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Short Communication

Phylogenetic evidence of the transfer of *nodZ* and *nolL* genes from *Bradyrhizobium* to other rhizobia

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ABSTRACT

Nod factor modifications mediated by *nodZ* and *nolL* gene products (fucosylation and acetylation of fucose residues, respectively) were probably later acquisitions in the nodulation process. Novel phylogenetic analyses suggest that *nodZ* and *nolL* genes were transferred from *Bradyrhizobium* to other nodule bacteria. These bradyrhizobial genes are highly diverse while rhizobial, sinorhizobial and mesorhizobial *nodZ* and *nolL* genes are represented by few branches among those from bradyrhizobia. These genes in novel rhizobial backgrounds may have favored efficient nodulation in legume hosts commonly associated with *Bradyrhizobium* strains.

1. Introduction

Few legumes may form nodules with rhizobia that do not produce Nod factors (Giraud et al., 2007) but in most legumes nodulation is dependent on Nod factors that are key molecules in rhizobia-plant interactions (Dénarié et al., 1996). Nod factors are produced by enzymes coded by *nod*, *noe* or *nol* genes (collectively referred here as *nod* genes) that are inducible by plant exudates such as flavonoids or other unrelated molecules (Hassan and Mathesius, 2012).

Some of the modifications occurring in Nod factors are sulfation, methylation, acetylation, carbamoylation, fucosylation, arabinosylation (Debellé et al., 1986; Horvath et al., 1986; Carlson et al., 1994; Stepkowski et al., 2003, 2005, 2007; D'Haeze et al., 1999; Quinto et al., 1997). Some of the genes responsible for such modifications have been designated as host specifity genes and some of them are patchily distributed among *Sinorhizobium* (officially called *Ensifer*), *Rhizobium* (Martinez et al., 1995) or *Bradyrhizobium* (Steenkamp et al., 2008; Stepkowski et al., 2003, 2005, 2007) strains. Both fucosylation and sulfation occur alternatively at the same position of Nod factors and maybe fulfill the same function. Sulfate seems to be involved in protecting Nod factors from chitinase degradation (Staehelin et al., 1994). A relationship of *nod* genes or Nod factor structure and specificity is however not direct (Perret et al., 2000; López-Lara et al., 1996) probably because other bacterial systems such as type III secretion systems or different exopolysaccharides contribute to host specificity (Djordjevic et al., 1987; Marie et al., 2001; Jones, 2012).

In rhizobia, incongruent phylogenies of symbiotic and core genes have been commonly observed (reviewed in Rogel et al., 2011 and in Martínez-Romero, 2009) and are explained by the lateral transfer of symbiotic genes among rhizobia, in relation to adaptation to different hosts (Rogel et al., 2011; Lindström et al., 2010). Nodulation genes are found in symbiotic plasmids or islands that may be mobilized among rhizobia (Rogel et al., 2001; Sullivan and Ronson, 1998). The existence of beta-rhizobia is explained by an ancient transfer event of symbiosis genes from alpha-rhizobia (Chen et al., 2003; Bontemps et al., 2010). Similarly, it is speculated that *nod* genes were transferred to *Azorhizobium*, an epiphytic bacterium (Lee et al., 2008), or to *Methylobacterium* (Jourand et al., 2004), *Devosia* (Rivas et al., 2002), *Phyllobacterium* (Valverde et al., 2005), and from *Burkholderia* to *Cupriavidus* (Andam et al., 2007).

Azorhizobium caulinodans Nod factors are fucosylated (Mergaert et al., 1996). In *Bradyrhizobium japonicum* fucosylation of Nod factors by *nodZ* (Sanjuan et al., 1992) has been linked to nodulation of siratro (*Macroptilium atropurpureum*) and *Vigna umbellata* (Cohn et al., 1999) but *nodZ* mutants were still capable of nodulating soybean (Stacey et al., 1994). *Sinorhizobium fredii* mutants in *nodZ* genes have decreased competitiveness to nodulate soybean (Lamrabet et al., 1999). A NGR234 *nodZ* mutant does not nodulate *Pachyrhizus tuberosus* (Quesada-Vincens et al., 1997). In *Mesorhizobium*

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loti nodZ mutants did not form nodules in *Lotus filicaulis* (Rodpothong et al., 2009) not in *L. pedunculatus* (Scott et al., 1996), with conflicting results on nodulation of *L. corniculatus* (Scott et al., 1996; Rodpothong et al., 2009). An extended capacity to nodulate novel hosts such as siratro and cowpea or *Lotus* was promoted by transferring *nodZ* genes to *R. leguminosarum* sv. viciae (López-Lara et al., 1996; Pacios Bras et al., 2000). In *R. etli* and *R. phaseoli nodZ* genes are found in relation to *Phaseolus vulgaris* nodulation and fucosylation was a preferred Nod factor modification for the cultivar tested (Laeremans et al., 1999). However we found *nodZ* pseudogenes in some *R. phaseoli* strains such as CIAT 652, and in *R. popolucense* Pop5 (new taxa submitted).

