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Opinion

A Resurrected Scenario: Single Gain and Massive Loss of Nitrogen-Fixing Nodulation

Robin van Velzen,¹ Jeff J. Doyle,² and Rene Geurts ^{1,*}

Root nodule endosymbiosis with nitrogen-fixing bacteria provides plants with unlimited access to fixed nitrogen, but at a significant energetic cost. Nodulation is generally considered to have originated in parallel in different lineages, but this hypothesis downplays the genetic complexity of nodulation and requires independent recruitment of many common features across lineages. Recent phylogenomic studies revealed that genes that function in establishing or maintaining nitrogen-fixing nodules are independently lost in non-nodulating relatives of nitrogen-fixing plants. In our opinion, these data are best explained by a scenario of a single gain followed by massively parallel loss of nitrogen-fixing root nodules triggered by events at geological scale.

Nitrogen-Fixing Nodule Endosymbiosis: A Complex Genetic Trait

Nitrogen is a critical limiting element for plant growth. It is predominantly present as atmospheric di-nitrogen gas (N₂), an unsuitable source for plants. Instead, plants rely on reduced nitrogen forms, such as ammonium (NH₄⁺) or nitrate (NO₃[−]) that they generally absorb from soil. Some plants, however, make specialized root organs called nodules where they intracellularly host **diazotrophic** (see [Glossary](#)) bacteria. Legumes (Fabales, Fabaceae) and the non-legume *Parasponia* (Rosales, Cannabaceae) host a polyphyletic group of diazotrophic α - and β -proteobacteria collectively referred to as **rhizobia**. Other nodulating species are known as ‘**actinorhizal**’ because they host diazotrophic filamentous Actinobacteria in the genus *Frankia*. Inside nodule cells, these microsymbionts find appropriate physiological conditions to catalyze the conversion of atmospheric N₂ to NH₄⁺ by the bacterial enzyme complex **nitrogenase** [1]. Fueled by plant photosynthates, such endosymbionts provide these plants with an additional supply of nitrogen.

Nitrogen fixation is an energy-demanding conversion, requiring 16 moles of ATP per mole of N₂ fixed [1]. Nodulating plants tightly regulate nodule numbers through autoregulation [2,3] and abolish nodulation altogether when sufficient nitrogen is available, such as in fertilized agricultural fields [4]. Similarly, nodulation has been found to be limited mainly by photosynthesis in tropical legumes [5]. Thus, it is clear that nodulation confers a fitness advantage only under environmental conditions in which growth is limited by nitrogen and when the benefit conferred by symbiotic nitrogen exceeds the cost of photosynthetic carbon.

Nitrogen-fixing nodules are the result of an intricate exchange of host plant and microsymbiont signals that simultaneously trigger nodule organogenesis and intracellular microbial infection. Within nodule cells, specialized symbiotic membranes allow for the exchange of sugars and ammonia, while defense responses are repressed [6,7]. Genetic dissection of nodulation in the legume models *Medicago truncatula* (medicago) and *Lotus japonicus* (lotus) uncovered more than 40 essential symbiosis genes. These include genes encoding LysM-type receptor kinases such as **NFP/NFR5** that perceive rhizobial lipo-chitooligosaccharides (**LCOs**; known as **Nod**

Highlights

N₂-fixing nodulation symbiosis is a complex and important agronomic trait. It occurs in phylogenetically separated lineages, and its evolution may be explained by two alternative hypotheses: (i) single gain followed by massively parallel loss, or (ii) parallel evolution and fewer losses. The latter hypothesis is widely accepted, but the first hypothesis is supported by recent phylogenomic data.

Molecular and developmental commonalities across distinct lineages support a common origin of nodulation. Moreover, recent comparative genomics studies revealed parallel loss of key nodulation genes in non-nodulating species.

These findings support a single gain of nodulation followed by massively parallel loss in most descendant lineages. Such massive loss may have been triggered by reductions in global atmospheric CO₂ levels.

