Molecular Phylogenetics and Evolution 63 (2012) 265–277

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Distinct *Bradyrhizbium* communities nodulate legumes native to temperate and tropical monsoon Australia

Tomasz Stępkowski [a,*], Elizabeth Watkin [b], Alison McInnes [c,1], Dorota Gurda [a], Joanna Gracz [a], Emma T. Steenkamp [d]

[a] Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61 704 Poznań, Poland
[b] School of Biomedical Sciences, Faculty of Health Sciences, Curtin University, Perth, Australia
[c] School of Natural Sciences, University of Western Sydney, Hawkesbury Campus, Locked Bag 1797, Penrith South DC, NSW 1797, Australia
[d] Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

ARTICLE INFO

Article history:
Received 24 September 2011
Revised 14 December 2011
Accepted 19 December 2011
Available online 3 January 2012

Keywords:
Australia
Biogeography
Bradyrhizobium
Climate change
Geographical isolation
Leguminosae
Multilocus sequence analysis
nodA gene

ABSTRACT

Geographic isolation and growing climate aridity played major roles in the evolution of Australian legumes. It is likely that these two factors also impacted on the evolution of their root-nodule bacteria. To investigate this issue, we applied a multilocus sequence analysis (MLSA) approach to examine *Bradyrhizobium* isolates originating from temperate areas of Western Australia (WA) and the tropical-monsoon area of the Northern Territory (NT). The isolates were mostly collected from the nodules of legumes belonging to tribes, genera and species endemic or native to Australia. Phylogenetic analyses of sequences for the housekeeping *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and 16S rRNA genes and nodulation *nodA* gene revealed that most isolates belonged to groups that are distinct from non-Australian *Bradyrhizobium* isolates, which is in line with earlier studies based on 16S rRNA gene sequence analyses. Phylogenetic analysis of the *nodA* data allowed identification of five major Clades among the WA and NT isolates. All WA isolates grouped in a subgroup I.1 of Clade I with strains originating from temperate eastern Australia. In contrast, the NT isolates formed part of Clades I (subgroup I.2), III (subgroup III.3), IV, V and X. Of these *nodA* clades, Clade I, Clade IV, Clade X presumably have an Australian origin. Overall, these data demonstrate that the impact of geographic isolation of the Australian landmass is manifested by the presence of numerous unique clusters in housekeeping and nodulation gene trees. In addition, the intrinsic climate characteristics of temperate WA and tropical-monsoon NT were responsible for the formation of distinct legume communities selecting for unrelated *Bradyrhizobium* groups.

1. Introduction

The Leguminosae, accounting for about 2500 species, represent the most speciose angiosperm family in Australia (Sjöström and Gross, 2006). Although the ancestral lineages of many of the native species probably migrated here from other continents (e.g., *Crotalaria*, *Desmodium*, *Hovea*, *Indigofera*, and *Templetonia*) (Schrire et al., 2005), a large proportion of the Australian legumes (e.g., the tribes Bossiaeeae and Mirbelieae) are endemic to the continent (Crisp and Cook, 2003). This is thought to be a direct consequence of the long-lasting geographic and climatic isolation of these legume taxa. Climate change late in the Eocene, which coincides with the radiation of Australian legume taxa (Lavin et al., 2005; Crisp and Cook, 2009) was triggered by the separation of Australia from Antarctica, leading to formation of the Antarctic Circumpolar Current. This, in tandem with the large-scale decrease in atmospheric carbon dioxide levels contributed to global climate cooling and aridification (DeConto and Pollard, 2003; Florindo et al., 2003). As a result, the glaciation of Antarctica (35–30 Mya) strengthened the barrier to intercontinental migration, effectively isolating Australia from South America. The increased aridity led to the extinction of many "Gondwanan" elements or reduction of their range, while expansion of arid habitats promoted diversification of sclerophyllus taxa (Crisp et al., 2004; Hill, 2004; Martin, 2006; Byrne et al., 2008). For example, the *ca.* 750 species comprising the endemic tribes Bossiaeeae and Mirbelieae radiated *ca.* 25–10 Mya during the transition period from wetter to semi-arid climatic conditions (Crisp and Cook, 2003, 2009; Crisp et al., 2004).

Most legumes are capable of establishing a symbiosis with soil bacteria collectively termed rhizobia, which are characterized by their capacity to induce the formation of nodules on the roots or stems of the plant, in which the bacterium fixes atmospheric nitrogen to ammonia (Sprent, 2007). Rhizobia represent diverse

* Corresponding author. Fax: +48 61 852 05 32.
E-mail address: sttommic@ibch.poznan.pl (T. Stępkowski).
[1] Present address: Environment and Sustainability Directorate, ACT Government, GPO Box 158, Canberra, ACT 2601, Australia.

1055-7903/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2011.12.020

bacteria from two classes of the phylum Proteobacteria, *i.e.*, the alpha- and beta-proteobacteria and have been termed α- and β-rhizobia. Both α-rhizobia and β-rhizobia group in assemblages comprising free-living species, together with plant and animal pathogens and symbionts (Masson-Boivin et al., 2009). The α-rhizobia currently comprise five major genera, *Azorhizobium*, *Bradyrhizobium*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium* and *Rhizobium* (Sawada et al., 2003; Martens et al., 2008). There is also a number of species that cluster primarily with non-symbiotic bacteria in the genera, *Devosia*, *Methylobacterium*, *Ochrobactrum* and *Phyllobacterium* (Sy et al., 2001; Rivas et al., 2002; Ngom et al., 2004; Valverde et al., 2005). The β-rhizobia have been included in two genera, *Burkholderia* and *Cupriavidus* (Moulin et al., 2001), which originate from tropical and subtropical parts of the Americas, Asia, Africa, and Australia. Based on our current understanding, the β-rhizobia predominantly originate from root nodules of the pantropical genus *Mimosa* (Chen et al., 2003; Parker et al., 2007; Bontemps et al., 2010).

