European Origin of *Bradyrhizobium* Populations Infecting Lupins and Serradella in Soils of Western Australia and South Africa†‡

Tomasz Stępkowski,^{1*} Lionel Moulin,² Agnieszka Krzyńska,¹ Alison McInnes,³
Ian J. Law,⁴ and John Howieson⁵

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland¹; Laboratoire des Symbioses Tropicales et Méditerranéennes, IRD-CIRAD-INRA-AGROM, 34398 Montpellier cedex 5, France²; School of Earth and Geographical Sciences, The University of Western Australia, 35 Stirling Hwy, Nedlands, WA 6009, Australia³; Plant Protection Research Institute, Private Bag X134, Queenswood 0121, South Africa⁴; and Centre for Rhizobium Studies, Murdoch University, Murdoch, WA 6150, Australia⁵

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We applied a multilocus phylogenetic approach to elucidate the origin of serradella and lupin *Bradyrhizobium* strains that persist in soils of Western Australia and South Africa. The selected strains belonged to different randomly amplified polymorphic DNA (RAPD)-PCR clusters that were distinct from RAPD clusters of applied inoculant strains. Phylogenetic analyses were performed with nodulation genes (*nodA*, *nodZ*, *nolL*, *noeI*), housekeeping genes (*dnaK*, *recA*, *glnII*, *atpD*), and 16S-23S rRNA intergenic transcribed spacer sequences. Housekeeping gene phylogenies revealed that all serradella and *Lupinus cosentinii* isolates from Western Australia and three of five South African narrow-leaf lupin strains were intermingled with the strains of *Bradyrhizobium canariense*, forming a well supported branch on each of the trees. All *nodA* gene sequences of the lupin and serradella bradyrhizobia formed a single branch, referred to as clade II, together with the sequences of other lupin and serradella strains. Similar patterns were detected in *nodZ* and *nolL* trees. In contrast, *nodA* sequences of the strains isolated from native Australian legumes formed either a new branch called clade IV or belonged to clade I or III, whereas their nonsymbiotic genes grouped outside the *B. canariense* branch. These data suggest that the lupin and serradella strains, including the strains from uncultivated *L. cosentinii* plants, are descendants of strains that most likely were brought from Europe accidentally with lupin and serradella seeds. The observed dominance of *B. canariense* strains may be related to this species' adaptation to acid soils common in Western Australia and South Africa and, presumably, to their intrinsic ability to compete for nodulation of lupins and serradella.

Lupins (*Lupinus* spp.) were introduced as grain and forage crops into Australia and South Africa at the end of the 19th and beginning of the 20th centuries (10). The introduced lupins were species native to the Mediterranean region, including white lupin (*Lupinus albus* L.), narrow-leaf lupin (*Lupinus angustifolius* L.), blue lupin (*Lupinus cosentinii* L.), and yellow lupin (*Lupinus luteus* L.). Major centers of lupin cultivation became established in the winter rainfall regions of Western Australia and the Western Cape province of South Africa. Today, Australia is the leading world producer of lupin seeds used mainly in stock feed. In addition to the lupins, serradella (*Ornithopus* spp.) was introduced as a pasture and forage crop in Western Australia. As soils of Western Australia and South Africa did not contain indigenous rhizobia able to form effective nodules on lupins and serradella, inoculation was initially required for successful cultivation of these legumes (40). Following the introduction of inoculum strains in Western Australia in the early 1930s, inoculation of lupins and serradella was regarded as essential for the next 30 to 40 years. The

widespread cultivation of lupins subsequently led to the establishment of lupin bradyrhizobia in soils of Western Australia. Effective nodulation of uninoculated narrow-leaf lupins was detected in the 1950s (12), and that of *Lupinus cosentinii* plants was detected even earlier (6). Effective populations became similarly established in soils of the Western Cape where inoculation of lupin is no longer considered necessary by many farmers. Interestingly, the naturally occurring populations found in both Western Australia and the Western Cape were dominated by strains differing from those used in commercial inoculants (4, 24, 40).

Lupins are nodulated mainly by slow-growing strains classified in the genus *Bradyrhizobium* (16). Although considerable diversity exists among lupin bradyrhizobia, all lupin strains cluster broadly with *Bradyrhizobium japonicum* but not with *Bradyrhizobium elkanii* (1, 19). Phylogenetic analyses of 16S rRNA (*rrs*) and *dnaK* gene sequences grouped the lupin *Bradyrhizobium* strain WM9 within the *B. japonicum* lineage, which corroborated the earlier studies. Large differences in gene composition were, however, detected in the symbiotic gene region of WM9 with respect to *B. japonicum* strain USDA 110 (38). The *nodA* gene sequence position of the lupin strain was also distant with respect to *nodA* sequences of other bradyrhizobia. Similar incongruence between the *nod* and *dnaK* and other housekeeping gene phylogenies was subsequently reported in a study of over 30 *Bradyrhizobium* strains, originating from a broad spectrum of legumes from different

* Corresponding author. Mailing address: Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61 704 Poznań, Noskowskiego 12/14, Poland. Phone: 48 61 852 85 03, ext. 149. Fax: 48 61 852 05 32. E-mail: sttommic@ibch.poznan.pl.

† We dedicate this work to Andrzej B. Legocki on the occasion of his 66th birthday.

‡ Supplemental material for this article may be found at <http://aem.asm.org/>.