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factors) and activate a transcriptional network initiating nodule organogenesis, of which **NIN** is a master regulator [6,8,9]. Together with a series of other genes, such as **RPG**, *NIN* is also essential for intracellular infection [8,10]. Considering the developmental and physiological complexity and the number of genes that are indispensable for nodule formation, it is clear that nitrogen-fixing nodulation symbiosis is a functionally and genetically complex trait.

Recent comparative genomic studies have provided insights into the evolution of the nodulation trait [11,12]. Here, we discuss the implications of these new findings and advocate a radical change in the current view on evolution of nitrogen-fixing nodulation.

Phylogenetic Perspective on Nitrogen-Fixing Root Nodules

Because all nodulating plant species occur in the monophyletic group comprising the orders Fabales, Fagales, Cucurbitales, and Rosales, these four orders have been referred to as the **nitrogen-fixing clade** [13] (Figure 1). To account for the observation that nodulating lineages in this clade are interspersed among lineages that do not possess the symbiosis, two general hypotheses have been postulated [13]: (i) a single gain of nodulation in an ancestor of the nitrogen-fixing clade followed by massively parallel loss of this trait in most descendants, or (ii) parallel evolution of nodulation in some descendants and fewer losses. The first hypothesis has been almost universally dismissed, whereas the latter is widely accepted on the basis of two main arguments [14–24]. First, it comprises scenarios that require fewer evolutionary events and that are supported by phylogenetic ancestral state reconstruction studies [14–16,20,22]. Second, there is considerable variation among nodulating lineages in the type of microsymbiont, nodule ontogeny, and physiology [25–28]. For example, legume nodules are all infected by rhizobia and share a unique ‘stem-like’ ontogeny with a peripheral vascular system and a large central zone of infected cells. In contrast, nodules of the non-legume *Parasponia* are infected with the same rhizobial strains but have a ‘lateral root-like’ ontogeny with a central vascular bundle and infected cells in the periphery, similar to actinorhizal nodules [23]. Furthermore, distinct strategies evolved to provide low oxygen pressure to protect nitrogenase from oxidation. For example, most actinorhizal plants rely on mechanisms provided by the *Frankia* microsymbiont. *Frankia* spp. can differentiate into rigid infection structures, known as vesicles, that have a physical oxygen barrier, produce hopanoid-derivatives forming protective lamellar lipid layers, and/or express truncated hemoglobin *HbO* that affect oxygen homeostasis [25,29,30]. In contrast, legumes, *Parasponia*, and the actinorhizal plant *Casuarina* (Fagales, Casuarinaceae, *Casuarina glauca*) evolved a plant-encoded mechanism to control oxygen homeostasis. This mechanism relies on adaptation of either class I (*Parasponia*) or class II hemoglobin genes (legumes and *Casuarina*) to function as oxygen transporters in infected nodule cells [26–28]. Taken together, these and other differences are often regarded as evidence that not all nodules are homologous and that nodulation therefore arose independently in different lineages [14,15,21,23,24,31].

Despite the general acceptance of the parallel evolution hypothesis, there are several issues that challenge this view. First, as parallel evolution can in principle occur in any plant, it does not explain why all nodulating species are confined to the nitrogen-fixing clade. To resolve this apparent conflict, it is commonly assumed that a genetic predisposition event leading to a precursor state for nodulation evolved in a common ancestor of the nitrogen-fixing clade around 110 million years ago (Mya). This precursor state facilitated the parallel evolution of nodulation in different descendant lineages [13,22]. Despite decades of research, this hypothetical precursor state for nodulation has remained elusive and lacks empirical support [14,16,19,22]. In our opinion, this renders the supposed predisposition a problematic explanation.

Glossary

Actinorhizal plants: plants that make nodules hosting a symbiosis with *Frankia* bacteria.

Diazotroph: a microorganism that is able to grow without external sources of fixed nitrogen by using nitrogenase.

Frankia: a genus of nitrogen-fixing, Gram-positive filamentous actinomycete bacteria that can engage a symbiosis with actinorhizal plants.