2. Materials and methods

Bradyrhizobium strains previously isolated from *Phaseolus lunatus* (Ormeño-Orrillo et al., 2006), *Lupinus mariae-josephae* (Durán et al., 2013), *Pachyrhizus erosus* (Ramírez-Bahena et al., 2009) and *Lablab purpureus* (Chang et al., 2011) were used. Additionally, strains isolated in this study from *Phaseolus microcarpus* and *Phaseolus leptostachyus* were also analyzed (Supplementary Tables S1 and S2). Fragments of the *nodZ* and *nolL* genes were PCR amplified using primers shown in Supplementary Table S3 and Sanger sequenced using methods described by Moulin et al. (2004) and Stepkowski et al. (2005), respectively, or retrieved from whole genome sequences obtained by us (Durán et al., 2013; Servín et al. unpublished). All *nodZ* and *nolL* gene sequences available in the Genbank database were retrieved including those of a *Phaseolus albescens* symbiont recently reported by our group (Servín-Garcidueñas et al., 2012).

Sequences were aligned by using Muscle 3.8.31 (Edgar, 2004). Alignment lengths after gap removal were 426 and 655 characters for the *nodZ* and *nolL* gene sets, respectively. jModelTest (Posada, 2008) was used to find the model of evolution that best fit the data for subsequent phylogenetic analyses using the Akaike Information Criterion (Posada and Buckley, 2004). The models selected were TPM3uf + I + G and GTR + I + G for *nodZ* and *nolL* sets, respectively. All three codon positions were used as no substitution saturation was found in the third codon position of any gene ($Iss < Iss_c$, $P < 0.001$) (Xia et al., 2003). Maximum likelihood trees were generated with PhyML (Guindon et al., 2010) with tree node support evaluated by bootstrap analysis based on 1000 pseudoreplicate datasets. Phylogenetic relationships were also assessed by Bayesian inference using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Analyses were initiated with random starting trees, run for 2,000,000 generations and three separate analyses were executed. Markov chains were sampled every 100 generations. We discarded 25% of trees as "burn in".

The *nodZ* and *nolL* gene sequences determined in this study were deposited in the GenBank database under accession numbers KC526990–KC527000 and KC527001–KC527013, respectively.

3. Results and discussion

nodZ bradyrhizobial genes are highly diverse while rhizobial, sinorhizobial and mesorhizobial *nodZ* genes are represented by a few branches related to bradyrhizobial clade IV and VII *nodZ* genes (Fig. 1A). Clade IV includes bradyrhizobial isolates LMTR13 and LMTR21 isolated from *P. lunatus* in Peru (Ormeño-Orrillo et al., 2006), *L. mariae-josephae* (Lmj) bradyrhizobia obtained from alka-