TABLE 1. *Bradyrhizobium* strains used in this study

Strain	Legume host	Geographic origin ^a	Nodulation of serradella ^b	Reference or source
IS20	<i>Lupinus angustifolius</i>	SA	Nod ⁺ Fix ⁺	4
3S16	<i>Lupinus angustifolius</i>	SA	Nod ⁺ Fix ⁺	4
2S9	<i>Lupinus angustifolius</i>	SA	Nod ⁺ Fix ⁺	4
4S16	<i>Lupinus angustifolius</i>	SA	Nod ⁺ Fix ⁻	4
5S10	<i>Lupinus angustifolius</i>	SA	Nod ⁺ Fix ⁺	4
BDV5029	<i>Bossiaea ensata</i> (Bossiaeeae)	NSW	Nod ⁻	18
BDV5057	<i>Bossiaea foliosa</i> (Bossiaeeae)	NSW	Nod ⁻	J. Burdon (CSIRO)
BDV5173	<i>Daviesia ulicifolia</i> (Mirbelieae)	NSW	Nod ⁻	J. Burdon (CSIRO)
BDV5325	<i>Gompholobium huegelii</i> (Mirbelieae)	NSW	Nod ⁻	J. Burdon (CSIRO)
BDV5419	<i>Hardenbergia violacea</i> (Phaseoleae)	NSW	Nod ⁻	J. Burdon (CSIRO)
BDV5493	<i>Mirbelia rubiifolia</i> (Mirbelieae)	NSW	Nod ⁻	J. Burdon (CSIRO)
USDA3001	<i>Acacia decurrens</i>	SA	ND ^d	26
USDA3475	<i>Acacia melanoxylon</i>	Brazil	ND	26
WSM1253	<i>Ornithopus compressus</i>	Kastro, Greece	Nod ⁺ Fix ⁺	J. Howieson (CRS) ^c
WSM1420	<i>Lupinus cosentinii</i>	WA	Nod ⁺ Fix ⁺	J. Howieson (CRS)
WSM1704	<i>Tephrosia gardneri</i> (Millettieae)	Pilbara, WA	Nod ⁻	53
WSM1735	<i>Rhynchosia minima</i> (Phaseoleae)	Pilbara, WA	Nod ⁻	53
WSM1743	<i>Indigofera</i> sp. (Indigofereae)	Pilbara, WA	Nod ⁻	53
WSM1790	<i>Indigofera colutea</i> (Indigofereae)	Pilbara, WA	Nod ⁻	53
WSM2148	<i>Ornithopus compressus</i>	Albany, WA	Nod ⁺ Fix ⁺	24
WSM2149	<i>Ornithopus compressus</i>	Albany, WA	Nod ⁺ Fix ⁺	24
WSM2150	<i>Ornithopus compressus</i>	Albany, WA	Nod ⁺ Fix ⁺	24
WSM2151	<i>Ornithopus sativus</i>	Carrabin, WA	Nod ⁺ Fix ⁺	24
WSM2152	<i>Lupinus angustifolius</i>	Carrabin, WA	Nod ⁺ Fix ⁺	24
WSM2153	<i>Lupinus angustifolius</i>	Carrabin, WA	Nod ⁺ Fix ⁺	24
WSM2154	<i>Lupinus angustifolius</i>	Carrabin, WA	Nod ⁺ Fix ⁺	24
WSM2155	<i>Daviesia nudiflora</i> (Mirbelieae)	Carrabin, WA	Nod ⁺ Fix ⁺	24
WSM2156	<i>Mirbelia floribunda</i> (Mirbelieae)	Carrabin, WA	Nod ⁺ Fix ⁺	24
WSM2157	<i>Ornithopus sativus</i>	Yelbeni, WA	Nod ⁺ Fix ⁺	24
WSM2158	<i>Ornithopus compressus</i>	Yelbeni, WA	Nod ⁺ Fix ⁺	24
WSM2159	<i>Ornithopus compressus</i>	Yelbeni, WA	Nod ⁺ Fix ⁺	24
WSM2458	<i>Lupinus angustifolius</i>	Carrabin, WA	Nod ⁺ Fix ⁺	24
WSM468	<i>Lupinus cosentinii</i>	Albany, WA	Nod ⁺ Fix ⁺	24
WSM469	<i>Lupinus cosentinii</i>	Albany, WA	Nod ⁺ Fix ⁺	24
WSM471	<i>Ornithopus pinnatus</i> (inoculum strain)	Oyster Harbour, WA	Nod ⁺ Fix ⁺	13
WSM866	<i>Ornithopus</i> sp.	Kastro, Greece	Nod ⁺ Fix ⁺	13
WU140	<i>Lupinus cosentinii</i>	WA	Nod ⁺ Fix ⁺	21
WU425	<i>Ornithopus compressus</i> (inoculum strain)	Esperance, WA	Nod ⁺ Fix ⁺	28
WU8	<i>Lupinus cosentinii</i>	WA	Nod ⁺ Fix ⁺	21
AI3	<i>Lupinus angustifolius</i> (inoculum strain)	Guildford, WA	Nod ⁺ Fix ⁺	21
VK7	Inoculum strain	SA	Nod ⁺ Fix ⁺	N. Amarger, France
W72	<i>Lupinus cosentinii</i> (inoculum strain)	WA	Nod ⁺ Fix ⁺	J. Howieson (CRS)

^a Abbreviations: SA, South Africa; NSW, New South Wales; WA, Western Australia.

^b Shoot dry weight of plants showing Nod⁺ Fix⁺ phenotype was significantly greater than that of uninoculated controls ($P < 0.05$).

^c CRS, Centre for Rhizobium Studies, Murdoch University, Australia.

^d ND, not defined.

geographical regions (26). This work has differentiated three major clades among the *Bradyrhizobium nodA* sequences, one of which, called clade II, was composed exclusively of the strains isolated from lupins, broom (*Cytisus scoparius*), and serradella. Similar phylogenetic patterns detected for *nodC* and *nifH* gene sequences corroborated the unique position of the symbiotic genes of lupin and serradella bradyrhizobia (15, 49, 50). These findings led to the delineation of bradyrhizobia infecting legumes of the Genistae and Loteae tribes in a new biovariety, genistearum. This single ecotype comprises bradyrhizobia that group with the species *B. japonicum* (*B. japonicum* bv. genistearum) together with the strains that form a related sister lineage, for which a new name, *Bradyrhizobium canariense* bv. genistearum, was recently proposed, and at least two other additional lineages called the alpha and beta genotypes (49, 50).

In the present study, we took advantage of the unique phylogenetic position characterizing lupin and serradella bradyrhi-

zobia symbiotic genes to elucidate the origin of the diverse naturalized populations persisting in soils of Western Australia and South Africa. To our knowledge, this is the first attempt to infer the origin of naturalized rhizobia using multilocus phylogenetic analyses based on both symbiotic and housekeeping genes.

MATERIALS AND METHODS

Bacterial strains. *Bradyrhizobium* strains included in the study and their characteristics are described in Table 1. A table describing reference strains used in the phylogenetic trees is presented in the supplementary material. Yeast extract mannitol agar medium (48) was used for growth and maintenance of strains. All *Bradyrhizobium* strains were grown at 28°C.