Rhizobia, like many free-living bacteria, are regarded as cosmopolitan microorganisms, which could be attributed to their capacity for long-distance dispersal and the global distribution of their Leguminosae hosts (Schrire et al., 2005; Kellogg and Griffin, 2006). However, the tenet of microbial cosmopolitism expressed in Baas-Becking's famous statement "*everything is everywhere, but the environment selects*" has recently been challenged in a series of studies, which revealed the existence of geographically isolated microbial populations (e.g., Papke et al., 2003; Whitaker et al., 2003; Schmidt et al., 2011). Geographic distance, as a barrier to dispersal, is most often invoked to explain such distribution patterns (Martiny et al., 2006). However, due to the controversy related to species definition in prokaryotes (Doolittle and Papke, 2006), there is a tendency to study biogeographic relationships by focusing on traits rather than taxonomic identities (Green et al., 2008). This approach has been justified in rhizobial biogeography, as it takes into consideration the disparate and rapidly evolving symbiotic traits (which are prone to lateral transfer) from non-symbiotic traits (Sullivan and Ronson, 1998). Indeed, various phylogenetic studies have revealed the geographic structure preserved in phylogenies of symbiotic genes, e.g., *nifD* (Parker et al., 2002; Andam and Parker, 2008) and *nodC* (Jarabo-Lorenzo et al., 2003; Gu et al., 2007; Lin et al., 2007). The rhizobial genus for which we currently have the best biogeographic understanding is *Bradyrhizobium* with its nine *nodA* clusters (referred to as Clade I–IX) (Moulin et al., 2004; Stępkowski et al., 2005, 2007; Steenkamp et al., 2008; Rodríguez-Echeverría, 2010; Muñoz et al., 2011). Clade I and Clade IV are mostly associated with Australian strains, Clade II strains are common in Europe and the Mediterranean, Clade V and Clade VII as well as the newly described Clade VIII and Clade IX (Muñoz et al., 2011) include strains native to the subtropical and tropical parts of the Americas, while Clade VI comprises photosynthetic bradyrhizobia nodulating *Aeschynomene* spp. Only the highly differentiated Clade III is apparently cosmopolitan and comprises strains from the Americas, sub-Saharan Africa, southern and eastern Asia, and Australia.

Although the biogeographic structure derived from symbiotic gene trees of rhizobia may reflect the impact of geographic distance as a dispersal limiting barrier, the underlying phylogenetic patterns may also be shaped by distribution of their host legumes (Martiny et al., 2006; Schrire et al., 2005). This is because the nodulation gene products are involved in biosynthesis of the so-called Nod factor signal molecule (Dénarié et al., 1996), which interacts with a specific NFR5 receptor protein during recognition of the rhizobium by its legume host (Radutoiu et al., 2003). This could imply that the evolution of nodulation loci follows legume evolution more closely than other gene categories in the rhizobial genome (Doyle, 1998). Thus, the presence (or lack) of legume hosts may influence rhizobial dispersal in the same way that an unsuitable climate constrains plant migration beyond its natural growing range. Given that the distribution of symbiotic alleles in a specific area depends to a large extent on the availability of legume hosts, it can be expected that the historical and ecological factors that drive legume evolution also contribute to the evolution of their rhizobial symbionts.

In this study we considered the hypothesis that the evolution of rhizobia is intricately linked with the forces that shaped the evolution of legumes they are associated with (Han et al., 2010). Our principal objective was thus to elucidate the extent to which historical processes related to geographic isolation, climate change and colonization of Australia by immigrant legume taxa have influenced the composition and evolution of Australian rhizobia. For this purpose, we utilized the DNA polymorphisms and phylogenetic information included in five housekeeping loci (*atpD*, *dnaK*, *glnII*, *gyrB* and *recA*) and the symbiotic locus *nodA*. We also focused on bacteria in the genus *Bradyrhizobium* that is known for its broad host-range and prevalence in the nodules of legumes native to Australia (Lange, 1961; Marsudi et al., 1999; Lafay and Burdon, 1998, 2001, 2007). Previous studies mostly targeted the symbionts of members of the tribes Bossiaeeae and Mirbelieae, and the genus *Acacia* from temperate areas in New South Wales, Victoria, and the Australian Capital Territory (Lafay and Burdon, 1998, 2001). In contrast, little information is available regarding the rhizobia from the Northern Territory (NT) and from Western Australia (WA), which encompasses the Southwest Australian Biodiversity Hotspot (Hopper and Gioia, 2004; Lafay and Burdon, 2007). Therefore, we specifically focused on isolates of *Bradyrhizobium* that were obtained from native legumes in temperate WA and the tropical-monsoon region of the NT, as both regions are thought to include major centers of legume diversification (Crisp et al., 2001; Crisp and Cook, 2003).

2. Materials and methods

2.1. Bradyrhizobium isolates

The *Bradyrhizobium* isolates included in the study originated from legumes growing in five different conservation areas in WA and one in the NT (Table 1). From WA the *Bradyrhizobium* isolates were obtained from Karijini National Park, Lesueur National Park, Stirling Range National Park, Fitzgerald River National Park and Tarin Rock Nature Reserve. These isolates were obtained from soil samples using a set of eight native legumes as traps, and their nodulation abilities were authenticated on seedlings of their respective legumes hosts (Marsudi et al., 1999). In brief, 10 soil samples were collected from each site (50 samples in total). Each of the eight hosts were grown in the 10 soil samples. All nodules were harvested and the root nodule bacteria isolated. A total of 865 slow growing isolates were obtained and subsequently grouped based on physiological characteristics (Marsudi et al., 1999). Representatives from each physiological grouping were selected for 16S sequencing. Finally, 27 *Bradyrhizobium* strains were selected following the comparison of their 16S rRNA gene sequences. The strains were grown at 28 °C on yeast extract-mannitol agar (YMA) medium (Vincent, 1970).

From the NT, we selected 30 *Bradyrhizobium* isolates from a collection of rhizobia that were obtained during the 1991 and 1992 wet seasons from the nodules of native legumes growing in Kakadu National Park, adjacent areas of the Alligator Rivers Region (ARR) and Arnhem Land (McInnes and Ashwath, 1992). For comparative purposes, the NT collection also included four isolates originating from exotic legumes growing in these areas. Plant species were named in accordance with Short et al. (2011), with unidentified

Table 1
Geographic origin, host, identity, *nodA* type and source of the isolates included in this study.

