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Diversification of Lupine *Bradyrhizobium* Strains: Evidence from Nodulation Gene Trees[▽]†

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Bradyrhizobium **strains isolated in Europe from Genisteae and serradella legumes form a distinct lineage, designated clade II, on nodulation gene trees. Clade II bradyrhizobia appear to prevail also in the soils of Western Australia and South Africa following probably accidental introduction with seeds of their lupine and serradella hosts. Given this potential for dispersal, we investigated *Bradyrhizobium* isolates originating from a range of native New World lupines, based on phylogenetic analyses of nodulation (*nodA*, *nodZ*, *noeI*) and housekeeping (*atpD*, *dnaK*, *glnII*, *recA*) genes. The housekeeping gene trees revealed considerable diversity among lupine bradyrhizobia, with most isolates placed in the *Bradyrhizobium japonicum* lineage, while some European strains were closely related to *Bradyrhizobium canariense*. The *nodA* gene tree resolved seven strongly supported groups (clades I to VII) that correlated with strain geographical origins and to some extent with major *Lupinus* clades. All European strains were placed in clade II, whereas only a minority of New World strains was placed in this clade. This work, as well as our previous studies, suggests that clade II diversified predominately in the Old World, possibly in the Mediterranean. Most New World isolates formed subclade III.2, nested in a large "pantropical" clade III, which appears to be New World in origin, although it also includes strains originating from nonlupine legumes. Trees generated using *nodZ* and *noeI* gene sequences accorded well with the *nodA* tree, but evidence is presented that the *noeI* gene may not be required for nodulation of lupine and that loss of this gene is occurring.**

The papilionoid legume genus *Lupinus* comprises ca. 275 species of annual and perennial herbs and shrubs with an amphi-Atlantic distribution. The majority of species are distributed in the New World, with ca. 100 species in the western part of North America and ca. 85 species in the Andes. Only 15 species are found in the Old World, mainly surrounding the Mediterranean (15). Lupines, in part due to their highly effective nitrogen-fixing symbiosis with root nodule bacteria, have been grown since antiquity as a green manure and are an important pulse crop. Their adaptation to nutrient-poor, often acid soils and arid climates means that lupines can be grown in areas where cultivation of more demanding crops, such as soybeans, is problematic (12).

Lupines constitute an isolated lineage within the tribe Genisteae sensu stricto (2). The remaining Genisteae form two assemblages, one comprising the genera *Anarthrophyllum*, *Argyrolobium*, *Dichilus*, and *Melolobium*, which diversified predominantly in the Southern Hemisphere, and the other (called Genistinae) comprising *Cytisus*, *Chamaecytisus*, *Genista*, *Retama*, *Spartium*, *Teline*, *Ulex*, and several other small genera that have their centers of diversity in the Mediterranean basin. The geographic origin of the genus *Lupinus* remains unclear. However, phylogenetic analyses reveal four robustly supported clades that are congruent with lupine geography and chromosome number (1, 2, 3, 15). Notably, all the Old World species are placed in a single clade, here labeled OW, while the New World species comprise three strongly supported lineages; a large western New World (WNW) group distributed in western North America, Mexico, and the Andes; a small group centered in the southeast United States; and a predominately lowland eastern New World (ENW) group distributed mainly in the south-central United States and eastern South America (Fig. 1). The two large WNW and ENW lineages have largely allopatric distributions, but species from both clades are sympatric in limited areas, notably, in the south-central Andes in Bolivia. The Andean species (81 out of 85), which are nested in a strongly supported subclade within the WNW clade, provide one of the most spectacular examples of recent explosive plant species diversification driven by the recent uplift of the Andes (15).

Cross-inoculation studies have shown that lupines share a common rhizobial pool with other legumes in the tribe Genisteae, including the genera *Cytisus*, *Genista*, *Retama*, and *Teline* (18, 19, 37, 38, 47). Additionally, lupines are effectively nodulated by rhizobia isolated from serradella (*Ornithopus*, a genus that belongs to the more distantly related tribe Loteae) and are ineffectively nodulated by rhizobia isolated from the genera *Lotus*, *Anthyllis*, and *Phaseolus* (8, 9, 25).

Lupines are nodulated by fast-growing rhizobia (that are poorly characterized), as well as by slow-growing strains of the

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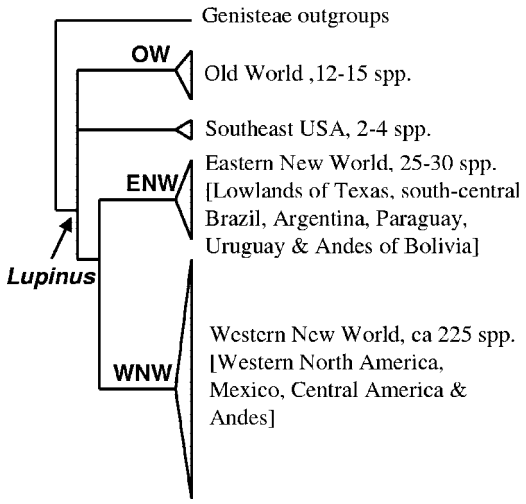


FIG. 1. Schematic *Lupinus* phylogeny showing major clades, which are robustly supported in individual and combined parsimony and Bayesian analyses of the nuclear DNA sequence loci internal transcribed spacer and LEGCYC1A that include up to 140 accession numbers (15). No rhizobia were available from lupine species in the small clade labeled Southeast USA for this study.

genus *Bradyrhizobium* (8, 25). Phylogenetic studies based on nonsymbiotic genes revealed significant heterogeneity among lupine bradyrhizobia, which group with several additional distinct lineages, including *Bradyrhizobium japonicum* and *Bradyrhizobium canariense*. Fewer lupine bradyrhizobia grouped with *Bradyrhizobium elkanii* in housekeeping gene studies (5, 17, 23, 28, 41, 42, 48).

In contrast to the housekeeping gene phylogenies, most lupine isolates form a single cluster, referred to as clade II, in *nodA* nodulation gene trees. Notably, *nodA* clade II comprises bradyrhizobia isolated from other Genisteae species and from serradella species, which corroborates cross-inoculation data (19, 28, 42). Similar grouping was observed in phylogenies of *nodC* and *nifH* genes (17, 48, 49), giving rise to the new biovar *genistearum* for *Bradyrhizobium* strains nodulating Genisteae legumes, which presumably correspond to clade II *Bradyrhizobium* strains.