The *Bradyrhizobium* strains were collected in the winter rainfall region of Western Australia and the South African Western Cape region. The selected Western Australian strains represented *Bradyrhizobium* populations isolated from three sites (Albany, Carrabin, and Yelbeni). Isolates were recovered either from the nodules of serradella plants growing at the sites (*Ornithopus compressus*

TABLE 2. Oligonucleotides used for PCR amplification^a

Primer	Sequence (5'–3')	Target gene (position)	Reference
TSnodA2b	GATTCCVWGBCCYTCVAGATC	<i>nodA</i> ^b (345–325)	This study
TSnodD1-1c	CAGATCNAGDCCBTTGAARCGCAT	<i>nodD1</i> ^b (24–1)	This study
TSnodB2N	CTGTGRTTHGCRAYCTYRTGYCC	<i>nodB</i> ^b (239–217)	This study
TSnodZ3	GGTTTCGGYGAYTGYCTBTGGTC	<i>nodZ</i> ^b (40–62)	26
TSnodZ4	AATRTCTTCGCCRTTRCCRTGCC	<i>nodZ</i> ^b (552–530)	26
TSnoeI1	CTGGATATCGGYTGCAATGATGG	<i>noeI</i> ^c (64–86)	26
TSnoeI2	TCGGCYCCCTGMACTCCATCCA	<i>noeI</i> ^b (452–430)	26
TSnoLlF	CTCTTTATGGCRATAAGYGGGA	<i>nolL</i> ^b (211–231)	This study
TSnoLlR	CAAATCCATGAGRCGAAACAC	<i>nolL</i> ^b (936–916)	This study
TSrecA	CAACTGCMYTGCGTATCGTCGAAGG	<i>recA</i> ^c (8–32)	This study
TSrecAr	CGGATCTGGTTGATGAAGATCACCATG	<i>recA</i> ^c (620–594)	This study
TSatpDf	TCTGGTCCGYGGCCAGGAAG	<i>atpD</i> ^c (189–208)	This study
TSatpDr	CGACACTTCGCGARCCGCCTG	<i>atpD</i> ^c (804–784)	This study
TSglnII	AAGCTCGAGTACATCTGGCTCGACGG	<i>glnII</i> ^c (13–38)	This study
TSglnIIR	SGAGCCGTTCCAGTCGGTGTCTG	<i>glnII</i> ^c (681–660)	This study
TS-ISR1	GTGAAGTCGTAACAAGGTAGCCGTAG	<i>rrn16S</i> ^c (1437–1462)	This study
TS-ISR2	GTGCTTTTACCTTTCCCTCAC	<i>rrn23S</i> ^c (590–569)	This study
TSdnaK2	GTACATGGCCTCGCCGAGCTTCA	<i>dnaK</i> ^c (1794–1772)	39
TSdnaK3	AAGGAGCAGCAGATCCGCATCCA	<i>dnaK</i> ^c (1468–1490)	39

^a All primers except TS-ISR2 were used for amplification and sequencing of PCR products.

^b Position of the primer in the corresponding sequence of *Bradyrhizobium* sp. strain WM9.

^c Position of the primer in the corresponding sequence of *Bradyrhizobium japonicum* USDA 110.

and *Ornithopus sativus*) or from the nodules of bait plants grown in soil collected from the sites (*O. compressus*, *O. sativus*, and *L. angustifolius*) (24). The Albany, Carrabin, and Yelbeni sites were characterized by acid (pH 4.6 to 6.4) soils. At all sites, either narrow-leaf lupin or serradella had been grown prior to the isolation of strains. At each site, the inoculant strain WU425 had been extensively applied by farmers for the 20 years prior to sampling, and additionally, at the Yelbeni site, the inoculant strain WSM471 had been introduced (13). The selected strains belonged to distinct randomly amplified polymorphic DNA (RAPD)-PCR clusters, as assessed using the combined datasets (24) for the primers RP04, RP05, and RP01 (35). The analysis of RAPD-PCR profiles revealed large diversity (>50% dissimilarity between most strain types), showing that all populations were composed of multiple strain types (24). The selected strains represent both dominant and unique strain types for the given site; however, all had RAPD-PCR profiles that were distinct from the WU425 and WSM471 inoculant strains isolated in Western Australia from serradella nodules (13, 28). Five strains, including WSM468, WSM469, WSM1420, WU8, and WU140, originated from uncultivated *Lupinus cosentinii* plants growing at Albany, Western Australia. Two strains, WSM2155 and WSM2156, were isolated from nodulated plants of *Daviesia nudiflora* Meissn. and *Mirbelia floribunda* Benth., respectively, growing at Carrabin within 20 m of serradella trials and a narrow-leaf lupin crop. We also included the A13 and W72 strains that had been used as inocula in Western Australia before strains WU425 and WSM471 were developed (5). Four strains originated from native Western Australian legumes, whereas six other strains were isolated by Lafay and Burdon (19) from native legumes growing in New South Wales (Table 1).

Unlike Western Australian strains, all five South African strains were isolated from *Lupinus angustifolius* nodules. The lupin plants were grown in four moderately acid (pH 5.5 to 5.8) soil samples collected at Hopefield, Langewens, Eendekuil, and Tygerhoek, and one moderately alkaline (pH 7.6) soil from Roodebloem. Strains 1S20, 2S9, 3S16, and 5S10 originated from the acid soils, whereas strain 4S16 came from the alkaline site. The acid soils hosted large, >5,000 rhizobia g of soil⁻¹, and diverse *Bradyrhizobium* populations, as RAPD-PCR analyses with BOXA1R, ERIC, M13 phage, and REP primers showed. On the other hand, the alkaline soil population was small, <400 rhizobium cells g of soil⁻¹, and homogenous. These strains were serologically distinct from the current inoculant strain WU425, imported from Australia, and the preceding strain VK7 isolated in the Western Cape from lupin (4).

All strains were tested for their ability to nodulate yellow serradella (*O. compressus* cv. Avila) plants as described elsewhere (38).

Molecular techniques. For PCR amplification experiments, total genomic DNA was isolated using the sodium dodecyl sulfate-proteinase K lysis procedure described by Moulin et al. (26) or using the QIAGEN QIAamp DNA mini kit following the recommendations of the producer. All primers used for PCR amplification are listed in Table 2. The *dnaK* sequences were amplified and

sequenced by following the procedure described elsewhere (39). Other markers—*nodA*, *nodZ*, *noeI*, *nolL*, intergenic transcribed spacer (ITS), *atpD*, *glnII*, and *recA* gene fragments—were amplified using the following procedure: 95°C for 2 min followed by 35 cycles of 95°C for 45 s, 58°C 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 producer (Roche Diagnostics GmbH, Germany). The annealing temperature for amplification of *nodA*, *nodZ*, and *noeI* was 53°C, whereas the annealing temperature for *nolL* was 50°C. The PCR products were purified with a QIAquick gel extraction kit (QIAGEN, Germany). The sequencing reactions were performed as described elsewhere (26). The sequences were deposited in the EMBL database under the accession numbers given in Table 3.