LCOs: lipo-chitooligosaccharides that act as symbiotic signals, that is, as Myc factors when produced by endomycorrhizal fungi or as Nod factors produced by rhizobia and *Frankia*.

NFP/NFR5: NOD FACTOR PERCEPTION/NOD FACTOR RECEPTOR 5 lysin motif (LysM) containing receptor kinase that is essential for Nod factor signaling in legumes.

NIN: NODULE INCEPTION transcription factor that is essential for nodule formation in legumes.

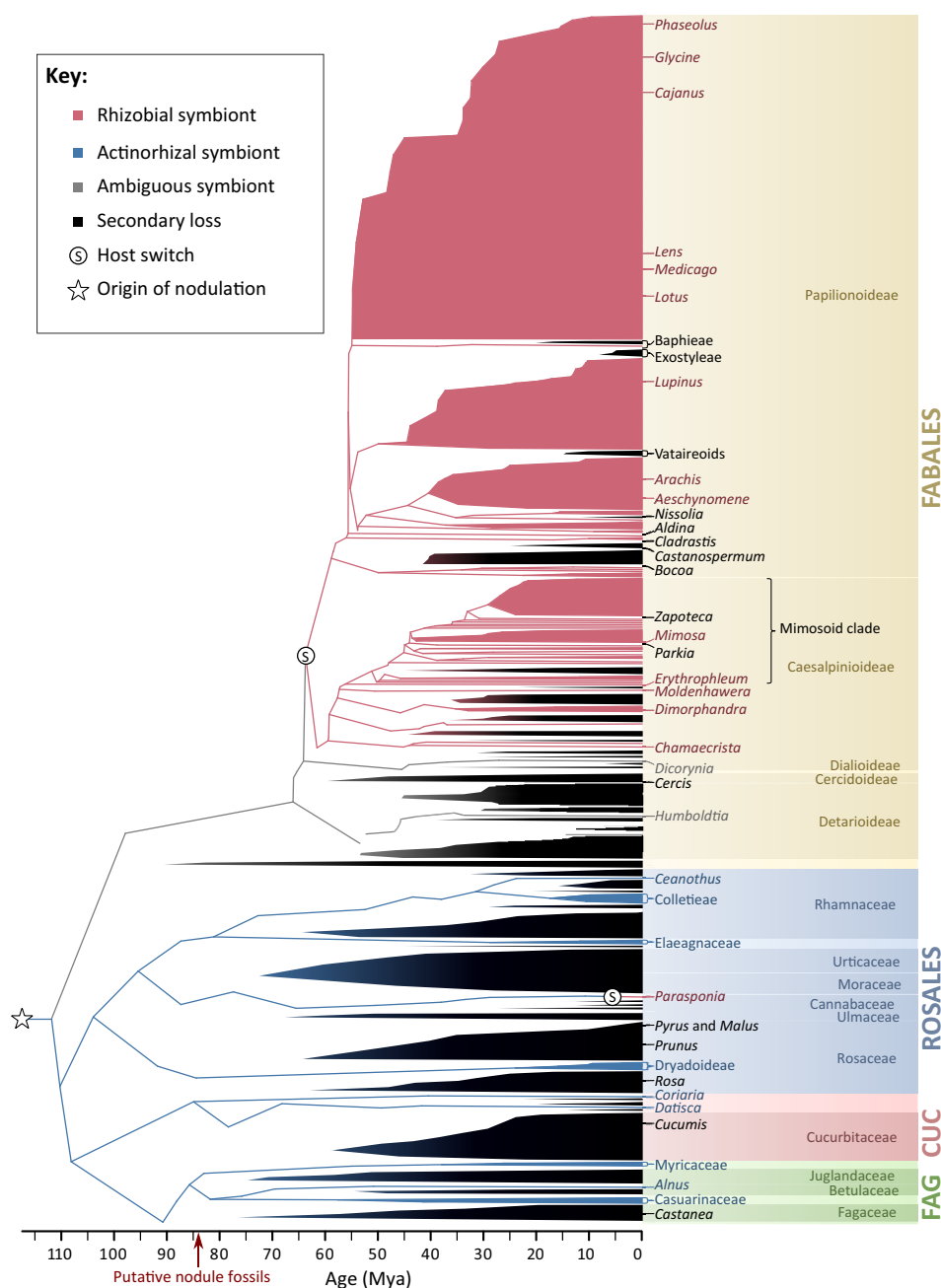
Nitrogenase: bacterial enzyme complex responsible for catalyzing the reduction of nitrogen (N_2) to ammonia (NH_3).