So far, most research has focused on *Bradyrhizobium* isolates from native Old World lupines growing in the Mediterranean (2) or from Old World species introduced into continental Europe, Australia, and South Africa. Considering that the four major lupine lineages occupy largely isolated present-day geographic distributions, the presumption is that they may be nodulated by rhizobia differing from European clade II strains. Our objective was to address this issue by searching for possible biogeographic patterns preserved in *nod* gene phylogenies. For this purpose, we selected *Bradyrhizobium* strains isolated mainly from lupine nodules collected from native Andean and lowland South American lupines (15).

MATERIALS AND METHODS

Bacterial strains. The *Bradyrhizobium* strains included in the study and their characteristics are described in Table 1. The majority of lupine strains were isolated from dried nodules collected in the Andes and Brazil. We also included *Bradyrhizobium* type strains representing *B. japonicum* (USDA 6^T), *B. elkanii* (USDA 76^T), and *B. yuanmingense* (CCBAU 10071^T), as well as several strains isolated from a range of legumes growing in Panama, Brazil, Japan, China, Mexico, and the United States. Yeast extract mannitol agar medium (46) was used for the growth and maintenance of the strains. All *Bradyrhizobium* strains were grown at 28°C. *Bradyrhizobium* strains were isolated from dried nodules as described by McInroy (26).

Molecular techniques. For PCR sequencing experiments, total genomic DNA was isolated using the sodium dodecyl sulfate-proteinase K lysis procedure described by Moulin et al. (28) or using the QIAGEN QIAamp DNA minikit by following the manufacturer's recommendations. The primers used in this study are listed in Table 2. Primers were designed using OligoAnalyzer 3.0 software (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). All PCR amplifications were made by following the procedures described previously (42). In brief, PCR samples were denatured at 95°C for 2 min, followed by 35 cycles of 95°C for 45 s, 58°C (*atpD*, *dnaK*, *glnII*, and *recA*) for 30 s, and 72°C for 1.5 min (2.5 min for *nodA*) and a final elongation step of 7 min at 72°C, as recommended for the FastStart high-fidelity PCR system by the manufacturer (Roche Diagnostics GmbH, Germany). The annealing temperature for amplification of *nodA*, *nodZ*, and *noeI* was 53°C, or 51°C for some strains in *noeI* amplification. PCR products were purified using the QIAquick gel extraction kit (QIAGEN, Germany) and sequenced using the BigDye Terminator v1.1 cycle sequencing kit on an ABI3100 automated capillary DNA sequencer (Applied Biosystems).

Phylogenetic analyses. The multiple nucleotide sequence alignments were generated using ClustalX (44) and optimized manually with GeneDoc (30). All phylogenetic analyses were performed using PAUP version 4.0b10 (43). Due to the large number of sequences, parsimony and maximum likelihood (ML) analyses were performed using the heuristic search option of PAUP.

For the *nodA*, *nodZ*, *noeI*, *atpD*, *dnaK*, *glnII*, and *recA* data sets, ML and neighbor-joining (NJ) analyses were performed using PAUP. The best-fit model for each gene was assessed using MODELTEST3.6 (34). The best-substitution models (by Akaike information criterion in MODELTEST) found for each marker were, for *atpD*, *dnaK*, *glnII*, *recA*, and *noeI*, GTR+I+G (number of substitutions [NST] = 6; general time reversible with invariant sites and a gamma rate distribution) and, for *nodZ*, TVM+I+G (transversional model with invariant sites and a gamma rate distribution). The *nodA* phylogeny was constructed using the same ML model as described by Moulin et al. (28), i.e., base frequencies, transition/transversion ratios, and substitution rates were estimated at each position from the data using ML analyses (GTR+G+I model); third-codon positions were excluded from the analyses as they accumulated too much saturation (28). Starting from a random tree, five independent heuristic searches were performed to reach the best ML tree. The *Bradyrhizobium-Methylobacterium-Burkholderia* branch extracted from the best ML tree found (score: $-\ln L$ = 8,372.71 at 362,916 rearrangements, where L is likelihood) is shown (see Fig. 4). For the *atpD*, *recA*, *dnaK*, *glnII*, and *nodZ* gene trees, bootstrap analyses were performed using heuristic searches under distance and ML models (using PAUP), with 1,000 and 100 replicates, respectively. Due to the large datasets, bootstrap values for *nodA* phylogeny were assessed using only NJ analyses.

A Shimodaira-Hasegawa (S-H) test of congruence of tree topologies (trees constructed with an ML model [NST = 2], gamma distribution of sites, and ML estimates of base frequencies) was performed using PAUP on a restricted data set of 12 *Bradyrhizobium* strains for which all markers were available (i.e., BLUH1, BC-C2, BLUT1, FTA6, USDA76, C8, CH2443, USDA6, CCAU10071, CH2509, USDA110, and CH2498), with *Sinorhizobium meliloti* strain 1021 as a root. The results of the tests are shown in Fig. 2; see also Fig. S2 in the supplemental data. S-H tests were also performed on *nodA*-constrained trees, using the best ML models to build the original tree shown in Fig. 4 and to test several hypotheses on strain origin (see Fig. 5).

Nucleotide sequence accession numbers. The accession numbers of sequences generated in this study are listed in Table 3.

RESULTS AND DISCUSSION

Strains of the diverse *Bradyrhizobium* lineages nodulate *Lupinus* spp. In this study, we characterized a collection of 52 *Bradyrhizobium* strains, of which 32 were isolated from lupine nodules sampled from three (OW, ENW, and WNW) of the four major *Lupinus* lineages, with 23 isolates originating from native New World lupines (Fig. 1). Prior to this study, it was known that *Bradyrhizobium* strains isolated in Europe from Genisteae and serradella legumes form a distinct group (clade