Sequence analyses. Multiple nucleotide sequence alignments were generated using ClustalX (45) and optimized manually using Genedoc software (27). All phylogenetic analyses were performed using PAUP, version 4.0b10 (44). Due to the large number of sequences, parsimony and maximum-likelihood (ML) analyses were performed using the heuristic search option of PAUP.

An intragenic recombination test was assessed on each alignment using recombination detection prediction (23), which combines the RDP, GeneConv, Bootscan, MaxChi, Chimaera, and SiScan programs. Intragenic recombination (by at least two programs) was detected only for strain 4S16 in the *glnII* phylogeny. This strain was retained in the phylogenetic trees, though its placement should be considered as unresolved in the *glnII* phylogeny.

For the *nodA*, *nodZ*, *noeI*, *nolL*, *atpD*, *dnaK*, *glnII*, and *recA* gene phylogenies, ML and neighbor-joining (NJ) analyses were performed using PAUP (44). The best-fit model for each gene was assessed using MODELTEST3.6 (33). ML phylogenies using a codon partition were also performed, i.e., specific substitution rates were evaluated following the codon structure of the DNA sequence (this model allows one to estimate the site-specific substitution rates and thus evaluate data saturation, which may disturb the phylogenetic signal). The best substitution models found for each marker were as follows: *dnaK*, TrN+G (Tamura-Nei plus Gamma); *glnII* and *recA*, GTR+I+G (General Time Reversible with invariant sites and a gamma rate distribution); *atpD*, codon site-specific rates.

The *nodA* phylogeny was constructed using the same ML model as described by Moulin et al. (26). The base frequencies, Ti/Tv ratios, and substitution rates were estimated at each position from the data using ML analyses. The third position in the codon was excluded from the analyses, as it accumulated too much saturation (26). The ITS phylogeny was assessed using parsimony analyses as described by Vinuesa et al. (49). Parsimony analyses allow gaps to be treated as informative (as a fifth base) and were preferred due to the high number of insertions/deletions found in the 16S-23S intergenic sequence. The ITS alignment consisted of a 1,056-bp fragment, of which 374 bp showed insertion/deletion events. This alignment can be obtained upon request. The ITS phylogeny was assessed using only *Bradyrhizobium* strains, as ITS from outer-group

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

Strain	Accession number assigned to amplified gene								
	<i>dnaK</i>	ITS	<i>recA</i>	<i>atpD</i>	<i>glnII</i>	<i>nodA</i>	<i>nodZ</i>	<i>noI</i>	<i>noeI</i>
1S20	AJ891170	AJ891238	AJ891263	AJ891286	AJ891294	AJ890296	AJ891184	+	—
3S16	AJ891171	AJ891239	AJ891264	AJ891287	AJ891295	AJ890297	AJ891185	+	—
2S9	AJ891172					AJ890308	+	+	—
4S16	AJ891173	AJ891240	AJ891265	AJ891288	AJ891178	AJ890289	+	AJ891196	—
5S10	AJ891174					AJ890299	+	+	—
BDV5029	AJ891175	AJ891241	AJ891266			AJ890291	—	ND	—
BDV5057	AJ891176	AJ891242	AJ891267			AJ890311	—	ND	—
BDV5173	AJ891177					AJ891168	—	ND	—
BDV5325	AJ891188		AJ891268			AJ890290	—	ND	—
BDV5493	AJ891199					AJ890312	—	ND	—
USDA3001	AJ919269								
USDA3475	AJ919270								
WSM1253	AJ891209					AJ890292	+	ND	—
WSM1420	AJ891210					AJ890316	+	ND	—
WSM1704	AJ891211	AJ891243	AJ891269			—	—	ND	—
WSM1735	AJ891212	AJ891244	AJ891270			AJ890293	AJ891186	ND	AJ891207
WSM1743	AJ891213	AJ891245	AJ891271			AJ890285	AJ891187	ND	AJ891208
WSM1790	AJ891214	AJ891246	AJ891272			AJ890286	+	ND	+
WSM2148	AJ891215	AJ891247	AJ891273			AJ890302	AJ891189	+	—
WSM2149	AJ891216	AJ891248	AJ891274			AJ890319	+	AJ891197	—
WSM2150	AJ891217	AJ891249	AJ891275	AJ891289	AJ891179	AJ890320	+	+	—
WSM2151	AJ891218	AJ891250				AJ890303	+	+	—
WSM2152	AJ891219	AJ891251	AJ891276			AJ890304	AJ891190	AJ891198	—
WSM2153	AJ891220					AJ890305	+	+	—
WSM2154	AJ891221	AJ891252	AJ891277	AJ891290	AJ891180	AJ890306	AJ891191	AJ891200	—
WSM2155	AJ891222	AJ891253	AJ891278			AJ890310	AJ891192	AJ891201	—
WSM2156	AJ891223	AJ891254				AJ890294	+	+	—
WSM2157	AJ891224	AJ891255	AJ891279	AJ891291	AJ891181	AJ890301	+	AJ891202	—
WSM2158	AJ891225					AJ891169	+	+	—
WSM2159	AJ891226					AJ890288	AJ891193	AJ891203	—
WSM2458	AJ891227					AJ890287	+	+	—
WSM468	AJ891228	AJ891256				AJ890315	+	+	—
WSM469	AJ891229					AJ890309	+	+	—
WSM471	AJ891230	AJ891257	AJ891280	AJ891292	AJ891182	AJ890307	+	AJ891204	—
WSM866	AJ891231					AJ890298	+	AJ891205	—
WU140	AJ891232	AJ891258	AJ891281			AJ890313	+	AJ891206	—
WU425	AJ891233	AJ891259	AJ891282	AJ891293	AJ891183	AJ890300	AJ891194	+	—
WU8	AJ891234					AJ890314	+	+	—
A13	AJ891235	AJ891260	AJ891283			AJ890318	AJ891195	+	—
VK7	AJ891236	AJ891261	AJ891284			AJ890295	+	+	—
W72	AJ891237	AJ891262	AJ891285			AJ890317	+	+	—

^a Amplification conditions of all genes listed are described in Materials and Methods. ND, not determined; +, amplification of PCR products not sequenced; —, lack of amplification.

bacteria would be impossible to align correctly due to their large sequence divergence. For the *atpD*, *recA*, *dnaK*, and *glnII* phylogenies, the bootstrapping analyses were performed using the heuristic searches under distance and ML models (using PAUP) with 1,000 and 100 replicates, respectively. Due to the large datasets, bootstrap values on *nodA* and ITS phylogenies were assessed using only parsimony and NJ analyses.