Geographic origin[a]	Legume host[b]	Legume tribe	Strain	Species identities[c]	*nodA* Clade[d]	Source
Bolivia	*Lupinus tominensis*	Genisteae	CH2437	*Bradyrhizobium* sp.	III	Stępkowski et al. (2007)
Brazil	*Lupinus albescens*	Genisteae	CH2509	*B. japonicum*	VII	Stępkowski et al. (2007)
China	*Lespedeza cuneata*	Desmodieae	CCBAU10071^T	*B. yuanmingense*	III	Yao et al. (2002)
Fitzgerald River National Park-WA	*Acacia acuminata*	Acacieae	WSM3976	Aus 1	I	This study
"	*Bossiaea eriocarpa*	Bossiaeeae	WSM3980	Aus 1	I	"
"	*Gastrolobium capitatum*	Mirbelieae	WSM3977	Aus 4	I	"
"	*Gompholobium polymorphum*	"	WSM3979	Aus 1	I	"
"	*Jacksonia sericea*	"	WSM3978	Aus 1	I	"
Kakadu National Park-NT	*Acacia conspersa*	Acacieae	ARR781	Aus 23	X	"
"	Acacia dimidiata	"	ARR487	Aus 22	V	"
"	*Acacia holosericea*	"	ARR410	Aus 17	III	"
"	*Acacia mimula*	"	ARR286	Aus 23	X	"
"	*Cajanus acutifolius*	Phaseoleae	ARR598	Aus 24	IV	"
"	*Calopogonium mucunoides*[b]	"	ARR494	Aus 8	I	"
"	*Crotalaria goreensis*[b]	Crotalarieae	ARR751	Aus 13	III	"
"	*Crotalaria medicaginea*	"	ARR312	Aus 24	IV	"
"	*Crotalaria medicaginea*	"	ARR681	Aus 24	IV	"
"	*Crotalaria medicaginea*	"	ARR699	Aus 24	IV	"
"	*Crotalaria montana*	"	ARR560	Aus 20	III	"
"	*Crotalaria* sp.	"	ARR867	Aus 10	III	"
"	*Desmodium brownii*	Desmodieae	ARR705	Aus 23	X	"
"	*Desmodium* sp.	"	ARR401	Aus 18	III	"
"	*Desmodium* sp.	"	ARR434	Aus 11	III	"
"	*Desmodium* sp.	"	ARR595	Aus 14	III	"
"	*Dunbaria singuliflora*	Phaseoleae	ARR551	Aus 21	III	"
"	*Dunbaria singuliflora*	"	ARR678	Aus 8	I	"
"	*Dunbaria singuliflora*	"	ARR679	Aus 8	I	"
"	*Flemingia parviflora*	"	ARR549	Aus 9	III	"
"	*Galactia megalophylla*	"	ARR603	Aus 8	I	"
"	*Galactia tenuiflora*	"	ARR394	Aus 8	I	"
"	*Galactia tenuiflora*	"	ARR696	Aus 16	III	"
"	*Indigofera colutea*	Indigofereae	ARR688	Aus 11	III	"
"	*Indigofera linifolia*	"	ARR859	Aus 11	III	"
"	*Jacksonia dilatata*	Mirbelieae	ARR534	Aus 8	I	"
"	*Senna obtusifolia*[b]	Cassieae	ARR709	Aus 23	-	"
"	*Stylosanthes viscosa*[b]	Aeschynomeneae	ARR65	Aus23	X	"
"	*Tephrosia leptoclada*	Millettieae	ARR660	Aus 8	III	"
"	*Tephrosia leptoclada*	"	ARR858	Aus 11	III	"
"	*Tephrosia remotiflora*	"	ARR766	Aus 24	IV	"
"	*Uraria lagopodoides*	Desmodieae	ARR862	Aus 15	III	"
"	*Vigna lanceolata*	Phaseoleae	ARR341	Aus 21	III	"
"	*Vigna radiata*	"	ARR375	Aus 19	III	"
Karijini National Park-WA	*Acacia acuminata*	Acacieae	WSM3982	Aus 5	I	"
"	*Acacia acuminata*	"	WSM3985	Aus 12	I	"
"	*Bossiaea eriocarpa*	Bossiaeeae	WSM2238	Aus 6	–	"
"	*Gastrolobium capitatum*	Mirbelieae	WSM3981	Aus 5	I	"
"	*Gompholobium polymorphum*	"	WSM3984	Aus 7	–	"
"	*Jacksonia sericea*	"	WSM3998	Aus 5	I	"
"	*Kennedia coccinea*	Phaseoleae	WSM3983	Aus 1	I	"
"	*Swainsona formosa*	Galegeae	WSM2241	Aus 7	–	"
Lesueur National Park-WA	*Acacia acuminata*	Acacieae	WSM3988	Aus 1	I	"
"	*Bossiaea eriocarpa*	Bossiaeeae	WSM3986	Aus 1	I	"
"	*Gastrolobium capitatum*	Mirbelieae	WSM3991	Aus 1	I	"
"	*Gompholobium polymorphum*	"	WSM2248	Aus 1	–	"
"	*Gompholobium polymorphum*	"	WSM3989	Aus 1	I	"
"	*Jacksonia sericea*	"	WSM3990	Aus 1	I	"
"	*Swainsona formosa*	Galegeae	WSM3987	Aus 1	I	"
South Africa	*Lupinus angustifolius*	Genisteae	1S20	*Bradyrhizobium* sp.	II	Stępkowski et al. (2005)
Stirling Range National Park-WA	*Jacksonia sericea*	Mirbelieae	WSM2249	Aus 1	I	This study
"	*Kennedia coccinea*	Phaseoleae	WSM3992	Aus 1	I	"
"	*Swainsona formosa*	Galegeae	WSM3993	Aus 1	I	"
Tarin Rock Nature Reserve-WA	*Acacia acuminata*	Acacieae	WSM3995	Aus 3	I	"
"	*Gastrolobium capitatum*	Mirbelieae	WSM3994	Aus 1	–	"
"	*Jacksonia sericea*	"	WSM3996	Aus 1	I	"
"	*Kennedia coccinea*	Phaseoleae	WSM3997	Aus 2	I	"
Western Australia	*Ornithopus compressus*	Loteae	WU425	*B. canariense*	II	Stępkowski et al. (2005)

[a] Geographic coordinates of Kakadu National Park (13°02′11″S, 132°26′23″E)), Fitzgerald River National Park (33°56′51″S, 119°36′55″E), Karijini National Park (22°15′2″S, 117°58′32″E), Lesueur National Park (30°8′4″S, 115°6′2″E), Stirling Range National Park (34°24′0″S, 118°9′0″E), and Tarin Rock Nature Reserve (33°06′00″S, 118°14′00″E).
[b] Legumes not indigenous to Australia.
[c] Species identities for the isolates from Western Australia (WA) and the Northern Territory (NT) are based on the DNA sequence information for the three housekeeping loci *glnII*, *recA* and *atpD* (see Fig. 1).
[d] Instances where the *nodA* region were not obtained are indicated with "–"(see Fig. 2).

Desmodium species distinguished according to Brennan (1992). The nodulation abilities of the isolates were verified under axenic conditions on siratro (*Macroptilium atropurpureum*) by E. Hartley and G. Gemmell (unpublished data; Australian Inoculants Research Group, NSW Department of Trade and Investment) who also lyophilized the isolates for long-term storage. Isolates were reconfirmed for nodulation on siratro commencing in 2006 (A. McInnes, unpublished data) using the method described in McInnes and Ashwath (1992) with the plant nutrient medium described by McInnes and Date (2005). The growth rates of isolates at 28 °C were compared on Yeast extract mannitol agar (YMA) medium (Vincent, 1970 or Dalton, 1980) at either room temperature (in the NT) or at 28 °C. The distribution of Australian legume flora was elucidated using Australia's Virtual Herbarium database (http://www.chah.gov.au/avh/).