Nitrogen-fixing clade: a monophyletic group comprising the orders Fabales, Fagales, Cucurbitales, and Rosales and including all plant lineages with nitrogen-fixing root nodules.

Nod factors: symbiotic signaling LCOs produced by rhizobia and some *Frankia* bacteria that have nodulation genes.

Rhizobia: a polyphyletic group of diazotrophic Gram-negative rod-like α - and β -proteobacteria that can engage a symbiosis with nodulating legumes (Fabales) and *Parasponia* (Rosales, Cannabaceae).

RPG: RHIZOBIUM-DIRECTED POLAR GROWTH, a long coiled-coil protein that is essential for rhizobial infection in legumes.



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Figure 1. Single Gain and Massive Loss Hypothesis. Representation of the phylogenetic distribution of nodulating and non-nodulating plants in the nitrogen-fixing clade under the single gain and massive loss hypothesis. Phylogenetic tree of the nitrogen-fixing clade based on [15,66,67]; distribution of nodulating and non-nodulating plants based on [63]. Branch colors indicate putative host status and type of microsymbiont; color gradients indicate uncertainty in the phylogenetic placement of secondary losses. The legumes *Dicorynia* (Dialioideae) and *Humboldtia* (Detarioideae) have been reported to nodulate and may therefore represent additional 'relict' nodulators [68,69]. The putative host switch depicted in an ancestral legume is a minimum age estimate as it may have occurred as early as in an ancestor of all Fabales; the putative host switch depicted in an ancestral *Parasponia* is based on genetic changes supporting adaptations in hemoglobin required for oxygen transport in rhizobium symbiosis [11,27]. CUC, Cucurbitales; FAG, Fagales; Mya, million years ago.

Second, scenarios with many parallel origins optimized on phylogenetic trees are in conflict with developmental and structural data [14]. For example, within legumes as many as four independent origins are reconstructed, even though all legumes share the same stem-like ontogeny, which is consistent with nodule homology [18,19,22,23]. Similarly, given their ~85-million-year divergence, the two nodulating lineages in Cucurbitales *Coriaria* (Coriariaceae) and *Datisca* (Datiscaceae) are consistently reconstructed to represent independent origins, even though their nodule anatomy is very similar [14,15,22,23]. Also, the distinctive root-hair infection process shared by several species representing all three nodulating Fagales lineages suggests that their common ancestor had a similar characteristic [14,23,32]. Consequently, despite the evident phenotypic divergence between the main nodulating lineages that separated more than 100 Mya, developmental and structural data suggest far fewer and much older origins than those predicted based on phylogenetic considerations.

Third, phylogenetic ancestral state reconstructions generally imply *a priori* equal weight for gains and losses. However, considering the complexity of nitrogen-fixing nodules, it has often been acknowledged that an evolutionary gain of this trait is genetically much more difficult than a loss [24,31,33]. For example, single inactivating mutations in *NFP/NFR5*, *NIN*, and *RPG* each result in loss of functional nodules [10,34,35]. Thus, it would be more realistic to incorporate higher rates of losses versus gains when modeling the evolution of nodulation.