TABLE 1. *Bradyrhizobium* strains used in this study

Strain	Lineage[a]	Legume host	*Lupinus* clade[c]	Sampling origin	Source
BLUH1	BC	*Lupinus angustifolius*	OW	Canary Islands, Spain	M. León Barrios
BLUT1	BC	*Lupinus albus*	OW	Canary Islands, Spain	M. León Barrios
C4	BJ1	*Lupinus mutabilis*	WNW	Ecuador	G. Bernal
C8	BJ1	*Lupinus mutabilis*	WNW	Ecuador	G. Bernal
CH2310	BJ1	*Lupinus bandelierae*	ENW	Bolivia	This study
CH2318	BJ1	*Lupinus bandelierae*	ENW	Bolivia	This study
CH2352	BJ1	*Lupinus* sp.	WNW	Peru	This study
CH2355	BJ1	*Lupinus misticola*	WNW	Peru	This study
CH2391	BJ1	*Lupinus* sp.	WNW	Peru	This study
CH2437	*B*. sp.[b]	*Lupinus tominensis*	WNW	Bolivia	This study
CH2438	BJ1	*Lupinus breviscapus*	WNW	Bolivia	This study
CH2443	BJ1	*Lupinus pycnostachys*	WNW	Bolivia	This study
CH2490	BJ2	*Lupinus paranensis*	ENW	Brazil	This study
CH2493	BE	*Lupinus paraguariensis*	ENW	Brazil	This study
CH2498	BJ2	*Lupinus rubriflorus*	ENW	Brazil	This study
CH2506	BJ1	*Lupinus uleanus*	ENW	Brazil	This study
CH2509	BJ2	*Lupinus albescens*	ENW	Brazil	This study
CH2510	BJ1	*Lupinus bracteolaris*	ENW	Brazil	This study
FTA6	*B*. sp	*Lupinus nootkatensis*	WNW	Alaska	M. Parker
FTA7	BJ1	*Lupinus nootkatensis*	WNW	Alaska	M. Parker
ICEA	BC	*Lupinus nootkatensis*	WNW	Iceland	M. Jóhannsson
ICEB	BC	*Lupinus nootkatensis*	WNW	Iceland	M. Jóhannsson
ICED	BC	*Lupinus nootkatensis*	WNW	Iceland	M. Jóhannsson
Lcamp1	BJ1	*Lupinus campestris*	WNW	Mexico	This study
Lcamp6	*B.* sp.	*Lupinus campestris*	WNW	Mexico	This study
Lcamp8	*B.* sp.	*Lupinus campestris*	WNW	Mexico	This study
Lpol9	BJ1	*Lupinus polyphyllus*	WNW	Poland	This study
MCLA07	BC	*Lupinus albus*	OW	Salamanca, Spain	E. Velazquez
PL41	BJ1	*Lupinus* sp.	WNW	Peru	P. Lezama
RLA08	BJ1	*Lupinus albus*	OW	Leon, Spain	E. Velazquez
Zaluz80	BC	*Lupinus polyphyllus*	WNW	Poland	This study
ORSAT6[c]	BJ1	*Ornithopus sativus*		Mexico	This study
ORSAT8[c]	BJ1	*Ornithopus sativus*		Mexico	This study
Cytisus11	BJ1	*Cytisus* sp.		Poland	W. Małek
Os9	BJ2	*Cytisus scoparius*		Japan	W. Małek
AEKY10	BE	*Amphicarpaea edgeworthii*		Japan	M. Parker
C10-2	*B*. sp.	*Inga oerstediana*		Mexico	J. Grossman
C8P-1	BJ2	*Inga pavoniana*		Mexico	J. Grossman
CCBAU10071^T	BY	*Lespedeza cuneata*		China	W.-X. Chen
CPAC-M9	BE	*Mucuna aterrima*		Brazil	M. Scotti Muzzi
Da3-1	*B*. sp.	*Desmodium axillare*		Panama	M. Parker
F3b	BE	*Lespedeza cuneata*		Japan	T. Ezawa
Jwc91-2	BE	*Amphicarpaea bracteata*		United States	M. Parker
KFR22	*B*. sp.	*Faidherbia albida*		Kenya	D. Odee
Mm1-3	*B*. sp.	*Machaerium millefloruml*		Panama	M. Parker
Ppau 3-41	*B*. sp.	*Phaseolus pauciflorus*		Mexico	M. Parker
Rp2-1	*B*. sp.	*Rhynchosia pyramidalis*		Panama	M. Parker
Q4A	BJ2	*Lespedeza* sp.		Japan	T. Ezawa
USDA3002	BJ2	*Acacia decurrens*		Brazil	P. Van Berkum
USDA3259	BE	*Phaseolus lanatus*		Illinois	P. Van Berkum
USDA6^T	BJ1	*Glycine max*		Japan	P. Van Berkum
USDA76	BE	*Glycine max*		California	P. Van Berkum

[a] Lineage based on *atpD-recA-dnaK-glnII* phylogenies (Fig. 2 and 3; see Fig S3 in the supplemental data).
[b] *B*. sp., *Bradyrhizobium* sp.
[c] See Fig. l.

II) in *nod* gene trees (19, 28). Furthermore we showed that bradyrhizobia of this clade have become established in acid soils of Western Australia and South Africa following their probably accidental introduction with lupine and serradella seeds (42). Similar transfer with seeds of introduced legumes could explain the presence of clade II *nodA* sequences in New Zealand strains isolated from invasive *Cytisus scoparius* and *Ulex europeus* plants (50, 51). Considering the dynamic dispersion of clade II strains we wondered whether strains from a much wider range of lupine species, including native New World lupines which are geographically distant from the core Genistineae area, could also fall predominately within clade II.

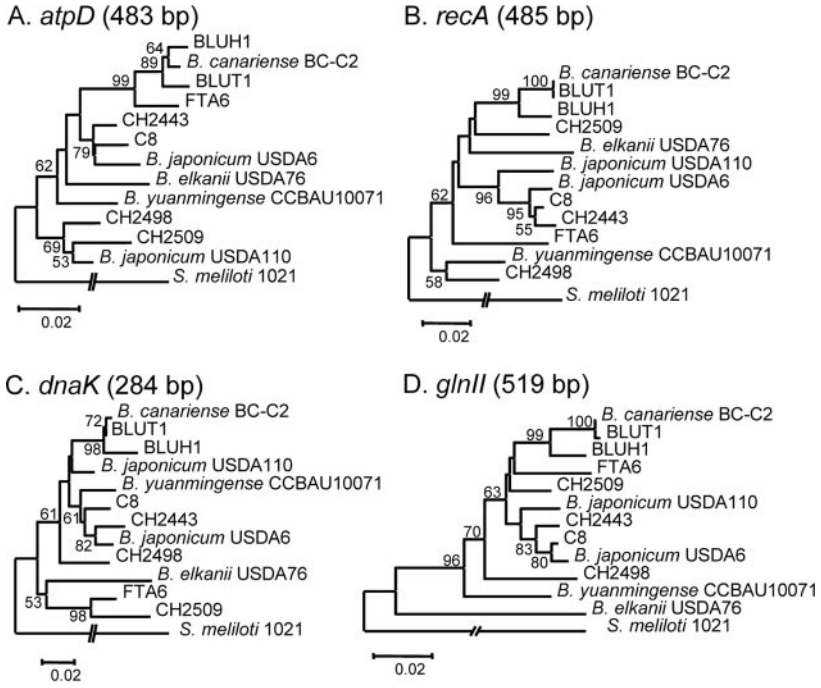
To determine the taxonomic status of the *Bradyrhizobium* lupine strains, we first evaluated the congruence of four phylogenetic markers (483 bp of *atpD*, 483 bp of *recA*, 284 bp of *dnaK*, and 519 bp of *glnII*) used in *Bradyrhizobium* classification studies (10, 40, 42, 49, 51) for a subset of 13 strains (Fig. 2A to D). We did not include the 16S rRNA gene marker in