2.2. DNA extraction, PCR and sequencing

Total genomic DNA was isolated using the SDS–proteinase K lysis procedure described by Moulin et al. (2004). The primers used in this study are shown in Supplementary Table 1S. All PCR amplification reactions were carried out following the procedures described previously (Stępkowski et al., 2005). 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, 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 the 16S ribosomal RNA (rRNA), *nodA* and *gyrB* were 58 °C, 53 °C and 55 °C, respectively. PCR products were purified with QIAquick gel extraction kit columns (QIAGEN, Germany), and sequenced using the BigDye® Terminator v3.1 cycle sequencing kit on an ABI3100 Automated Capillary DNA Sequencer (Applied Biosystems, USA). The nucleotide sequences were corrected manually with BioEdit v7.0.5 (Hall, 1999) and MultAlin software (Corpet, 1988). The GenBank accession numbers of sequences are listed in Supplementary Table 2S.

2.3. DNA sequence analysis

The sequences generated in this study were aligned as described previously (Steenkamp et al., 2008) by using BioEdit v7.0.5 (Hall, 1999) and the ClustalW (Larkin et al., 2007) tool included in this package. In addition to the sequences for the isolates examined, the alignments included sequences obtained from GenBank to enable capturing of the known diversity within the genus *Bradyrhizobium*. The polymorphism within and among groups of isolates was analyzed using DnaSP 5.10 (Librado and Rozas, 2009). This software was also used to calculate nucleotide diversity (π) for the individual sequence sets (Nei, 1987; Librado and Rozas, 2009). Additionally, the significance of population subdivision was evaluated with DnaSP by making use of permutation tests based on 10,000 replicates.

Maximum likelihood (ML) phylogenies were inferred with PhyML 3 (Guindon and Gascuel, 2003) using the best-fit nucleotide or amino acid substitution models as indicated by jModelTest 0.1.1 (Posada, 2008) and ProtTest 2.4 (Abascal et al., 2005). In addition to single gene trees, we also inferred trees using the combined sequence information for *glnII*, *recA* and *atpD* and for the combined information for *glnII*, *recA*, *atpD*, *gyrB* and *dnaK*, as has been done previously (e.g., Rivas et al., 2009; Vinuesa et al., 2005b). ML analysis of the *nodA* amino acid alignment utilized the JTT (Jones et al., 1992) substitution model. The *glnII*, *recA*, *glnII* + *recA* + *atpD* and *glnII* + *recA* + *atpD* + *gyrB* + *dnaK* nucleotide datasets all utilized the GTR (Tavaré, 1986) substitution model, while the TIM1, TIM2 and TPM3uf (Posada, 2008) models were used for the 16S rRNA, *atpD* and *nodA* nucleotide datasets, respectively. Because of the substitution saturation associated with third codon positions in the *nodA* data (Moulin et al., 2004), these positions were excluded from the DNA-based phylogenetic analysis of this region. All ML analyses accounted for among-site rate variation using gamma correction based on four rate categories. Except for the *nodA* amino acid dataset that harbored no invariant sites, all the analyses also included a proportion of invariable sites. Branch support was estimated using nonparametric bootstrap (bs) analyses based on 500 replicates.

The *glnII* + *recA* + *atpD*, *glnII* + *recA* + *atpD* + *gyrB* + *dnaK* and *nodA* (1st and 2nd codon positions only) nucleotide datasets were also subjected to Bayesian Inference (BI) with MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) using the GTR model with gamma correction for among-site rate variation and a proportion of invariable sites. The two multiple locus analyses also utilized unlinked or separate parameters across the respective gene partitions. For the analyses of the three- and five-gene datasets we used 4,000,000 and for the *nodA* dataset 10,000,000 Markov Chain Monte Carlo generations (three heated chains and a single cold chain) and tree sampling every 100 generations. Posterior probabilities (pp) were determined using the trees that were generated after stationarity was achieved and that were sampled post-burnin (*i.e.*, 400, 500 and 2000, respectively).

3. Results

3.1. Bradyrhizobium isolates

More than 60% of the isolates from WA and NT displayed the slow-growing phenotype, suggesting relatedness with the genus *Bradyrhizobium*. Based on 16S rRNA gene sequence comparisons against those in GenBank (Watkin, unpublished data) these isolates were confirmed to represent *Bradyrhizobium*. These comparisons also revealed that the faster-growing isolates were highly diverse and assigned to the genera *Rhizobium*, *Sinorhizobium*, *Azorhizobium* and *Burkholderia*, except for isolates WSM2238, WSM2241 and WSM2248, which also represented *Bradyrhizobium* (Watkin, unpublished data). Only the isolates from WA and NT that represented *Bradyrhizobium* were selected for further investigation.

3.2. Amplification and sequence analysis of glnII, recA, gyrB, dnaK, atpD, 16 rRNA, and nodA genes

For all 61 of the isolates, fragments of 519, 559 and 485 base pairs (bp) in length were amplified and sequenced for the genes *glnII*, *recA* and *atpD*, respectively. The only exceptions were isolates ARR603, ARR781, ARR859 and WSM3997. Sequence analysis of the *atpD* PCR products from isolates ARR603, ARR781, ARR859 and WSM3997 revealed similarity to protein products other than the expected ATP synthase beta chain. For a set of 29 isolates, 707 and 723 bp portions of the genes encoding *dnaK* and *gyrB* were also sequenced, while 16S rRNA gene sequences were determined for 18 representing the various housekeeping gene lineages (see below). Nucleotide comparisons using BLAST revealed that the five housekeeping genes analyzed in all of the isolates were most similar to corresponding sequences for known *Bradyrhizobium* strains in the GenBank database.

The complete *nodA* gene was amplified and sequenced in 56 isolates (34 from NT and 22 from WA) using the procedures developed previously (Moulin et al., 2004; Stępkowski et al., 2005). Despite numerous attempts, this approach did not allow amplification of the *nodA* gene from any of the three *Bradyrhizobium* isolates with the fast-growing phenotype (WSM2238, WSM2241 and WSM2248). Overall the *nodA* gene sequences of our *Bradyrhizobium*

isolates were longer than those of their counterparts in the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. Although some isolates of Clade III (see below) carry a second ATG codon that overlaps with the start codon of the *nodA* gene of fast growing rhizobia, the majority of the differences observed in this study were located in the inferred N-terminus of the NodA protein (Moulin et al., 2004; Stępkowski et al., 2007). Similar to previous observations for *Bradyrhizobium*, the *nodA* gene for all of the WA and NT isolates examined included a N-terminal segment that coded for additional 11–28 amino acid residues, giving rise to a deduced protein composed of 208–225 amino acid residues. For the majority of the isolates, the deduced NodA protein consisted of 210 amino acid residues of which 13 residues were included in the N-terminal segment. The longest *nodA* sequences were found in isolates ARR341 and ARR696, which both code for NodA proteins composed of 225 amino acids. It appears that this could be their minimal length, given that the *nodA* gene in both starts with a GTG codon and both lack alternative start codons overlapping with start codons in the fast growing rhizobia. The deduced NodA for other isolates (ARR434, ARR688, ARR751, ARR858, and ARR859) was composed of 208 residues, due to a 2-amino acid residue deletion in theN-terminal segment. The deduced NodA proteins in isolates ARR394, ARR494, ARR603, ARR678, and ARR679, as well as all of the isolates from WA were composed of 211 amino acid residues, which was due to a single codon insertion (aspartate 183) in the C-terminal part that appears to be a distinctive characteristic of the whole Clade I.