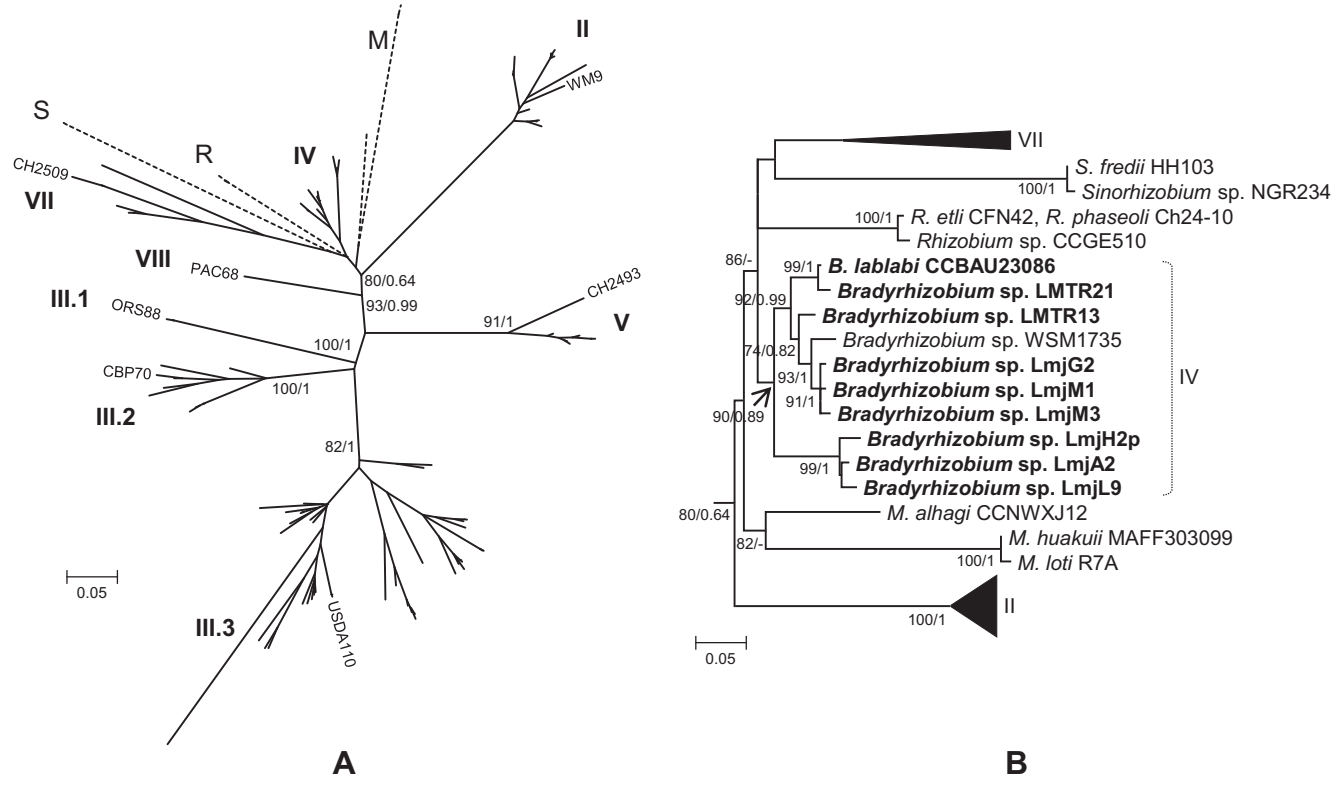

Fig. 1. (**A**) Maximum likelihood (ML) phylogeny of *nodZ* gene sequences. Roman numerals indicate bradyrhizobial *nodZ* clades as defined by Steenkamp et al. (2008) except clade VIII which comprises the gene of *B. jicamae* PAC68 obtained in this study. A representative strain is shown in each clade. R, *Rhizobium*; S, *Sinorhizobium*; M. *Mesorhizobium*. (**B**) Amplification of subtree including clades II, IV and VII. Sequences determined in this study are shown in bold. A similar tree was obtained by Bayesian inference (BI). Bootstrap supports values higher than 70% for the ML analysis as well as Bayesian posterior probabilities are indicated at tree nodes in the order ML/BI. Tree node support is indicated only for major clades in (A).

line soils in Spain (Durán et al., 2013), Chinese strain CCBAU 23086 from *Lablab purpureus* (Chang et al., 2011), and Australian isolate WSM1735 isolated from *Rhynchosia minima* (Stepkowski et al., 2005) (Fig. 1B). Clade VII comprises sequences from bradyrhizobia isolated from tropical legumes in America (Steenkamp et al., 2008; López-López et al., 2013). The *nodZ* phylogeny suggested that this gene was transferred from *Bradyrhizobium* to other rhizobia. In contrast, phylogeny of other *nod* genes like *nodA*, shows that *Rhizobium*, *Sinorhizobium* or *Mesorhizobium* genes are clearly separated from those of bradyrhizobia (Stepkowski et al., 2007; Martínez-Romero et al., 2010; Menna and Hungria, 2011). Interestingly, *nodZ* genes of symbiovar phaseoli *Rhizobium* strains CFN 42 and Ch24-10 isolated from *P. vulgaris* and of *Rhizobium* sp. CCGE510 isolated from *Phaseolus albescens* were related to clade IV *nodZ* that includes genes from *Phaseolus* bradyrhizobia (Fig. 1B). In most *Rhizobium* strains, *nodZ* genes are located in the symbiotic plasmids in close vicinity to a remnant of an IS21 transposase gene. Intriguingly, a remnant of an IS21 is also present close to *nodZ* in clade IV *P. lunatus* strain LMTR13 (our own unpublished data). Transfer of symbiotic genes was suggested to occur between bradyrhizobial strains based on phylogenetic analyses (Moulin et al., 2004) but there had not been any suggestion that there has been a transfer of *nodZ* genes from bradyrhizobia to *Rhizobium*. Previously it was suggested that *Bradyrhizobium* symbiotic genes were transferred to a Brazilian *Sinorhizobium* strain (Barcellos et al., 2007) but this looks like a recent event in view of sequence similarity. Remarkably, phylogenies of *ackA* and *pta* genes (Fournier and Gogarten, 2008) that served as the basis to recognize the lateral transfer of acetoclastic methanogenesis genes from clostridium to methanosarcinales resemble *nodZ* gene phylogenies (Fig. 1A) as methanosarcinales are a single branch among diverse clostridial sequences.