TABLE 2. Oligonucleotides used for PCR amplification[a]

Primer	Sequence (5′–3′)	Target gene (primer position)	Reference or source
TSnodA2b	GATTCCVWGBCCYTCVAGATC	*nodA* (345–325[b])	42
TSnodD1-1c	CAGATCNAGDCCBTTGAARCGCAT	*nodD1* (24–1[b])	42
TSnodB2N	CTGTGRTTHGCRAYCTYRTGYCC	*nodB* (239–217[b])	42
TSnodZ3	GGTTTCGGYGAYTGYCTBTGGTC	*nodZ* (40–62[b])	28
TSnodZ4	AATRTCTTCGCCRTTRCCRTGCC	*nodZ* (552–530[b])	28
TSnoeI1	CTGGATATCGGYTGCAATGATGG	*noeI* (64–86[c])	28
TSnoeI2	TCGGCYCCCTGMACRTCCATCCA	*noeI* (452–430[c])	28
TSrecAf	CAACTGCMYTGCGTATCGTCGAAGG	*recA* (8–32[c])	42
TSrecAr	CGGATCTGGTTGATGAAGATCACCATG	*recA* (620–594[c])	42
TSatpDf	TCTGGTCCGYGGCCAGGAAG	*atpD* (189–208[c])	42
TSatpDr	CGACACTTCCGARCCSGCCTG	*atpD* (804–784[c])	42
TSglnIIf	AAGCTCGAGTACATCTGGCTCGACGG	*glnII* (13–38[c])	42
TSglnIIr	SGAGCCGTTCCAGTCGGTGTCG	*glnII* (681–660[c])	42
TSdnaK2	GTACATGGCCTCGCCGAGCTTCA	*dnaK* (1794–1772[c])	40
TSdnaK4	GGCAAGGAGCCGCAYAAGG	*dnaK* (1057–1075[c])	This study

[a] All primers were used for amplification and sequencing of PCR products.
[b] Position of the primer in the corresponding sequence of lupine *Bradyrhizobium* sp. strain WM9.
[c] Position of the primer in the corresponding sequence of *B. japonicum* USDA 110.

our phylogenetic analyses as this gene is too conserved to resolve genospecies in *Bradyrhizobium* (49, 52). The congruence of the tree topologies was evaluated using S-H tests, and the results are shown in Fig. 2E. Congruence ($P > 0.05$) was found between *recA* and *atpD* trees ($P = 0.164$), *recA* and *glnII* trees ($P = 0.186$), and *atpD* and *glnII* trees ($P = 0.305$), but there was no congruence between the *dnaK* tree and any other tree. We thus decided to evaluate the taxonomic status of the



E. Topology Congruence tests

SH test	*recA*	*atpD*	*glnII*	*dnaK*
recA	-	**0.164**	**0.186**	0.001
atpD		-	**0.305**	0.001
glnII			-	0.000
dnaK				-

FIG. 2. Comparison of the *atpD* (A), *recA* (B), *dnaK* (C), and *glnII* (D) gene fragment phylogenies (size of the alignment used for each marker is in parentheses). Trees shown were constructed by NJ, from a Kimura 2P distance matrix. Node robustness was evaluated by using 1,000 bootstrap replications. (E) Summary of S-H tests of congruence of tree topologies among ML phylogenies of the four markers (trees not shown). ML settings for each marker were of two types: a gamma distribution of site substitution and an estimating-base-frequency substitution. Boldface values indicate *P* values greater than 5% between two tree topologies, meaning the topologies are not statistically different by the S-H test.

TABLE 3. PCR amplification and accession numbers of sequenced PCR products[a]

Strain	Accession no for gene:						
	atpD	*recA*	*dnaK*	*glnII*	*nodA*	*nodZ*	*noeI*
BLUH1	AM168275	AM168321	AM168344	AM168365	AM117529	AM168375	AM168396[b]
BLUT1	AM168276	AM168322	AM168345	AM168366	AM117525	AM168376	AM168397[b]
C4	AM168277	AM168323	AM168346		AM117512	AM168377	−
C8	AM168278	AM168324		AM168367	AM117513	AM168378	AM168398[b]
CH2310	AM168279	AM168325	AM168347		AM117514	AM168379	−
CH2318	AM168280	AM168326	AM168348		AM117515	+	−
CH2352	AM168281				AM117520	+	−
CH2355	AM168282				AM117561	+	−
CH2391	AM168283				AM117521	+	−
CH2437	AM168284	AM168327	AM168349	AM168368	AM117539	+	−
CH2438	AM168285				AM117522	AM168380	−
CH2443	AM168286	AM168328	AM168350	AM168369	AM117529	+	−
CH2490	AM168287				AM117530	AM168381	−
CH2493	AM168288	AM168329			AM117562	AM168382	−
CH2498	AM168289	AM168330	AM168351	AM168370	AM117531	+	−
CH2506	AM168290	AM168331			AM117540	AM168383	−
CH2509	AM168291	AM168332	AM168354	AM168371	AM117556	AM168384	AM168399
CH2510	AM168292	AM168333	AM168355	AM168372	AM117533	+	−
FTA6	AM168293	AM168334	AM168356	AM168373	AM117523	AM168385	AM168400
FTA7	AM168294	AM168335	AM168357		AM117524	AM168386	−
ICEA	AM168295				AM117549	+	−
ICEB	AM168296				AM117550	+	−
ICED	AM168297				AM117551	+	−
Lcamp1	AM168298	AM168336	AM168358		AM117532	AM168387	+
Lcamp6	ND[c]		AM168359		AM117552	+	ND
Lcamp8	AM168299	AM168337	AM168360		AM117560	+	AM168401[b]
Lpol9	AM168300				AM117555	+	ND
MCLA07	AM168301				AM117535	+	−
PL41	AM168302				AM117519	+	−
RLA08	AM168303				AM117518	+	−
Zaluz80	AM168304				AM117546	+	ND
ORSAT6	AM168305				AM117526	+	+
ORSAT8	AM168306				AM117543	+	+
Cytisus11	AM168307				AM117548	+	−
Os9	AM168308				AM117536	+	ND
AEKY10	AM168309				AM117538	+	AM168402
C10-2	AM168310				AM117563	AM168388	AM168403
C8P-1	AM168311				AM117547	+	AM168404
CCBAU10071^T		AM168343	AM168361		AM117557	AM168389	AM168405
CPAC-M9	AM168312				AM117537	+	AM168406
Da3-1	AM168313				AM117541	+	−
F3b		AM168339			AM117517	AM168390	AM168407
Jwc91-2	AM168314				AM117527	+	AM168408
KFR22		AM168340			AM117542	+	AM168409
Mm1-3	AM168315				AM117534	AM168391	AM168410
Ppau 3-41	AM168316				AM117559	+	AM168411
Rp2-1	AM168317				AM117544	+	−
Q4A	AM168318				AM117553	AM168392	AM168412
USDA3002	AM168319				AM117516	−	−
USDA3259			AM168364		AM117558	AM168393	AM168413
USDA6^T	AM168320	AM168341	AM168362		AM117545	AM168394	AM168414
USDA76		AM168342	AM168363		AM117554	AM168395	AM168415