The partition homogeneity tests (100 random trees; 1,000 replicates) and Shimodaira-Hasegawa tests of congruence of tree topologies were performed using PAUP on a restricted data set of 13 *Bradyrhizobium* strains (USDA94, USDA76, 1S20, 4S16, USDA110, WSM2150, WSM2154, WSM2157, WSM471, WSM2150, 3S16, BC-C2, and WU425) for which sequences were available for all markers. The result of this analysis is presented in the supplemental material.

Nucleotide sequence accession number. Sequences were deposited in the EMBL database under the accession numbers given in Table 3.

RESULTS

***Bradyrhizobium canariense* dominates serradella and lupin isolates from Western Australia and South Africa.** The nodulation and nitrogen fixation abilities of the strains were evaluated on yellow serradella (*O. compressus* cv. Avila). Pink nod-

ules were formed on the plants inoculated with Western Australian serradella strains and with four of the five South African narrow-leaf lupin strains. The exception was strain 4S16 (the alkaline soil isolate), which formed only small, white nodules. Effective nodules were also found on serradella plants inoculated with the blue lupin (*L. cosentinii*) isolates and with strains WSM2155 and WSM2156 (isolated from two Western Australian native species). No nodules were found on plants inoculated with other Western Australian and New South Wales native legume isolates (Table 1).

The taxonomic position of each *Bradyrhizobium* strain used in this study was initially verified by phylogenetic analysis of *dnaK* gene sequences. The *dnaK* tree was constructed with partial (278 bp) sequences, encoding the variable 3' end gene fragment (Fig. 1A). This part of the 70-kDa chaperone gene accumulates a large number of substitutions, giving a tree topology that corroborates trees of other gene phylogenies, including 16S rRNA, *recA*, and *glnII* gene markers (39). We

FIG. 1. (A) ML tree based on partial *dnaK* sequences; (B) maximum-parsimony tree based on complete 16S-23S rRNA intergenic transcribed sequences. New strains from this study are indicated in boldface type. Host plant and geographical origin are indicated between brackets. The scale bar (only for the ML *dnaK* tree) indicates the number of substitutions per site. The bootstrap values indicated were calculated under distance criteria (1,000 replicates). Abbreviations: B., *Bradyrhizobium*; BC, *B. canariense*; B. jap., *B. japonicum*; B. liaon., *B. liaoningense*; B. yuan., *B. yuanmingense*; B. IX, *Bradyrhizobium* genospecies IX (defined by Willems et al.) (52); Rho., *Rhodopseudomonas*; NSW, New South Wales Australia; WA, Western Australia; CI, Canary Islands; SA, South Africa; USA, United States; La, *Lupinus angustifolius*; Lc, *Lupinus cosentinii*; Oc, *Ornithopus compressus*; Op, *Ornithopus pinnatus*; Os, *Ornithopus sativa*; Cham., *Chamaecytisus proliferus*; Ad, *Adenocarpus*; Te. and Tel., *Teline*; Lesp., *Lespedeza*; L., *Lupinus*; G., *Glycine*; F., *Faidherbia*; inoc., inoculum.

decided to use additional markers, taking into account the uncertainty related to phylogenetic inference based on a single gene marker analysis, especially for the genus *Bradyrhizobium* (29). The selected gene markers included the ITS region between the 16S rRNA (*rrs*) and 23S rRNA (*rrl*) genes, ATP synthase beta chain (*atpD*), glutamine synthetase II (*glnII*), and recombination protein *recA* genes. All of these gene markers have been used by other workers for phylogenetic reconstructions of *Rhizobiaceae* (9, 46, 49, 50, 51). It is noteworthy that the selected genes occupy distinct positions on the chromosome of *Bradyrhizobium japonicum* strain USDA 110 (17). Thus, assuming that synteny occurs between *Bradyrhizobium* chromosomes (31), we expected that these gene markers would yield phylogenetic reconstructions indicative of the whole genome (not just single genes).

ML and NJ phylogenies of *dnaK* revealed that, apart from two lupin isolates, 1S20 and 4S16 from South Africa, the remaining lupin and serradella strains formed a well-defined

branch (78% bootstrap support), that was related to (but distinct from) the *Bradyrhizobium japonicum* sequences (Fig. 1A). The only sequence that was determined earlier (39), and which clustered in this new branch, was the sequence of *B. canariense* strain BC-C2. Two isolates from Australian native legumes (WSM2155 and WSM2156) were also located in this cluster. Pairwise sequence similarity within this cluster ranged from 94% to 100%, although the majority of sequences were 98 to 99% related. The sequence of the inoculant strain W72 was identical to the sequences of strains WSM866, WSM2153, WSM2154, and 5S10. Similarly, two widely used inoculant strains, WU425 and VK7, showed identical *dnaK* sequences, as well as strains WSM2150, WSM2156, and WU140; WSM468, WSM2149, and WU8; and WSM2151, WSM2155, WSM2158, WSM2159, and WSM2458.