3.3. The isolates from Western Australia and the Northern Territory represent distinct populations

The average nucleotide diversity (Nei, 1987; Librado and Rozas, 2009) associated with *glnII*, *recA*, *atpD* and *nodA* for the isolates from the two collection areas differed markedly. Based on the combined *glnII + recA + atpD* dataset, the average π-value for the WA isolates was 0.05662 (standard deviation [sd] = 0.00307), while that of the NT isolates was 0.08928 (sd = 0.00325). Based on the *nodA* data, π-values of 0.03941 (sd = 0.00502) and 0.21595 (sd = 0.00796) were obtained for the respective WA and NT isolates.

Analysis of the polymorphisms within and among the different isolate collections revealed that the isolates obtained from the Kakadu National Park and surrounding areas (NT) are genetically differentiated from those originating in WA (Table 2). This separation was most pronounced in the *nodA* data where the average number of nucleotide substitutions per site between the NT and WA populations (*Dxy*-values of 0.2319–0.2373) were much higher than those observed among different WA localities (*Dxy*-values of 0.0317–0.0424). Also, the tests for population subdivision allowed confident rejection ($P < 0.001$) of the null hypothesis that the WA and NT populations are not genetically differentiated (*i.e.*, that they are genetically homogenous), which was not the case for the comparisons among individual WA populations. The increased number of fixed polymorphisms and subdivision between the NT the WA populations were also evident in the estimates of F_{ST} and *Nm*. The F_{ST}-values for the comparisons among WA populations were generally an order of magnitude lower than those obtained for the comparisons between the NT and WA populations (0.0644–0.1241 *vs.* 0.4472–0.4821), while the *Nm*-values were much higher for the former and lower for the latter comparisons (3.53–7.26 *vs.* 0.54–0.62).

The distinctness of the WA and NT populations was also evident in the *glnII + recA + atpD* dataset, although not to the same extent as with the *nodA* data (Table 2). In general the WA populations were less diverged (*Dxy*-values of 0.0493–0.0678) than when the NT population were made to include WA populations (*Dxy*-values of 0.0836–0.1002). In fact, the significant population subdivision estimates were lowest for the comparisons between NT and WA populations, thus supporting the idea that the isolates from these two areas are genetically distinct. Therefore, to achieve a more detailed view of the structure within the WA and NT populations, we subjected our DNA sequence data to ML and BI-based phylogenetic analysis.

3.4. Bradyrhizobium isolates from Western Australia and Northern Territory form separate housekeeping gene clusters

ML phylogenies were inferred for each of the three housekeeping loci, *atpD*, *glnII* and *recA*, separately (Supplementary Fig. S1) and from the combined *glnII + recA + atpD* dataset (Fig. 1). Among the isolates examined, 16 strains from WA and NT did not consistently group with any other taxon included in the various analyses and were designated Aus2–4, Aus6, Aus9–10, Aus12–20 and Aus22. However, all three of these datasets and the combined dataset supported the grouping of 45 WA and NT isolates into eight well-supported groups (Aus1, Aus5, Aus7–8, Aus11, Aus21 and Aus23–24). The only exception was group Aus1, which was split into two subgroups in the *recA* tree (Supplementary Fig. S1). In the *atpD* tree clade Aus1 was also split, but in this case across three sub-clades and two single-isolate lineages (Supplementary Fig. S1) that had no obvious correlation with those in the *recA* and *glnII + recA + atpD* trees. In *Bradyrhizobium* such variations in the grouping of isolates among different single gene trees have been attributed to horizontal gene transfer among distantly related taxa and/or incomplete lineage sorting among closely related taxa (e.g., Vinuesa et al., 2008; Rivas et al., 2009). Additional research is thus needed to resolve the observed incongruence and to determine whether the taxa included in Aus1 indeed share a most recent common ancestor. Nevertheless, the isolates originating from WA and from NT were separated into lineages that did not overlap (Fig. 1). For example, the isolates obtained from WA formed part of lineages Aus1–7 and Aus12, while those originating from the NT formed part of Aus8–11 and Aus13–Aus24. For the most part, the WA and NT lineages also did not cluster together – the only exception was WA lineage Aus12, which formed part of a cluster that also included NT lineages Aus9–11 and *Bradyrhizobium yuanmingense* (Fig. 1).

Some of the WA and NT isolates associated loosely with known isolates and species of *Bradyrhizobium* (Fig. 1). Group Aus1 included WA isolates, as well as isolate 1S20 that was previously characterized from lupin in South Africa (Stępkowski et al., 2005). In all of our housekeeping gene trees, Aus5 and Aus7 tended to group with Aus6 isolate WSM2238, which together appears to be associated with *Bradyrhizobium liaoningense*, albeit with low to no bootstrap support (Fig. 1, Supplementary Fig. S1). In addition, the WA single-isolate lineages Aus3 (WSM3995) and Aus4 (WSM3977) appear to have some association with *Bradyrhizobium* genospecies beta (Vinuesa et al., 2005b), although a clade including these taxa were again not supported by our single gene trees (Supplementary Fig. S1). Aus9–12, displayed an association with *B. yuanmingense*, but the single gene datasets did not support the monophyly of these clades/lineages with *B. yuanmingense*. Finally, Aus15–21 formed part of an assemblage that included *Bradyrhizobium elkanii* and the recently described *Bradyrhizobium lablabi* (Chang et al., 2011), *Bradyrhizobium pachyrhizi* and *Bradyrhizobium jicamae* (Ramirez-Bahena et al., 2009), although none of our isolates appeared to be conspecific with these known species. Therefore, despite the fact that more research is needed to resolve the taxonomic status of the isolates, we are confident that the groups and single-isolate lineages identified among the WA and NT isolates represent distinct species taxa. A large proportion of them are probably new to science as they did not consistently group with previously studied isolates or species of *Bradyrhizobium*.

Table 2
Estimates[a] for genetic differentiation and gene flow based on the sequence data for the isolates studied.