A Single Gain of Nodulation

Based on our current understanding of the phylogeny of the nitrogen-fixing clade, the hypothesis of a single gain of nodulation requires at least 7 losses in Fagales, 5 in Cucurbitales, 17 in Rosales, and 36 in Fabales (Figure 1). Assuming a single common nodulating ancestor readily explains why all nodulating species occur in one clade and, consequently, eliminates the need to hypothesize the highly speculative predisposition for nodulation, replacing it with the evolution of nodulation itself. Clearly, accurate reconstruction of evolutionary events that occurred ~110 Mya is difficult. Nevertheless, there are two main lines of evidence suggesting that the origin of nodulation is much older than generally assumed.

The first line of evidence comes from structural and developmental similarities across nodules from distantly related plants. *Parasponia* and some legume species host rhizobia in fixation threads, which show strong resemblance with infection structures found in actinorhizal nodules [36,37]. Fixation threads have been considered ‘primitive’, suggesting a common ancestral state [36]. Furthermore, the ontogeny of all nodules from Cucurbitales, Fagales, and Rosales species is very similar [23,24,28]. Given that these three orders form a clade (Figure 1), we believe that these similarities may be more readily explained by homology than by parallel or convergent similarity. The latter requires that developmental constraints favoring the evolution of a certain type of nodules arose in the common ancestor of this clade. This echoes the predisposition for nodulation in the entire nitrogen-fixing clade required by multiple gain scenarios, and for which there is no evidence. We therefore argue that these similarities have a stronger phylogenetic signal than what may be expected based on parallel evolution.

The second line of evidence can be found in the commonalities in symbiotic gene function between diverse nodulating lineages. Assuming that all nodules arose from a single gain, it can be predicted that the genes recruited for nodulation are orthologous [24]. For example, expression of the *NIN* transcription factor is essential to induce nodule organogenesis in legumes as well as in the actinorhizal plant *Casuarina* [9,35,38]. Moreover, *Casuarina NIN* can functionally complement a legume *nin* knockout mutant [9]. A recent study comparing genes with nodule-enhanced expression from medicago and *Parasponia* revealed a set of 290

putatively orthologous genes that are used in both species [11]. These include key genes that are specifically required for nodulation such as *NIN* and *RPG*. Similarly, comparative transcriptomic studies revealed extensive commonalities in nodule-expressed genes between legumes and the actinorhizal plants *Casuarina* and *Alnus* (Fagales), although orthology was not assessed rigorously for these genes [17]. Such commonalities have been interpreted as cases of ‘deep homology’ or as leads to the supposed predisposition [17–19,39]. We rather advocate the simpler interpretation that these commonalities are the result of a single recruitment of all of the components of a homologous nodulation symbiosis [14,24]. In our opinion, these structural, molecular, and genetic data together suggest that the various nodulation phenotypes have a single origin.

The fossil record would provide clear evidence for the single gain hypothesis, if definitive fossilized roots bearing nodules were found that pre-dated ancestors in which independent origins of nodulation are hypothesized under the parallel origins hypothesis. Several fossilized root structures that strongly resemble multi-lobed nodules were dated at ~84 Mya [40]. Given that nodule fossils are rare/absent even from more recent sediments in diverse and widespread lineages such as legumes, this date should be considered as a minimum bound for the age of nodulation if the fossils are truly nodules. Notably, it is much older than the crown age of any nodulating lineages (Figure 1) [15,22,41]. For example, legumes are the oldest and most diverse nodulating lineage, and abundant in the fossil record; but the earliest fossils that can be definitively assigned to the legume family appeared in the late Paleocene (~65 Mya) [42]. Because nitrogen-fixing symbiotic nodules can be very difficult to distinguish from other root structures such as nematode root galls and ectomycorrhizal nodules [43], more and better fossil evidence are needed to confirm an early origin of nodulation (see Outstanding Questions).