[a] +, amplification of PCR products that were not sequenced; −, lack of amplification. Amplification conditions for all genes are described in Materials and Methods.
[b] *noeI* pseudogene.
[c] ND, not determined.

strains by sequencing 485 bp of the *atpD* gene in all 52 strains included in the study (except for strain Lcamp6, for which no amplification product could be obtained) and to sequence fragments of *recA* (559 bp) and *glnII* (594 bp) genes in several randomly selected strains to confirm the *atpD* phylogeny clustering (see Fig. S1 of the supplemental material).

The *atpD* ML tree of all strains (Fig. 3) reveals several clades, usually with limited internal resolution, placed within the major *Bradyrhizobium* branch. Some of these clades correspond to the previously delineated species *B. japonicum*, *B. canariense B. liaoningense*, and *B. yuanmingense*, as well as to the genotypes α and β (Fig. 3; see Fig. S1 in the supplemental material). *B. japonicum* is polyphyletic in the *atpD* phylogeny, with sequences placed in two separate groups, one containing strain USDA 6^T, which forms a group with various *Lupinus* strains, and the other containing *B. japonicum* USDA 110,

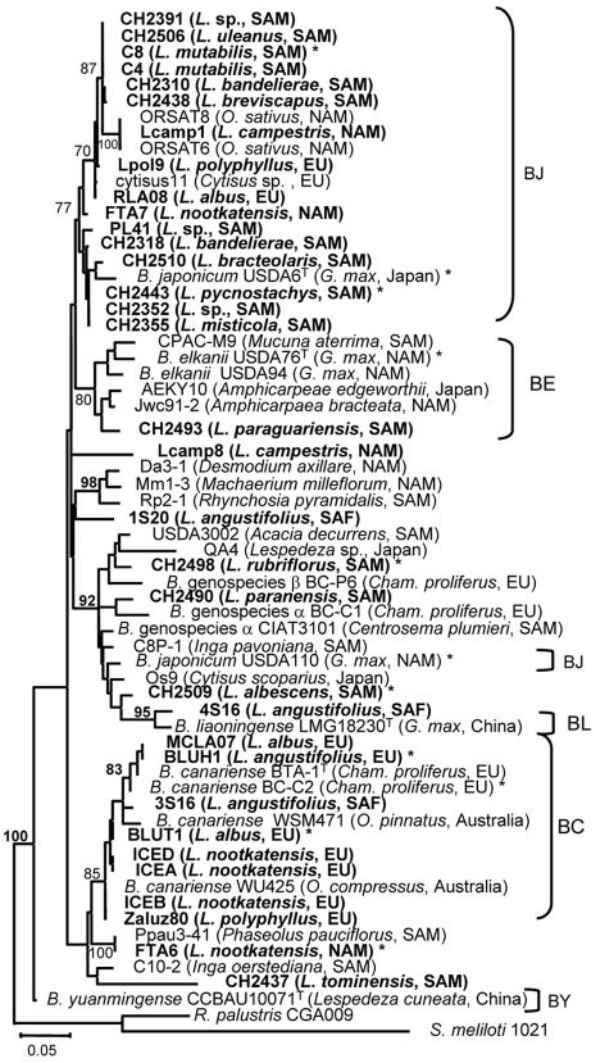


FIG. 3. ML trees based on partial *atpD* sequences. The tree shown was constructed unrooted. Lupine strains are indicated in boldface. Host plants and geographical origins of the strains are in parentheses. The scale bar indicates the number of substitutions per site. Bootstrap values indicated are percentages of 1,000 replicates (calculated under distance criteria); they are in boldface when a bootstrap value >70% was found under ML criteria (100 replicates). Abbreviations: BC, *B. canariense*; BJ, *B. japonicum*; BL, *B. liaoningense*; BY, *B. yuanmingense*; EU, Europe; NAM, North America; SAM, South America; SAF, South Africa. Asterisks indicate strains used for S-H tests (strains for which all markers were sequenced). Reference strains and their accession numbers were as follows: USDA 110, NP_767080; 1S20, AJ891286; BTA-1, AY386739; WSM471, AJ891292; 3S16, AJ891287; WU425, AJ891293; BC-C2, AY386736; BC-P6, AY653751; LMG18230^T, AY386752; 4S16, AJ891288; BC-C1, AY386735; CIAT3101, AY653762; *Rhodopseudomonas palustris* CGA009, NC_005296).

which groups together with the type strains representing the genospecies α and β and with the *B. liaoningense* type strain LMG18230. Similar grouping of *B. japonicum* strains has recently been observed by Vinuesa et al. (49) in their *atpD* tree. The *B. yuanmingense* strain CCBAU 10071^T forms a single clade at the base of the *atpD* tree (Fig. 3).

These results corroborate earlier reports describing great diversity among lupine *Bradyrhizobium* strains, which were found to group in several distinct branches in housekeeping gene trees (5, 23, 28, 42, 48, 49). The majority of lupine strains in all trees grouped in the *B. japonicum* cluster or with *B. canariense*. In the *atpD* tree, 16 lupine strains plus 2 strains from serradella and 1 from *Cytisus* form a moderately supported clade (77% bootstrap) containing *B. japonicum* strain USDA 6^T. Seven lupine strains, all originating from Europe and Iceland, form a well-supported cluster (85% bootstrap) with *B. canariense* strains. Notably, only one lupine strain groups consistently with *B. elkanii*, which implies that *B. elkanii* is seemingly rare among lupine isolates. Six lupine strains and one strain from *Cytisus* isolated in Japan occupy variable or weakly supported positions in the trees, albeit distinct from *B. japonicum*, *B. canariense*, or *B. elkanii*. These strains may therefore be regarded as unresolved. Despite the differences found between trees, all gene marker phylogenies presented here clearly show that lupine strains constitute a heterogeneous phylogenetic group within the genus *Bradyrhizobium*.