Populations compared [b]	Number of differences between populations		*Dxy* [c]	Genetic differentiation (K_{ST}^{*}) [d]	Gene flow [e]	
	Fixed	Shared			F_{ST}	*Nm*
glnII + recA + atpD						
NT *vs.* WA (35, 27)	0	175	0.0947	0.04319 (0.0000***)	0.2306	1.67
NT *vs.* Krn (35, 8)	0	94	0.0836	0.03232 (0.0000***)	0.2276	1.70
NT *vs.* Ftz (35, 5)	0	79	0.1002	0.02168 (0.0000***)	0.3121	1.10
NT *vs.* Lsr (35,7)	8	65	0.1000	0.03834 (0.0000***)	0.3767	0.83
Krn *vs.* Ftz (8, 5)	3	72	0.0664	0.08956 (0.0010**)	0.3318	1.01
Krn *vs.* Lsr (8, 7)	3	60	0.0678	0.09246 (0.0000***)	0.3879	0.79
Ftz *vs.* Lsr (5, 7)	0	70	0.0493	0.03286 (0.0190*)	0.1410	2.97
Groups 1 *vs.* 8 (16, 7)	36	9	0.0809	0.14275 (0.0000***)	0.6903	0.22
Groups 1 *vs.* 11 (16, 4)	35	6	0.0786	0.10368 (0.0000***)	0.6997	0.21
Groups 1 *vs.* 23 (16, 5)	85	2	0.1268	0.17148 (0.0000***)	0.8256	0.11
Groups 1 *vs.* 24 (16, 5)	63	16	0.1191	0.14629 (0.0000***)	0.7367	0.18
Groups 8 *vs.* 11 (7, 4)	49	0	0.0628	0.27180 (0.0020**)	0.8315	0.10
Groups 8 *vs.* 23 (7, 5)	109	1	0.1153	0.36290 (0.0000***)	0.9213	0.04
Groups 8 *vs.* 24 (7, 5)	75	3	0.1022	0.30658 (0.0020**)	0.8207	0.10
Groups 11 *vs.* 23 (4, 5)	104	0	0.1077	0.41881 (0.0030**)	0.9291	0.04
Groups 11 *vs.* 24 (4, 5)	73	1	0.0965	0.33708 (0.0050**)	0.8252	0.11
Groups 23 *vs.* 24 (5, 5)	96	1	0.1190	0.38425 (0.0060**)	0.8709	0.07
nodA						
NT *vs.* WA (33, 22)	4	80	0.2345	0.26422 (0.0000***)	0.4553	0.60
NT *vs.* Krn (33, 6)	5	37	0.2373	0.11396 (0.0000***)	0.4472	0.62
NT *vs.* Ftz (33, 5)	6	30	0.2319	0.11608 (0.0000***)	0.4630	0.58
NT *vs.* Lsr (33,6)	9	26	0.2335	0.13872 (0.0000***)	0.4821	0.54
Krn *vs.* Ftz (6, 5)	0	16	0.0424	0.08221 (0.1060^{ns})	0.0726	6.39
Krn *vs.* Lsr (6, 6)	0	15	0.0317	0.08458 (0.0370*)	0.1241	3.53
Ftz *vs.* Lsr (5, 6)	0	13	0.0414	0.02099 (0.1470^{ns})	0.0644	7.26
Clades I *vs.* III (59, 103)	8	169	0.2774	0.11910 (0.000***)	0.5728	0.37
Clades I *vs.* IV (59, 7)	46	33	0.2410	0.08918 (0.0000***)	0.7157	0.20
Clades I *vs.* X (59, 4)	67	8	0.2346	0.06288 (0.0000***)	0.8372	0.10
Clades III *vs.* IV (103, 7)	9	82	0.2549	0.09434 (0.0000***)	0.7226	0.19
Clades III *vs.* X (103, 4)	15	14	0.2626	0.01677 (0.0000***)	0.6211	0.30
Clades IV *vs.* X (7, 4)	86	2	0.2221	0.24537 (0.0020**)	0.7741	0.15

[a] All estimates were determined with DnaSP 5.10 (Librado and Rozas, 2009) and only those for populations that included 4 or more isolates are indicated.
[b] Populations were defined in terms of either geographic origin (WA = Western Australia; NT = Northern Territory; Ftz = Fitzgerald River National Park; Lsr = Lesuer National Park; Krn = Karinini National Park) of the isolates examined or the clades to which they were assigned (see Figs. 1 and 2). For the definitions based on geographic origin and the *glnII + recA + atpD* phylogeny, the analyses included only those sequences that were determined for the isolates from NT and WA (Table 1), while the *nodA* clade-based tests also included *nodA* sequences from previous studies (particularly those of Stepkowski et al. (2005, 2007) and Moulin et al., 2004). The number of samples in each comparison is indicated in parentheses.
[c] Divergence between populations were expressed as the average number of nucleotide substitutions per site between populations using (Nei, 1987).
[d] Population subdivision was evaluated using the sequence-based statistic K_{ST}^{*} (Hudson et al., 1992a), which utilizes the nucleotide differences between sequences in populations. This statistic has been shown to be a powerful measure of population differentiation when the markers under investigation are selectively neutral [see Supplementary Table 3S and Vinuesa et al. (2008) for data on *glnII*, *recA* and *atpD*] and subject to recombination, as is the case for the datasets analyzed here (see Supplementary Table 4S). Probability (*P*)-values for rejecting the null hypothesis that two populations are genetically homogenous are indicated in parentheses. These *P* values were estimated with permutation (10,000 replicates) tests. ns = not significant.
[e] DNA sequence-based estimates described by Hudson et al. (1992b).
* $0.01 < P < 0.05$.
** $0.001 < P < 0.01$.
*** $P < 0.001$.

The overall topologies of the three-gene and five-gene phylogenies generated in this study resembled those generated previously (Vinuesa et al., 2005b; Rivas et al., 2009). Both of the *glnII + recA + atpD* and *glnII + recA + atpD + gyrB + dnaK* datasets support the deep divergence between the *B. elkanii* assemblage and the remainder of the known *Bradyrhizobium* species and lineages (Figs. 1 and 2). However, some of the isolates examined in this study appear to form additional deep divergences within the *Bradyrhizobium* phylogeny. This is particularly true for the isolates from NT where Aus22–23 and Aus24 represent novel lineages near the base of the *glnII + recA + atpD* and *glnII + recA + atpD + gyrB + dnaK* trees.