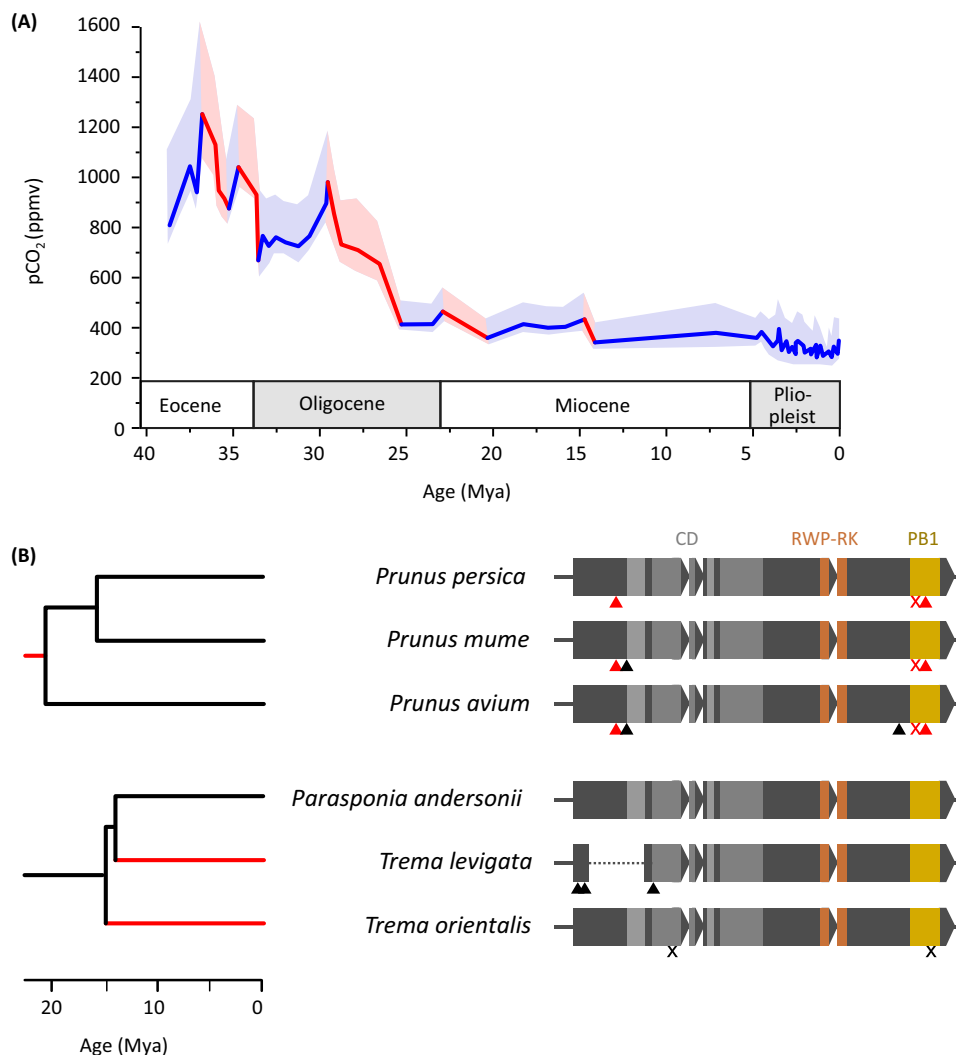
Parallel Loss of Symbiosis Genes in Non-nodulating Species

Given the phylogenetic framework, a single gain hypothesis implies a massively parallel loss of the nodulation trait within the nitrogen-fixing clade [13,18,24,33]. It was previously hypothesized that the genomes of non-nodulating taxa could harbor ‘fossil’ evidence of nodulation, such as pseudogenization of genes functioning only in nodulation similarly as was found for loss of arbuscular mycorrhizal symbiosis [19,44]. Two recent phylogenomic studies found exactly that [11,12]. The first study compared the genome of *Parasponia* with its closest non-nodulating relative, *Trema*, and revealed that three genes that are essential for establishing nitrogen-fixing root nodules in legumes and actinorhizal plants, namely, *NFP/NFR5*, *RPG*, and *NIN*, are lost or pseudogenized in the *Trema* genome as well as in those of relatively distantly related non-nodulating Rosales species [11]. The second study compared genomes of nodulating and non-nodulating plants across the nitrogen-fixing clade and revealed a similar pattern of gene loss for *NIN* and *RPG* [12]. Orthologs of these genes occur outside the nitrogen-fixing clade where they must have non-symbiotic functions that remain unknown. Nevertheless, given our current understanding, within the nitrogen-fixing clade these genes are exclusively associated with nodulation, strongly suggesting that loss of these genes resulted from the loss of the nodulation trait. Consequently, absence of nodulation in these sampled species across the nitrogen-fixing clade likely represents a secondary loss rather than an ancestral state.