ML and NJ phylogenetic trees of the ITS sequences of 25 strains confirmed the relationships found for lupin and serradella strains using the *dnaK* phylogeny. Each tree contained

FIG. 2. ML phylogenetic trees based on partial *recA* (A), *glnII* (B), and *atpD* (C) sequences. New strains from this study are indicated in boldface type. Scale bars indicate the number of substitutions per site. The bootstrap values indicated were calculated under distance criteria (1,000 replicates); they are in boldface type when a bootstrap value of $\geq 80\%$ was obtained also using ML models (100 replicates). Abbreviations are defined in the legend to Fig. 1.

a well-supported branch which, in addition to the lupin and serradella strains under investigation, was composed exclusively of previously sequenced *B. canariense* strains (Fig. 1B). The *B. canariense* branch essentially showed the same grouping as the *dnaK* tree. Thus, strains A13, W72, WU425, VK7, and WSM2154 had identical ITS sequences, as did WSM2150 and WU140, WSM468 and WU8, and WSM2151 and WSM2155. *B. canariense* strain ISLU16 was identical to strains A13, W72, WU425, VK7, and WSM2154. The remaining strains from Western Australia shared $\geq 98\%$ similarity with the other *B. canariense* strains. Similar topology was revealed in the *recA* phylogenetic tree obtained with 23 *recA* strain sequences (Fig. 2A), confirming the distinct position of lupin and serradella isolates and their grouping with *B. canariense*. The grouping with the *B. canariense* branch was further supported by ML and NJ trees of *glnII* (Fig. 2B) and *atpD* (Fig. 2C), although the latter two genes were sequenced in only eight strains.

Strains 1S20 and 4S16 were the only exceptions among the lupin and serradella isolates that consistently grouped outside the *B. canariense* branch. However, their placement differed

between the phylogenetic trees, according to the marker gene used. On the *dnaK* tree (Fig. 1A), 1S20 formed a distinct clade with BDV5029, BDV5493, and USDA3001, whereas on the *recA* tree (Fig. 2A), it grouped with BDV5029. Strain 1S20 also occupied distinct positions on the *atpD* and *glnII* trees, although the placement was weakly supported (Fig. 2B and C). On the ITS tree, strain 1S20 grouped with 4S16, close to *B. japonicum* type strain USDA 6. On the *recA* and *atpD* trees, strain 4S16 grouped close to *Bradyrhizobium liaoningense* strain LMG18230, while on the *dnaK* tree strain 4S16 occupied a distinct position. These phylogenies suggested that strain 1S20 may represent a new lineage, whereas 4S16 may belong to *B. liaoningense* or be related to this species.

The *dnaK* and *recA* trees showed that native Australian legume bradyrhizobia (other than strains WSM2155 and WSM2156) were either situated within the *B. japonicum* clade (BDV5325), occupied a position close to the *Bradyrhizobium yuanmingense* cluster (WSM1743), or formed a cluster with *B. elkanii* strains (WSM1704). Strain BDV5057 occupied a distinct position on the *dnaK* tree, whereas it was placed in the *B. yuanmingense* cluster on the ITS tree and closer to *B. japonicum* on the

FIG. 3. ML phylogenetic tree of *nodA* sequences. The tree was constructed by the ML approach with a model described in Materials and Methods. Details of the model are summarized in the inset. Only the *Bradyrhizobium*-*Burkholderia*-*Methylobacterium* branch is shown. The scale bar indicates the number of substitutions per site. Bootstrap values (percentage of 1,000 replicates under distance criteria, only values >75%) are given at the branching nodes. *Bradyrhizobium* country of origin and host plant are in brackets. New strains from this study are indicated in boldface type. Clade numbers correspond to the *nodA* phylogeny defined by Moulin et al. (27). Abbreviations are defined in the legend to Fig. 1.

recA tree (Fig. 1 and 2). Two other strains, WSM1735 and WSM1790, formed a separate clade on the ITS and *recA* trees, whereas on the *dnaK* tree, they grouped with *B. japonicum*.

Partition homogeneity (incongruence length difference) tests and Shimodaira-Hasegawa tests of congruence of tree topologies (see the supplemental material) confirmed in part the analogy of topologies obtained in the ITS, *dnaK*, *recA*, *atpD*, and *glnII* phylogenies but only on restricted datasets. Topology congruence was found between the ITS, *atpD*, and *dnaK* trees but not between the *glnII* and *recA* trees. Partition homogeneity tests

also reflected homogeneity between markers on small partitions (2 to 3 markers) but not on partitions implementing all markers (see the supplemental material). Such statistical incongruence between housekeeping genes has already been reported, even if trees show identical topologies (9, 46). We thus relied on bootstrap values to distinguish clades in the phylogenetic trees. Our data indicated that the Western Australia serradella isolates and three South African narrow-leaf lupin isolates grouped with *B. canariense* strains with high bootstraps and thus represent authentic *B. canariense* isolates, although

FIG. 4. ML trees of *nodZ* (A), *nolL* (B), and *noeI* (C) sequences. New strains from this study are indicated in boldface type. Scale bars indicate the number of substitutions per site. Bootstrap values (percentage from 100 replications) are indicated at nodes when higher than

DNA-DNA hybridizations would be needed to confirm our results.

Similarity of lupin and serradella *nodA* gene sequences to clade II bradyrhizobia. Following the strategy described earlier (26), we amplified in all strains from lupin and serradella nodules a DNA fragment that comprised the intergenic region between *nodD* and *nodA*, the complete *nodA* sequence, and part of the *nodB* gene. An exception was WSM2158, for which only a partial sequence covering the 5' half of the gene was obtained. Moreover, we amplified the *nodA* gene in three strains from legumes native to Western Australia, except strain WSM1704, for which we could not obtain a *nodA*, nor any other *nod* gene (*nodZ*, *nolL*, and *noeI*) product. Complete *nodA* gene sequences were, however, amplified in five (of the six) *Bradyrhizobium* strains that originated from New South Wales native legume species.

All *nodA* sequences of the strains isolated from lupins and serradella were closely related, with identities ranging from 90 to 100%. In the ML tree, all sequences formed a common branch (100% bootstrap support) with other lupin and serradella strains of European or North American origin as well as with strains from other species of the tribe Genisteae isolated in Europe and Japan (Fig. 3) and strains WSM2155 and WSM2156, isolated from Western Australian native legumes. This branch was earlier referred to as clade II (26). However, only *nodA* sequences of *L. cosentinii* isolate WU140 and the narrow-leaf lupin 4S16 were intermingled with formerly sequenced strains. The remaining lupin and serradella strains from Western Australia and South Africa formed a distinct subgroup within clade II, with 95% bootstrap support. This subgroup is composed of very similar sequences (identity ranging from 97% to 100%). The strains WSM2151, WSM2152, WSM2155, WSM2157, and WSM2159 had identical sequences. Similarly, strains WU425, WSM2153, and 3S16; WSM2148 and WSM2156; and WSM2154 and WSM2458 had 100% similarity. This branch included *nodA* sequences of strains WSM866 and WSM1253 originating from the Mediterranean (Greece) which have never been applied as inocula in Western Australia.