The evolution of the *nodA* nodulation gene in the genus *Bradyrhizobium*. Previous studies indicated that the *nodA* genes in strains of *Bradyrhizobium* are longer than *nodA* genes from the genera *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*, due to the presence of codons for an additional 13 amino acid residues that form the N-terminal part of the deduced NodA protein (28, 42). Such a segment is also present in *nodA* sequences of *Methylobacterium nodulans* ORS2060 and *Burkholderia tuberum* STM678, which group with *Bradyrhizobium* sequences. This segment is present in the majority of the *Bradyrhizobium* strains studied here. However, in 14 American strains, the aligned *nodA* sequences reveal that the predicted NodA proteins could be shorter, as their sequences carry a second ATG (ATG2) codon that matches the start codon of the fast-growing rhizobia. In three strains isolated from lupine and serradella, Lcamp1, ORSAT6, and ORSAT8, the first putative codon is GTG, while the second is ATG. Moreover, six other strains, including strain CH2509, originating from a native Brazilian lupine, may produce a NodA protein identical to those of the fast-growing species. These strains carry only one ATG codon, which overlaps with the start codon for NodA proteins of the fast-growing strains (see Fig. S5 in the supplemental material). This finding strengthens our earlier interpretation that the *nodA* gene may be undergoing an evolutionary progression towards a shorter *nodA* sequence, which is typical for the majority of rhizobia (28). Strain CH2509, which belongs to new clade VII, as well as photosynthetic bradyrhizobia that here have been grouped in clade VI (Fig. 4), illustrates the loss of this N-terminal segment. On the other hand, the finding that some strains of clade III carry the second ATG codon, which in sequence alignment overlaps with start codons of other rhizobia (and potentially could be an alternative start codon), suggests that in some strains evolution toward a shorter *nodA* gene is ongoing.

Biogeographic structure shown by the *nodA* gene phylogenetic tree. This study supports previous reports showing the dissimilarity between the nodulation and housekeeping gene trees (Fig. 3 and 4), which invoke the hypothesis of multiple lateral transfers of symbiotic loci among *Bradyrhizobium* lineages (17, 28, 41, 42, 48, 49). Previous studies showed that, despite their great diversity, the *nodA* gene sequences in the