Loss of Nodulation and Decreasing Global Atmospheric CO₂

Nodulation is an example of phenotypic plasticity in plants [45], turned on and off in nodulating species depending on the benefit provided by symbiotic bacteria relative to the cost of their maintenance. Although the relationship between phenotypic plasticity and adaptive evolution is

complex [46], it seems reasonable to predict that, should abiotic and biotic conditions consistently disadvantage symbiosis in any nodulating plant species over a long period, the ability to nodulate would be lost. This is because, when nodulation is turned off, genes dedicated solely to the symbiosis, thus freed from purifying selection, would accumulate



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Figure 2. Decrease of Atmospheric CO_2 and Pseudogenization of *NIN* in Non-nodulating *Prunus* and *Trema* Species. (A) Global atmospheric CO_2 levels decreased over a period of 40 million years [56]. Significant drops are marked red and are most prominent in the Eocene–Oligocene transition ~34 million years ago (Mya), through Late Oligocene ~29–23 Mya, and the Middle Miocene ~14 Mya. Shaded areas indicate confidence intervals based on [56]. $p\text{CO}_2$, partial pressure of carbon dioxide in parts per million volume; Plio-Pleist, Plio-Pleistocene. (B) Preliminary data on timing of parallel loss events based on mutations in the *NIN* gene. Shared loss-of-function mutations (marked in red) in *NIN* from *Prunus* species indicate that the associated loss event occurred before their divergence ~21 Mya [70–73]. Independent loss-of-function mutations in *NIN* from two *Trema* species indicate that the associated loss events occurred after their divergence ~17 Mya [11,15]. Phylogenetic branches along which putative loss events may have occurred are in red; *NIN* exon structure given in arrows (introns not in scale). CD, four conserved domains in gray; RWP-RK, conserved amino acid domain in orange; PB1, Phox and Bem1 domain in yellow. Triangles indicate frameshift mutations; X's indicate nonsense mutations resulting in premature in-frame stop codons.

inactivating mutations through neutral processes and become pseudogenes. The major challenge for postulating many independent losses is therefore to identify a common factor that would cause nodulation, once an asset to plant growth, to become a liability in numerous unrelated nodulating plant lineages. Increased availability of fixed nitrogen, or development of ‘cheating’ bacteria that enter root nodules, but do not deliver nitrogen, have been postulated as possible factors driving loss of nodulation [12]. However, such scenarios would most probably act only locally, whereas widespread parallel loss is explained best by a factor acting globally and at geological timescales. One such factor is atmospheric CO₂, which is the basis for photosynthesis. In contrast to other primary growth factors such as light, water, and nutrient availability, CO₂ is relatively evenly distributed throughout the Earth’s atmosphere, rendering it an inescapable selective force [47]. Changes in atmospheric CO₂ levels have been postulated to drive the evolution of plant anatomy and physiology, such as C4 photosynthesis [48–51], stomatal density [52,53], and chemical defense [54,55]. Global atmospheric CO₂ levels have generally been decreasing in the last 100 million years, since the time when nodulation is hypothesized to have arisen under the single origin scenario. During this general decrease, there have been periods of particularly steep decline during the Eocene–Oligocene transition ~34 Mya, the Late Oligocene ~29–23 Mya, and the Middle Miocene climate transition ~14 Mya (Figure 2A) [56]. Consequently, given the high photosynthetic demands of symbiotic nitrogen fixation [1,5] and the variability in photosynthetic efficiency of different species, CO₂ could have become a limiting factor for plant growth for different lineages during these climatic transitions [51,53]. Decreasing CO₂ would account not only for the loss of nodulation in diverse lineages but also for differences in the timing of loss, from very recent (e.g., *Trema*) to more ancient (e.g., *Prunus*) (Figure 2B).