In *Sinorhizobium* NGR234 *nodZ* gene is part of *hsn* gene cluster 1 that includes *noeL* and *nolK* as part of the same operon neighbor to *noeK*, *noeJ* and *nodD1* (Freiberg et al., 1997). This gene organization, including *nodZ*, *noeL* and *nolK* is similar to that found in *Mesorhizobium loti* and both, sinorhizobia and mesorhizobia, are also related in *nodZ* gene phylogenies, such gene organization is not observed in the symbiovar phaseoli plasmids (not shown). *nodZ* gene evolution could have taken place around 60 million years ago maybe driven by fungal proliferation (Vajda and McLoughlin, 2004), if Nod factor modifications are related to chitinase defense (Staehelin et al., 1994; Stacey, 1995) that acts against fungi. Nod factor modifications could be an example of co-evolution of rhizo-

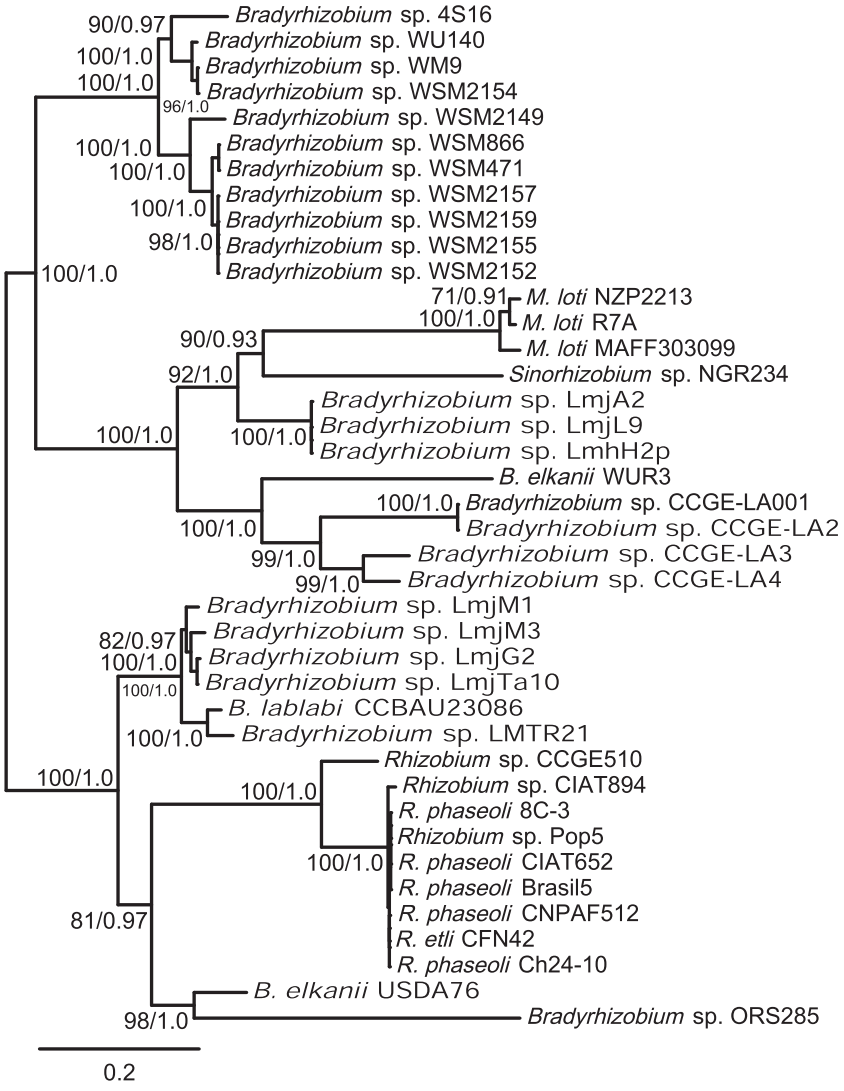

Fig. 2. Maximum likelihood (ML) phylogeny of *nolL* gene sequences. R, *Rhizobium*; S, *Sinorhizobium*; M. *Mesorhizobium*; B, *Bradyrhizobium*. Sequences determined in this study are shown in bold. A similar tree was obtained by Bayesian inference (BI). Bootstrap supports values higher than 70% for the ML analysis as well as Bayesian posterior probabilities are indicated at tree nodes in the order ML/BI. *nolL* pseudogene sequences of CIAT652 and Brasil5 were included in the analysis.

bia and their host-legume in response to fungal pathogens. *nodZ* gene transfer from bradyrhizobia could have occurred to an ancestor of *Rhizobium* and *Sinorhizobium* (around 50–45 million years ago) or alternatively, more recently to any rhizobial species and then transferred among bacteria followed by gene loss and rearrangements in some rhizobial lineages. An ancient transfer of *nod* genes has been considered to have occurred from alpha to beta-rhizobia (Chen et al., 2003; Bontemps et al., 2010) and later from *Burkholderia* to *Cupriavidus* (Andam et al., 2007). Gene rearrangements and losses account for the present organization of *nodZ* in different bacteria. *Sinorhizobium*, *Rhizobium* and *Agrobacterium* are closely related and the phylogenetic analysis of common *nod* genes showing intermixed *Rhizobium* and *Sinorhizobium nod* genes are suggestive of their lateral transfer between these genera (reviewed in Martínez-Romero, 2009).