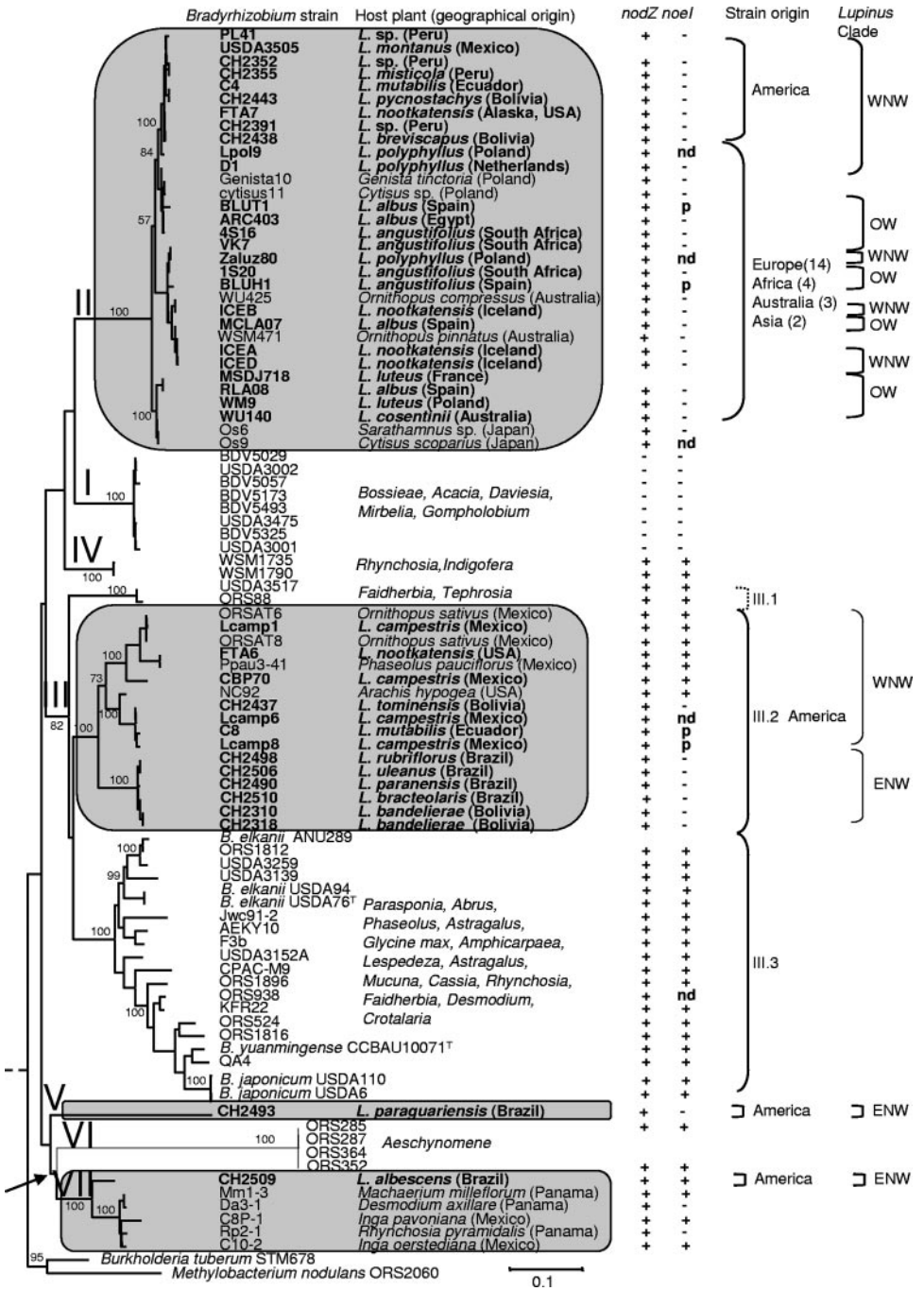


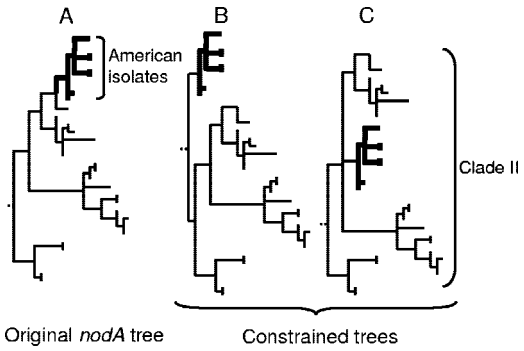
FIG. 4. ML phylogenetic tree of *Bradyrhizobium nodA* genes. The tree was constructed by a ML approach described in Materials and Methods. *Lupinus* strains are indicated in boldface; clades to which they belong are in shaded blocks. The scale bar indicates the number of substitutions per site. Bootstrap values >70% (percentage of 1,000 replicates calculated under distance criteria) are given at the branching nodes. Clades I to IV correspond to the *nodA* phylogenies described elsewhere (28, 42). Host plant species are indicated on the right side of the tree together with geographical origins in *nodA* clades containing *Lupinus* strains. Succeeding columns show results of *nodZ* and *noeI* PCR amplifications on test strains, geographical origin of lupine strains, *nodA* subclades defined by bootstrap values >80%, and finally *Lupinus* clades corresponding to the phylogeny presented in Fig. 1 including WNW, ENW, and OW. Accession numbers of strains included in this tree are as follows: *Bradyrhizobium japonicum* USDA 110, NC_004463; *B. elkanii* USDA 94, U04609; *Bradyrhizobium* sp. strain ARC403, AJ430731; BDV5029, AJ890291; BDV5057, AJ890311; BDV5173, AJ891168; BDV5325, AJ890290; BDV5493, AJ890312; CBP70, AJ430730; D1, AJ430727; Genista10, AJ430726; NC92, U33192; ORS88, AJ430716; ORS285, AF284858; ORS287, AJ437607; ORS352, AJ438775; ORS364, AJ437613; ORS524, AJ430717; ORS938, AJ430715; ORS1812, AJ430723; ORS1816, AJ430722; ORS1896, AJ430718; 1S20, AJ890296; 4S16, AJ890289; USDA 3001, AJ430713; USDA 3139, AJ430712; USDA 3152A, AJ430711; USDA 3475, AJ430710; USDA 3505, AJ430709; USDA 3517, AJ430708; WSM471, AJ890307; WSM1735, AJ890293; WSM1790, AJ890286; WU140, AJ890313; WU425, AJ890300; VK7, AJ890295; *Methylobacterium nodulans* ORS2060, AF266748; *Burkholderia phymatum* STM678, AJ302321. Abbreviations: +, amplified by PCR; −, no PCR product; nd, not determined; p, pseudogene.

genus *Bradyrhizobium* formed a monophyletic group that splits into four major branches, referred to as clades I, II, III, and IV. Clades I and IV comprise strains isolated from legumes native to Australia. Clade II includes Genisteae and serradella isolates of mainly European origin, while clade III is a large group comprising *nodA* sequences of mainly subtropical strains (28, 42). Here, we reveal even greater complexity in the *nodA* tree, with seven clades being distinguished (Fig. 4). Among these, the new clade VI represents photosynthetic strains that were previously classified in clade III.4.

The *nodA* gene tree included data from rhizobia isolated from 134 legume taxa that represented all genera and species for which a *nodA* sequence was available (alignment is available as supplemental material). In the ML *nodA* tree shown in Fig. 4, all Old World lupine strains isolated in Europe, the Canary Islands, and Iceland, as well as strain cytisus11, are placed in clade II, whereas only eight New World lupine strains are placed in clade II, and these are all from lupine species belonging to the WNW clade. The majority of New World lupine strains are found in clade III, which includes strains from both of the large New World *Lupinus* lineages (ENW and WNW) but no Old World lupine strains. Two new clades that include lupine strains are evident in the *nodA* tree of Fig. 4: clades V (strain CH2493) and VII (strain CH2509). Clade VII, apart from the Brazilian lupine strain CH2509, comprises strains, Da3-1, C10-2, C8P-1, Mm1-3, and Rp2-1, originating from Central America (Panama) and Mexico, which were isolated from *Desmodium axillare* (Desmodieae), *Inga* spp. (Ingeae), *Machaerium milleflorum* (Dalbergieae), and *Rhynchosia pyramidalis* (Phaseoleae), respectively (Table 1).

The New World lupine clade III strains form a strongly (100% bootstrap) supported subgroup, designated subclade III.2. Also included in subgroup III.2 are strains ORSAT6 and ORSAT8, from a Mexican soil sample (trapped using *Ornithopus sativus*), Ppau3-41, isolated in Mexico from *Phaseolus pauciflorus* (35), and peanut strain NC92, originating from North Carolina (11). Therefore, III.2 cannot be regarded as an exclusively lupine group. The *nodA* sequences in subclade III.2 show high sequence divergence (pairwise similarity ranges from 83% to 100%), with the lupine strains divided between two subgroups of closely related sequences (Fig. 4). It is notable that strains from the two independent ENW and WNW *Lupinus* lineages fall into separate subgroups. This could be interpreted as an example of codivergence of the New World lupine *Bradyrhizobium* strains with their lupine hosts. These New World lupine lineages both include elements from North and South America, suggesting that the two independent dispersals between North and South America postulated for lupines may have been accompanied by similar dispersal of their symbiotic *Bradyrhizobium* partners (15).

Although four strains of subclade III.2 originate from nonlupines, all strains originate from the Americas, and therefore it seems justified to name clade III.2 the "Pan-American" subgroup. Notably, a geographical structure in symbiotic gene trees was reported for *nodA* and *nifH* genes in *Sinorhizobium* isolates (14) and later for *nifD* (but not for the 16S rRNA locus) among *Bradyrhizobium* strains isolated in Australia, Central America, and eastern Asia (31, 35). Likewise, we previously showed that clade I isolates in the *nodA* tree have an Australian affinity, comprising strains isolated either in Australia or from native Australian legumes growing elsewhere (42). Clade I strains were also described among isolates collected in Papua New Guinea from the nodules of the nonlegume *Parasponia andersonii* and from legumes indigenous to New South Wales (22), which further strengthened our belief in the Australasian origin of this group. In our study, the clade VII strains originated exclusively from Central and South America, although only strain CH2509 was isolated from lupine. Thus, the four *nodA* clades I, II, III.2, and VII each reflect the geographical origins of their respective strains (Fig. 4).



Tree	−ln L	Diff −ln L	P(sh)
A	8372.71715	(best)	
B	8375.34420	2.62705	0.465
C	8376.32390	3.60675	0.478

FIG. 5. S-H tests on several constrained topologies of clade II of the *nodA* phylogeny. The American isolate subclade II branch is in boldface, and its place in clade II was constrained in two alternative topologies. S-H tests were performed with the original tree. Tree A, best ML tree obtained; trees B and C, two alternative constrained topologies in which American isolates are at the base of clade II (tree B) or mixed with clade II European and African isolates (tree C). ML tree and S-H test scores are summarized below trees. *P* values greater than 0.05 indicate tree congruence.

