-root systems on photosynthate partitioning to roots and nodules. *Plant Physiology* 83: 552-556.

Soltis DE, CJ Visger, PS Soltis. 2014. The polyploidy revolution then and now: Stebbins revisited. *Am J Bot* 101(7): 1057-1078.

Soltis PS, DE Soltis. 2016. Ancient WGD events as drivers of key innovations in angiosperms. *Curr Opin Plant Biol* 30: 159-165.

Sprent JI. 2007. Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. *New Phytol* 174(1): 11-25.

Sprent JI, J Ardley, EK James. 2017. Biogeography of nodulated legumes and their nitrogen-fixing symbionts. *New Phytol* 215(1): 40-56.

Stacey G, M Libault, L Brechenmacher, J Wan, GD May. 2006. Genetics and functional genomics of legume nodulation. *Curr Opin Plant Biol* 9(2): 110-121.

Stalker HT, ML Nickum, JC Wynne, GH Elkan, TJ Schneeweis. 1994. Evaluation of biological nitrogen fixation capacity in *Arachis* species and the possible role of polyploidy. *Peanut Science* 21: 55-60.

Sudova R, H Pankova, J Rydlova, Z Munzbergova, J Suda. 2014. Intraspecific ploidy variation: A hidden, minor player in plant-soil-mycorrhizal fungi interactions. *Am J Bot* 101(1): 26-33.

Sudova R, P Kohout, Z Kolarikova, J Rydlova, J Voriskova, J Suda, P Mraz. 2018. Sympatric diploid and tetraploid cytotypes of *Centaurea stoebe* s.l. do not differ in arbuscular mycorrhizal communities and mycorrhizal growth response. *Am J Bot* 105(12): 1995-2007.

Sugawara M, B Epstein, BD Badgley, T Unno, L Xu. 2013. Comparative genomics of the core and accessory genomes of 48 *Sinorhizobium* strains comprising five genospecies. *Genome Biology* 14(2): R17.

te Beest M, JJ Le Roux, DM Richardson, AK Brysting, J Suda, M Kubesova, P Pysek. 2012. The more the better? The role of polyploidy in facilitating plant invasions. *Ann Bot* 109(1): 19-45.

Tesitelova T, J Jersakova, M Roy, B Kubatova, J Tesitel, T Urfus, J Suda. 2013. Ploidy-specific symbiotic interactions: Divergence of mycorrhizal fungi between cytotypes of the *Gymnadenia conopsea* group (Orchidaceae). *New Phytol* 199(4): 1022-1033.

Thompson JN, SL Nuismer, K Merg. 2004. Plant polyploidy and the evolutionary ecology of plant/animal interactions. *Biological Journal of the Linnean Society* 82: 511-519.

Thompson JD, KF Merg. 2008. Evolution of polyploidy and the diversification of plant-pollinator interactions. *Ecology* 89(8): 2197-2206.

Thilakarathna RMMS, YA Papadopoulos, SAE Fillmore, B Prithiviraj. 2012. Genotypic differences in root hair deformation and subsequent nodulation for red clover under different additions of starter N fertilization. *Journal of Agronomy and Crop Science* 198(4): 295-303.

Trojak-Goluch A, U Skomra. 2013. Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. *Breed Sci* 63(4): 393-399.

Valladares F, D Sanchez-Gomez, MA Zavala. 2006. Quantitative estimation of phenotypic plasticity: Bridging the gap between the evolutionary concept and its ecological applications. *Journal of Ecology* 94(6): 1103-1116.

Van Drunen WE, BC Husband. 2018. Immediate vs. evolutionary consequences of polyploidy on clonal reproduction in an autopolyploid plant. *Ann Bot* 122(1): 195-205.

Vitousek PM, K Cassman, C Cleveland, T Crews, CB Field, et al. 2002. Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* 57/58: 1-45.

Vitousek PM, DNL Menge, SC Reed, CC Cleveland. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society B* 368: 1-9.

Walsh KB, JK Vessey, DB Layzell. 1987. Carbohydrate supply and N_2 fixation in soybean: The effect of varied daylenth and stem girdling. *Plant Physiology* 85: 137-144.

Wang D, S Yang, F Tang, H Zhu. 2012. Symbiosis specificity in the legume: rhizobial mutualism. *Cell Microbiol* 14(3): 334-342.

Warner DA, GE Edwards. 1993. Effects of polyploidy on photosynthesis. *Photosynthesis Research* 35: 135-147.

Watson RJ, R Heys, T Martin, M Savard. 2001. *Sinorhizobium meliloti* cells require biotin and either cobalt or methionine for growth. *Appl Environ Microbiol* 67(8): 3767-3770.

Wei N, R Cronn, A Liston, TL Ashman. 2019. Functional trait divergence and trait plasticity confer polyploid advantage in heterogeneous environments. *New Phytol* 221(4): 2286-2297.

Weir JB. 1961a. The effect of colchicine and indolyl acetic acid on diploid and tetraploid strains of red and white clovers in aseptic and pot culture. *Plant and Soil* 14: 187-196.

Weir JB. 1961b. A comparison of the nodulation of diploid and tetraploid varieties of red clover inoculated with different rhizobial strains. *Plant and Soil* 14: 85-89.

Weir JB. 1964. The effect of inositol on the growth and nodulation of diploid and tetraploid white clover grown in pot culture. *Plant and Soil* 20: 175-183.

Wendlandt CE, JU Regus, KA Gano-Cohen, AC Hollowell, KW Quides, JY Lyu, JL Sachs. 2019. Host investment into symbiosis varies among genotypes of the legume *Acmispon strigosus*, but host sanctions are uniform. *New Phytol* 221(1): 446-458.

Werner GD, WK Cornwell, JH Cornelissen, ET Kiers. 2015. Evolutionary signals of symbiotic persistence in the legume-rhizobia mutualism. *Proc Natl Acad Sci U S A* 112(33): 10262-10269.

Wood CW, BL Pilkington, P Vaidya, C Biel, JR Stinchcombe. 2018. Genetic conflict with a parasitic nematode disrupts the legume-rhizobia mutualism. *Evol Lett* 2(3): 233-245.

Young ND, F Debelle, GED Oldroyd, R Geurts, SB Cannon, et al. 2011. The *Medicago* genome provides insight into the evolution of rhizobial symbioses. *Nature* 480: 520-524.

Zhan SH, M Drori, EE Goldberg, SP Otto, I Mayrose. 2016. Phylogenetic evidence for cladogenetic polyploidization in land plants. *American Journal of Botany* 103(7): 1252-1258.

Zheng W, P Seguin, MS Beauregard. 2004. Diversity of *Trifolium ambiguum* nodulating rhizobia from the lower Caucasus. *Biology and Fertility of Soils* 40(2), 128-135.