The *nodA* sequence of WSM1743 grouped within clade III (26), which includes sequences of strains mainly of (sub)tropical origin. Two other native legume isolates from Western Australia, WSM1735 and WSM1790, formed a separate clade that occupied a sister position with respect to clade II. This new clade is termed clade IV. The *nodA* sequences of the two strains were 98% similar. All five New South Wales native legume isolates grouped in a branch called clade I together with *nodA* sequences of two strains isolated in South Africa and Brazil, respectively, from *Acacia* spp. native to Australia (26). All *nodA* sequences within clade I showed 98 to 99% identity. All deduced NodA proteins were composed of 211 amino acid residues (unlike the majority of *Bradyrhizobium* NodA proteins built of 210 residues), with a single codon

50% (using ML). Strains or groups of strains belonging to clades defined in the *nodA* phylogeny (Fig. 3) are indicated on nodes. Abbreviations: *L.*, *Lupinus*; *O.*, *Ornithopus*; *A.*, *Aeschynomene*; *Ast.*, *Asragalus*; *R.*, *Rhizobium*; *M.*, *Mesorrhizobium*; *C.*, *Crotalaria*.

insertion (aspartate 183) in the C-terminal part, which seems a distinctive characteristic of this clade (data not shown).

PCR amplification revealed the presence of the *nodZ* gene in all lupin and serradella isolates. Additionally, this gene was amplified in strain WSM1743 of clade III, and in strains WSM1735 and WSM1790 of clade IV. The *nodZ* gene was not amplified from the template DNAs of the New South Wales isolates which represented clade I (Table 3). This gene is responsible for fucosylation of Nod factor reducing end glucosamine, which appears to be a common Nod factor modification in the genus *Bradyrhizobium* (8, 34, 36). Phylogenetic sequence analysis of the *nodZ* sequences of strains belonging to clades II, III, and IV yielded essentially the same branching pattern as that of the *nodA* tree. The above data were consistent with the previous study (26), which showed that strains belonging to clades II and III carry the *nodZ* gene, whereas the strains of clade I do not (Fig. 4A).

Further analysis showed that all of the lupin and serradella bradyrhizobia except WSM1420 (for which no PCR product was obtained) carried the *nolL* gene, which is involved in acetylation of Nod factor fucose residues (2). In lupin strain WM9, a *nolL* homologue is located 5 kb downstream of the *nodW* gene (38). Considering that in soybean strain USDA 110, the common *nodABC* genes are separated from the *nodVW* operon by 300 kb (17), *nolL* may be similarly distant in lupin strains and may represent a distinct part of the symbiosis island. Previously, *nolL* was detected in all strains belonging to *nodA* clade II and in two strains of clade III but not in clade I (26). In the present work, we found it difficult to amplify this gene even in lupin and serradella bradyrhizobia, although we used more specific primers. Thus, we decided to exclude the strains of the remaining branches (I, III, and IV) from PCR amplification assays. The *nolL* sequences obtained were more divergent than those of the *nodA* and *nodZ* genes, with pairwise comparisons showing sequence similarities ranging from 87% to 100%. Nevertheless, the *nolL* phylogenetic tree displayed similar topology to that of trees derived from the *nodA* and *nodZ* genes (Fig. 4B).

The *noeI* gene is involved in the methylation of the Nod factor fucose residue, a widespread modification in broad-host-range rhizobia (14). The *noeI* in the genome of *B. japonicum* strain USDA 110 is located nearly 40 kb downstream of the common *nod* gene operon (17). Although this gene is widespread in clade III bradyrhizobia, it appears to be missing in clades I and II (26). Our present study confirmed the earlier data. The *noeI* gene was found in a strain belonging to clade III (WSM1743) and in strains WSM1735 and WSM1790 of the new clade IV. It was not, however, detected in the lupin and serradella strains nor in the clade I strains. Phylogenetic analysis of the *noeI* sequences confirmed the distant position of clade IV strain WSM1735 with respect to the strains of clade III. Although this corroborated the *nodA*-derived phylogeny, the position of WSM1735 on the *noeI* tree remained unresolved (Fig. 4C).

DISCUSSION

Application of legume inoculants may lead to the establishment of inoculated strains of rhizobia in soils, especially when a new crop is introduced. Well-established soil populations

often, however, contain rhizobia belonging to serologic and genomic types different from those of applied inoculum strains. For example, appreciable proportions of *Bradyrhizobium* populations infecting lupins and serradella in Western Australia and South Africa differ from the inoculum strains WU425 and WSM471 or VK7 used in the respective countries (4, 24). This genetic heterogeneity among naturalized rhizobia could have resulted from three different processes: (i) accidental introduction on soil-contaminated seeds (32) or through inoculation using soil from fields of legumes established elsewhere (40), (ii) acquisition by native strains of symbiosis genes from introduced inoculum strains by lateral gene transfer (41), or (iii) the presence of indigenous rhizobia capable of infecting the new legume species (20).

Examination of the phylogenetic relationships of nodulation and housekeeping genes showed that serradella and lupin bradyrhizobia in Western Australia and South Africa are closely related to strains originating from southern Europe. Together with the related finding that *Rhizobium etli* strains are carried by bean seeds (32), this evidence favors the first possibility, that they were introduced on imported seeds from Europe. Our data show remarkable consistency in phylogenetic placement of the majority of lupin and serradella strains within the respective gene trees, in all of which they form discrete branches (Fig. 1 and 4). This corresponds well with the present evidence that lupin and serradella bradyrhizobia originating from Europe form separate clades on *nodA* and *nodC* trees (15, 26, 38, 49, 50). Their unique position on symbiotic gene trees allows lupin and serradella bradyrhizobia of mainly European origin to be distinguished from bradyrhizobia nodulating legumes other than Genisteae and Loteae. The *nodA* sequences within clade II show $\geq 90\%$ identity, which is much higher, for instance, than identity within divergent clade III (Fig. 3 and 4). Excluding strain WU140, which shows 90% sequence identity, the remaining *nodA* sequences of the lupin and serradella strains from Western Australia and South Africa showed 97 to 100% identity and were practically indistinguishable from the two strains from Greece included in this study. This additionally contradicts the hypothesis of an indigenous origin of *nod* gene sequences in the lupin and serradella symbionts. In Western Australia, for example, one would expect higher divergence between European strains nodulating lupins and serradella and native Australian strains infecting these legumes. Likewise, the general similarity of *nod* gene sequences of the South African lupin isolates to the lupin and serradella strains from Western Australia and Greece and their dissimilarity with respect to other sequenced African isolates (26) makes it unlikely that their sequences are of native origin.