A further Nod factor modification found in some rhizobia is mediated by *NolL* that acetylates the fucose residue (Berck et al., 1999). This Nod factor decoration seems to be related to nodulation efficiency as an *R. etli nolL* mutant formed a reduced number of nodules in comparison to the wild type on *P. vulgaris* and on *Vigna umbellata* (Corvera et al., 1999). Although relatively few sequences are available, *nolL* phylogeny also showed that *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* sequences are found within those of *Bradyrhizobium* supporting the transfer of this gene from bradyrhizobia (Fig. 2 and Supplementary Fig. 1). All the symbiovar phaseoli *R. etli* and *R. phaseoli* sequences were found clustered, irrespective of the different geographical origin of the strains, and they were highly related to the *nolL* sequence of *Rhizobium* sp. CCGE510. Farther, but still related, are *nolL* sequence from *Bradyrhizobium* sp. LMTR21 isolated from cultivated *P. lunatus*. *Sinorhizobium* and *Mesorhizobium* sequences were related to those of *Bradyrhizobium* strains isolated from wild *P. leptostachyus* (CCGE-LA001, CCGE-LA2) and *P. microcarpus* (CCGE-LA3, CCGE-LA4), and of some *Lupinus mariae-josephae* (Lmj) bradyrhizobia (Fig. 2). Phylogenies of *nodZ* and *nolL* do not seem to be congruent (Supplementary Fig. S1), indicating that perhaps they were independent acquisition from bradyrhizobia. It is worth noting that *nolL* and *nodZ* genes are not contiguous in bradyrhizobia.

4. Conclusions

The two Nod factor modification genes analyzed here seem to have initially developed in the *Bradyrhizobium* genus and independently transferred to a few different fast-growing rhizobial genera. In comparison to the common *nodABC* genes, *nodZ* and *nolL* were probably later acquisitions in the nodulation process. In fast-growing rhizobia these genes may have favored efficient nodulation in legume hosts normally associated with *Bradyrhizobium* strains, like *Glycine* and *Pachyrhizus* for *nodZ*-bearing sinorhizobia. Analogously, transfer in the laboratory of *nodZ* genes to *R. leguminosarum* allowed the transconjugants to form nodules in soybean (López-Lara et al., 1996). Some *Lotus* species nodulating with mesorhizobia which possess *nodZ* genes also establish efficient symbiosis with *Bradyrhizobium* spp. (Vance et al., 1987). Finally, although *Rhizobium* strains are the preferred symbiont of *P. vulgaris*, it has been suggested that nodulation with *Bradyrhizobium* is the ancestral condition of the *Phaseolus* genus (Parker, 2002).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.03.003.

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