Concluding Remarks and Future Perspectives

Based on the single gain and massive loss hypothesis, we predict loss of nodulation-specific genes in most non-nodulating species in the nitrogen-fixing clade. Testing this prediction requires a more comprehensive genome-scale analysis of additional non-nodulating lineages from the nitrogen-fixing clade as well as from related orders. Nevertheless, it is already clear that the patterns of maintenance and loss of nodulation genes are not consistent. For example, the non-nodulating plant jujube (*Ziziphus jujuba*) retains *NIN* [11,57], whereas the nodulating legume *Arachis ipaensis* lost *RPG* [12,58]. This suggests that nodulation genes that are maintained in non-nodulating species encode unknown non-symbiotic functions, whereas such genes can become dispensable in some nodulating lineages. In-depth understanding of the molecular functioning of these genes is required to obtain insights in such deviant evolutionary trajectories.

Assuming a single gain of nodulation, at least two switches between *Frankia* and rhizobial microsymbiotic partners must have occurred. We hypothesize that nodulation first evolved with an LCO-producing *Frankia* species rather than rhizobia, since *Frankia* has the intrinsic characteristics to protect nitrogenase from oxidation [25]. Subsequently, different proteobacteria obtained LCO biosynthesis genes by horizontal gene transfer [59–61], allowing them to compete with *Frankia* and independently infect ancestors of legumes and of *Parasponia* (Figure 1), where *Burkholderia* spp. (β-proteobacteria) are considered to be more ancient microsymbionts than Rhizobiales (α-proteobacteria) [62]. Microsymbiont switches between *Frankia* and rhizobia almost certainly required genetic adaptations of the putative host plant. One such adaptation is the recruitment of hemoglobin genes to control oxygen homeostasis in the nodule, as rhizobia lack the oxygen-regulating features of *Frankia* [11,27]. It can be anticipated that several more adaptations, for example, in resistance responses, may have been essential.

Outstanding Questions

When, and how frequent, did parallel loss of nodulation occur?

Can nodulation be restored in non-nodulating plants by reintroducing lost symbiosis genes?

What was the ancestral nodule micro-symbiont and in which lineages did host switches occur?

Can fossil evidence be found to substantiate: (i) occurrence of the nodulation trait >100 Mya, and (ii) subsequent massively parallel loss of this trait?

Under a massive loss scenario, what factor(s) determined that the currently symbiotic lineages retained the nodulation trait?

What are the functions of symbiosis genes such as *NIN* and *RPG* in non-nodulating species both inside and outside the nitrogen-fixing clade?

Under a single gain and massively parallel loss scenario, the origin of nitrogen-fixing root nodulation should be further re-evaluated. Given massively parallel loss, the origin may have been even earlier than the nitrogen-fixing clade. Some indirect evidence supporting this hypothesis can be found in related orders within the fabid clade. First, there are unconfirmed studies on nodulating Zygo-phyloideae species (Zygophyllales, Zygophyllaceae) [63]. Second, stable isotope measurements of fossil wood from the Oligocene (23–34 Mya) suggested nitrogen fixation in *Magnistipula* (Malpighiales, Chrysobalanaceae) [50]. Experimental, phylogenomic, and fossil evidence is essential to assess whether these cases represent(ed) cases of nitrogen-fixing nodulation.

Based on our assumption that loss of nodulation genes is directly related to loss of nodulation, we predict that functional ancestral variants of these nodulation genes confer symbiotic potential to plants in the nitrogen-fixing clade. This can be tested by re-introducing such genes in non-nodulating plants and measuring symbiotic responses. Given their putatively recent loss of nodulation genes, close relationship with nodulating *Parasponia* [11], and availability of transformation protocols [64,65], *Trema* species are ideal candidates for such a re-engineering approach.

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