Earlier studies of bradyrhizobia nodulating Australian native legumes were limited to the analyses of 16S rRNA gene sequences (Lafay and Burdon, 1998, 2001, 2007). In order to relate our data to these earlier works we sequenced this gene in 18 isolates, representing various housekeeping gene groups. The 16S rRNA gene sequences for Aus1 and Aus2 isolates were identical to those of *Bradyrhizobium* genomospecies A strain BDV5028 originating from *Bossiaea ensata* (Lafay and Burdon, 1998). Furthermore, despite the fact that our inferred 16S rRNA phylogenetic tree generally lacked statistical support (as been noted before; e.g., see Vinuesa et al., 2005a; Willems et al., 2001b), certain WA and NT isolates consistently grouped with specific lineages of *Bradyrhizobium* (Supplementary Fig. S2). These included ARR312 (Aus24) that grouped with *Bradyrhizobium* genomospecies Y isolate ARRI218 originating from *Indigofera linifolia* (Lafay and Burdon, 2007), Aus23 isolates ARR65 and ARR286 that grouped with *Bradyrhizobium* genomospecies Z isolate ARRI709 originating from *Senna obtusifolia* (Lafay and Burdon, 2007), and Aus15 isolate ARR862 that grouped with *Bradyrhizobium* isolate Wal19 originating from *Wallaceodendron celebicum* (Andam and Parker, 2008).

3.5. nodA gene phylogeny supports the geographic distribution of Bradyrhizobium

The *nodA* datasets (nucleotides and inferred amino acids residues) analyzed included the sequences generated in this study, together with earlier published *Bradyrhizobium nodA* sequences (Moulin et al., 2004; Stępkowski et al., 2005, 2007; Lafay et al.,

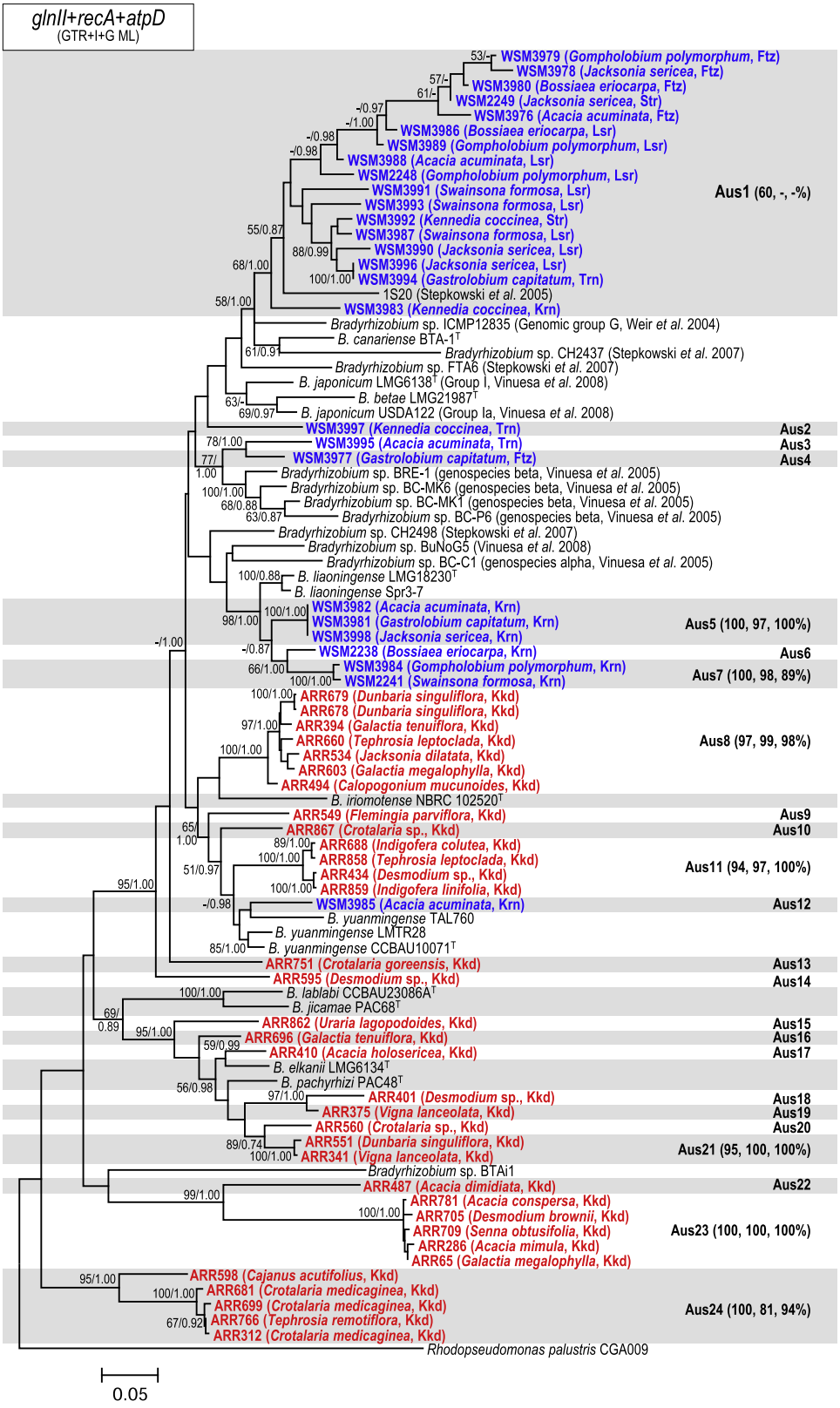

Fig. 1. Maximum likelihood (ML) phylogeny of the *Bradyrhizobium* based on the combined nucleotide information for the genes *glnII*, *recA* and *atpD*. Host and geographic origin for the isolates from Western Australia (WA, indicated in bold blue) and the Northern Territory (NT, indicated in bold red) are provided in parentheses (Kakadu National Park = Kkd; Fitzgerald River National Park = Ftz; Karijini National Park = Krn; Lesueur National Park = Lsr; Stirling Range National Park = Str; Tarin Rock Nature Reserve = Trn). ML bootstrap support (>50%) and Bayesian Inference (BI) posterior probabilities (>0.65) are indicated at the branches in the order: ML/BI; and those branches not receiving support by either of the two analysis procedures are indicated with "-". Clade/lineage designations (1–24) for the WA and NT isolates are indicated in on the right. For the latter clades, ML bootstrap support based on the analysis of single genes (Supplementary Fig. S1) are indicated in brackets next to the clade designations in the order *glnII*, *recA* and *atpD* (lack of support is also indicated with "-"). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