There is evidence that lateral gene transfer is responsible for dispersal of symbiosis genes among diverse bacteria of the α - and β -*Proteobacteria* groups (25, 37, 42, 43). The second possibility of transfer to indigenous strains is extremely unlikely, however, considering the observed relatedness between housekeeping genes of the Australian and South African isolates and the European lupin and serradella strains (discussed in the next paragraph). Lateral gene transfer of the symbiosis loci provides, however, an acceptable explanation for the presence of similar *nodA* sequences of clade II in different *Bradyrhizobium* lineages (26). In the present study, for example, we found that the *nodA* clade II sequences are carried by strains of

B. canariense sp. nov. and two *Bradyrhizobium* lineages (as shown by the housekeeping gene phylogenies), one of which (4S16) may be related to *B. liaoningense*, while the other strain (1S20) may form a new clade in this genus.

Rhizobia from many native legumes in Western Australia effectively nodulated several lupin species (*L. albus*, *L. cosentinii*, and *L. pilosus*) but were unable to infect *L. angustifolius*, *O. compressus*, or *O. sativus* (11, 20, 21, 22). The present study does not, however, support the third possibility that the bradyrhizobia isolated from *L. cosentinii* have an Australian origin. First, housekeeping gene phylogenies reveal that the blue lupin (*L. cosentinii*) isolates belong to the same *Bradyrhizobium canariense* clade as strains of serradella from Western Australia and three of five South African narrow-leaf lupin strains (Fig. 1 and 2). Second, the nodulation genes of the blue lupin isolates group within the same clade II as the strains isolated from serradella species and narrow-leaf lupin (Fig. 3 and 4). Furthermore, most of the strains isolated from Australian native legumes showed no phylogenetic affinity of their nodulation genes to the lupin and serradella isolates (with the exception of strains WSM2155 and WSM2156). Instead, they group in the distinct *nod* gene clusters I, III, and IV. It appears possible that the *nodA* gene clade I may be of Australian origin despite the fact that the first two clade I *nodA* sequences originated from strains isolated in South Africa and Brazil. This clade comprises 98 to 99% similar *nodA* sequences, whereas the housekeeping gene phylogenies indicate various genomic types. Moreover, the New South Wales strains were collected in different places from the nodules of native legumes representing various legume tribes, including two, *Bossiaeeae* and *Mirbelieae*, that are endemic to Australia (Table 1). Likewise, clade IV may represent another set of strains that coevolved with native Australian legumes. This implies that geographical separation preventing strain migration and recombination of symbiotic loci may be an essential factor responsible for the observed divergence patterns of nodulation genes. This would agree with recent observations that *nifD* sequences group according to a strain's geographical origin, unlike 16S rDNA sequences, for which such a correlation has not been found (30). It remains to be established whether the phylogenetic pattern shown by the *nodA* tree is due to a vicariance related to tectonic isolation of Australia some 32 million years ago or climatic changes contributing to rapid radiation of Australian sclerophyllous flora (including legumes) in the mid-Cenozoic period between 25 and 10 million years ago (7).

Although phylogenetic evidence in this study does not indicate that our lupin and serradella strains are of indigenous origin, two isolates from native Western Australian legumes (strains WSM2155 and WSM2156) were capable of nodulating serradella (Table 1). Both shared a high level of *dnaK*, ITS, *recA*, *nodA*, *nodZ*, and *nolL* sequence homology with the lupin, serradella, and other *B. canariense* strains and probably represent lupin and serradella bradyrhizobia. As the respective legumes from which these strains were isolated (*Daviesia nudiflora* and *Mirbelia floribunda*) were located within 20 m of serradella trials and a lupin crop, we believe that *Daviesia nudiflora* and *Mirbelia floribunda* are promiscuous legumes that can be nodulated by lupin and serradella bradyrhizobia as well as indigenous rhizobia. This would accord with the observation that five native legumes (seed harvested from the Carrabin

site) were nodulated by a cocktail of lupin and serradella strains originating from the Carrabin and Albany sites in Western Australia (24). The presence of alternate hosts may have facilitated the spread of lupin and serradella rhizobia throughout Western Australia after introduction from Europe.

Previous RAPD-PCR studies detected considerable genomic diversity among the lupin and serradella bradyrhizobia populations found in Australia and South Africa (4, 24). Although gene sequences, especially *nod* sequences of the lupin and serradella strains, were shown to be closely related and sometimes identical, well-defined distinct groups were detected. This diversification is unlikely to have occurred in the relatively short period of about a century following their introduction into soils of Western Australia and South Africa, since the divergence rate of conserved housekeeping genes is too low to explain the observed sequence diversity (46). It rather appears that these soils were initially infected and colonized by a variety of related strain types. Interestingly, all of the lupin and serradella strains from Western Australia and three of five South African strains group with *Bradyrhizobium canariense* sp. nov. *B. canariense* is a newly described species that originates from the Mediterranean region and is well adapted to acid soils common in Western Australia and South Africa (49, 50). Soil conditions often determine strain dominance. For example, soybean serocluster 135 (strains classified as *B. liaoningense*) is dominant in soils of high pH in the United States and South Africa (3, 47). It is noteworthy, therefore, that lupin strain 4S16 from the alkaline Roodebloem soil grouped with *B. liaoningense* in the *recA* and *atpD* trees (Fig. 2). Presumably, soil environment influences the survival of certain phylogenetic types. Dominance of the *B. canariense* strains in soil may also be related to compatibility with lupin and serradella, as reflected by the position of the *nod* genes of lupin and serradella strains on the phylogenetic trees presumably resulting from an evolutionary process interwoven with that of their natural hosts.

In conclusion, this study provided evidence that accidental cotransfer of effective lupin *Bradyrhizobium* strains, possibly by means of soil-contaminated seeds, accompanied the introduction of lupin and serradella from Europe to soils of Western Australia and South Africa. As in the transfer of seed-borne diseases, precautions against similar occurrences should be taken when introducing new legume crops to a region. Efforts to optimize nitrogen fixation through the inoculation of select rhizobial strains may be fruitless if less-effective and highly competitive strains are cointroduced.

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