The present study with much wider sampling confirms the earlier findings that all "European" strains isolated from Genisteae (and serradella) group exclusively in *nodA* gene clade II, implying the predominance of clade II strains among Genisteae bradyrhizobia in European soils (19, 28, 41, 42). Conversely, only 8 of the 23 lupine isolates from New World lupines are placed in clade II, while the remainder are either in clade III or in two new clades, V and VII (Fig. 4). Notably, the American clade II strains form a strongly supported (100% bootstrap) subclade of sequences with very low divergence, despite the fact that they originate from an extensive area spanning Alaska to Bolivia. This area coincides with the distribution of the WNW *Lupinus* clade and may imply that *nodA* gene sequences of all these strains diverged from a common ancestor more recently than those in the European strains and then spread rapidly across the Americas. Although the S-H test cannot discriminate between alternative placements of the American clade II strains (Fig. 5), clade II strain predominance in European soils and higher sequence diversity argue for a European rather than American origin. This would imply possible long-distance dispersal of the clade II strains, presumably across the Atlantic Ocean to North and South America, or, alternatively, their transfer from Europe following coloni-

zation of the Americas in the late 15th century. A similar explanation was earlier proposed for the spread of common bean (*Phaseolus vulgaris*) rhizobia outside the Americas (32) and recently for clade II bradyrhizobia in Australia, South Africa, and New Zealand (42, 50).

Clade II is characterized by low sequence divergence and a lack of resolution compared to clade III, which implies that the divergence in clade II was relatively recent compared to that in clade III. We hypothesize that clade II bradyrhizobia diversified initially in the Mediterranean basin, possibly in parallel with the divergence of their legume hosts belonging to the tribes Genisteae and Loteae, which radiated in this region (4). It is likely that radiation of these tribes was triggered by climatic changes leading to formation of the Mediterranean climate during the Pliocene epoch. It appears that lower temperatures and increased aridity after the end of the Miocene eliminated mesophytic boreotropical flora, giving rise to the expansion of cold- and drought-tolerant genera, such as those in the Genisteae and Loteae tribes (21, 33, 45). Thus, the predominance of clade II strains in Europe's soils might be perceived as a consequence of climatic changes that eliminated tropical elements from the European legume flora, while formation of the Sahara Desert prevented migration of subtropical legumes (and their rhizobia) from Africa. In contrast, the closure of the Isthmus of Panama enabled a wide-scale migration of tropical legumes and their rhizobia from South America to the north (21, 27, 45), which could explain the presence of clade III strains among the American lupine isolates.

Amplification of nodulation genes conferring modifications of the Nod factor reducing end. The structure of the nodulation (Nod) factor is an important determinant of rhizobial host specificity and can be related to the activity of genes involved in modification of the Nod factor reducing end, such as *nodZ*, *noeI*, *noeE*, and *nolL* (6, 13, 16, 36). Previous studies indicated that the *nodZ* gene, which is involved in fucosylation of the Nod factor reducing end (36, 39), is present in all strains belonging to *nodA* clades II, III, and IV but is probably missing in strains from clade I. Moreover, it was shown that the *nodZ* gene trees are congruent with the *nodA* gene phylogenies (28, 42). The present study further supports these findings. We were unable to amplify the *nodZ* gene from new strain USDA 3002 (isolated from *Acacia decurrens* in Brazil), which was placed in clade I in the *nodA* gene tree, whereas *nodZ* was detected in all strains belonging to *nodA* clades II and III, as well as in the strains of the new clades V and VII (Fig. 4). The *nodZ* gene tree is similar to the *nodA* tree, forming separate branches that group in a similar pattern and are composed of the same strains as the *nodA* clades (Fig. 4; see Fig. S3A in the supplemental material).

Methylation of carbon C-2 of the fucose molecule bound to the Nod factor reducing end is conferred by the *noeI* gene (16). This modification is particularly common among clade III strains, but the *noeI* gene has so far not been detected in clades I and II (28, 42). The use of additional isolates in the present study partially contradicts this finding. The *noeI* gene was missing in the majority of the clade II strains, although faint PCR bands were visible in some strains. For these strains we repeated the PCR amplification using a lower annealing temperature (51°C instead of 53°C). In strains BLUH1 and BLUT1 we sequenced the PCR products, which proved to be two *noeI* sequences carrying one nucleotide deletion that disrupted the coding sequence continuity. The *noeI* gene was detected in 17 of the 24 clade III strains (Fig. 4). However, a notable exception was the lack of the PCR products in a group of strains within subclade III.2 that were isolated from the ENW *Lupinus* clade (strains CH2310, 2318, 2490, 2498, 2506, and 2510). In two other lupine strains belonging to III.2 (C8 and Lcamp8), the amplified *noeI* fragments harbor an identical nonsense mutation and, additionally, strain C8 harbors a deletion, suggesting that these are nonfunctional copies (see Fig. S4 in the supplemental material). The *noeI* gene was also amplified and sequenced in the clade VII lupine strain CH2509 but not the clade V lupine strain CH2493 (Fig. 4; see Fig. S3B in the supplemental material).

Unlike *nodA* and *nodZ* genes, for which we have not detected pseudogenes, mutated and potentially inactive *noeI* gene copies suggest the loss of this Nod factor modification gene. In all these strains, the mutated sequences preserved extensive similarity to functional *noeI* gene sequences, implying that these mutations must have occurred recently. Interestingly, mutated *noeI* sequences were reported in lupine clade II strain WM9 and on the symbiosis plasmid of *Rhizobium etli* strain CFN42 (41). Similarly, *noeE* pseudogenes were detected in soybean modulating *Bradyrhizobium* strains USDA 110 and USDA 94, both of which are known to produce Nod factors that bear 2-O-methylated, but not 3-O-sulfated, fucose (20, 39). The presence of *noeI* pseudogenes indicates that the loss of these two genes reflects a more general evolutionary trend related to adaptation to the legume host requirements. We assume that the American clade III.2 strains diverged together with legumes other than lupines. The loss of *noeI* could therefore be a relatively recent adaptation to their new hosts that has occurred independently in various strains (28, 41).

Our conclusion that the *nodA* nodulation gene trees show strong geographical structure implies that divergence in geographical isolation, in parallel with host plant diversification, plays a significant role in the evolution of symbiotic loci (24). Comparison of *nod* gene trees with those of housekeeping genes shows that the latter retain much less geographic structure. Thus, geographic structure appears to be preserved in phylogenies of rapidly evolving accessory genes, such as nodulation genes, rather than in conserved housekeeping genes. In the latter, geographic structure was presumably erased by a series of dispersal and recombination events (49). Given the difficulty of defining bacterial species according to ecological or biological concepts (7, 29), it could be concluded that the biogeography of bacteria relates to genes rather than species, which appears to be corroborated by our results.

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