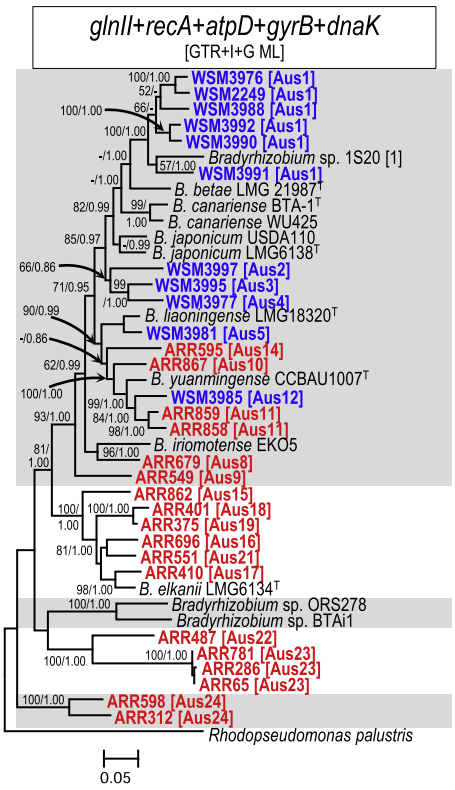

Fig. 2. ML phylogeny of *Bradyrhizobium* using the combined information for *glnII*, *recA*, *atpD*, *gyrB* and *dnaK*. The isolates from WA are indicated in bold blue and those from NT in bold red. The clade designations based on the *glnII + recA + atpD* phylogeny (Fig. 1) are indicated in square brackets. Alternate white and gray shading are used to indicated deep divergences within the genus. Branch support are indicated as in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2006; Steenkamp et al., 2008). ML and BI analyses of these datasets produced a well-supported phylogeny (Fig. 3; Supplementary Fig. S3), which separated the taxa included into a number of major clades that have previously been designated *nodA* Clades I–IX with Clade III further subdivided into subclades III.1, III.2 and III.3 (Stępkowski et al., 2007; Muñoz et al., 2011). The majority of the isolates originating from WA and NT formed part of four of these existing clades (Clades I, III, IV, V), while four NT isolates formed a new clade (Clade X) within the existing *nodA* phylogenetic framework.

All of the isolates from WA and six isolates from the Kakadu National Park (NT) grouped in Clade I, which splits into two well-supported subclades referred to as I.1 and I.2. The little differentiated subclade I.1 comprised all of the WA isolates (*glnII + recA + atpD* groups/lineages Aus1–5 and Aus12; see Fig. 2) examined in this study together with published *nodA* sequences of strains originating in New South Wales (Australia) (Stępkowski et al., 2005; Lafay and Burdon, 2006), as well as the isolates from native Australian *Acacia* species growing in Brasil, South Africa, and Portugal (Moulin et al., 2004; Rodríguez-Echeverría, 2010). Isolate BDV5111, which originated from New South Wales from *Daviesia leptophylla* (Lafay et al., 2006) was situated at the base of this clade. The six NT isolates (ARR394, ARR494, ARR534, ARR603, ARR678, ARR679) representing *glnII + recA + atpD* Aus8 formed part of subclade I.2. Two isolates (CP315 and NGR231) that were characterized previously (Lafay et al., 2006) occupied the basal position and were respectively isolated from *Parasponia rigida* and *Parasponia rugosa* plants in Papua New Guinea.

Both the ML and BI analyses of the *nodA* nucleotide dataset and ML analysis of the amino acid dataset recovered Clades X and IV as sister groups, although with marginal support (*nodA* MLbs = 53% and BIpp = 0.88, NodA MLbs = 51%; Fig. 3; data not shown). Five NT isolates (ARR312, ARR598, ARR681, ARR699, ARR766), all representing *glnII + recA + atpD* group Aus24, formed a well-supported group with two known strains (WSM1735 and WSM1790) in *nodA* Clade IV (Fig. 3). These strains were isolated from *Rhynchosia minima* and *Indigofera colutea* in the Pilbara region of WA (Stępkowski et al., 2005). Clade X also comprised NT isolates (ARR65, ARR281, ARR705 and ARR781), which all formed part of group Aus23 based on the housekeeping sequence information (Fig. 3).

In the *nodA* phylogeny isolate ARR487 (*glnII + recA + atpD* lineage Aus22; Fig. 1) grouped with strain CH2493. This strain was isolated from the root nodule of *Lupinus paraguariensis*, which is a species endemic to south-eastern Brazil (Stępkowski et al., 2007). Deep branching of these two isolates and the moderate support (Fig. 3) suggest that Clade V may represent a provisional assemblage, the composition and placement of which might change with additional sampling.

The majority of the isolates obtained from the NT formed part of the diverse third subclade of *nodA* Clade III (Fig. 3). Sublcade III.3 comprised known strains representing *Bradyrhizobium japonicum*, *B. yuanmingense*, *B. elkanii*, the majority of the recently characterized isolates from cowpea and peanut in southern Africa (Steenkamp et al., 2008), and strains isolated earlier from native legumes in Senegal (Moulin et al., 2004). Out of the eight isolates that formed part of the *B. elkanii* assemblage based on the housekeeping gene sequences (*i.e.*, lineages Aus15–21; Fig. 1), four (ARR375, ARR410, ARR560, ARR862; indicated as III.3a on the *nodA* tree) grouped with known isolates of *B. elkanii*, including the type strain (LMG6134) of this species (*nodA* MLbs = 50% and BIpp = 1.00 NodA MLbs = 59%; Fig. 3, data not shown). The other four isolates (ARR341, ARR410, ARR551, ARR696 indicated as III.3b on the *nodA* tree) formed a well-supported and distinct lineage within subclade III.3. The remaining nine isolates (ARR434, ARR549, ARR595, ARR660, ARR688, ARR751, ARR858, ARR859, ARR867; indicated as III.3c on the *nodA* tree) grouped with an isolate from WA (WSM1743) that were obtained from an *Indigofera* sp. (Stępkowski et al., 2005) and isolates that have previously been designated as *B. japonicum* (*nodA* MLbs = 53% and BIpp = 1.00).

The isolates (ARR65, ARR494, ARR709 and ARR751) originating from exotic legumes growing in the NT all either grouped closely with isolates from the native legumes, or were no more distantly related than some individual isolates from native legumes. This was true for both the housekeeping gene datasets and the *nodA* data (Figs. 1 and 3). Isolates ARR65 and ARR709 formed part of Aus23, while isolate ARR65 formed part of the new *nodA* Clade X. Based on the *glnII + recA + atpD* data, isolate ARR494 was part of Aus8, while it groups with other NT isolates in Clade I.2 of the *nodA* tree. Isolate ARR751, which represented Aus13 in the housekeeping gene trees, groups with other NT isolates in *nodA* Clade III.3. These data thus suggest that the exotic tropical legumes are nodulating with Australian isolates of *Bradyrhizobium*, in contrast to previous work with the introduced pasture legume serradella (*Ornithopus* spp.), which only nodulated with isolates originating from the Mediterranean region (Stępkowski et al., 2005).

4. Discussion

4.1. The majority of the examined isolates represent novel *Bradyrhizobium* lineages

Within the collection of isolates included in this study, we identified 24 distinct groups or lineages, the majority of which do not appear to be conspecific with known members of the genus *Bradyrhizobium* (Jordan, 1982). This genus is known for its ability to

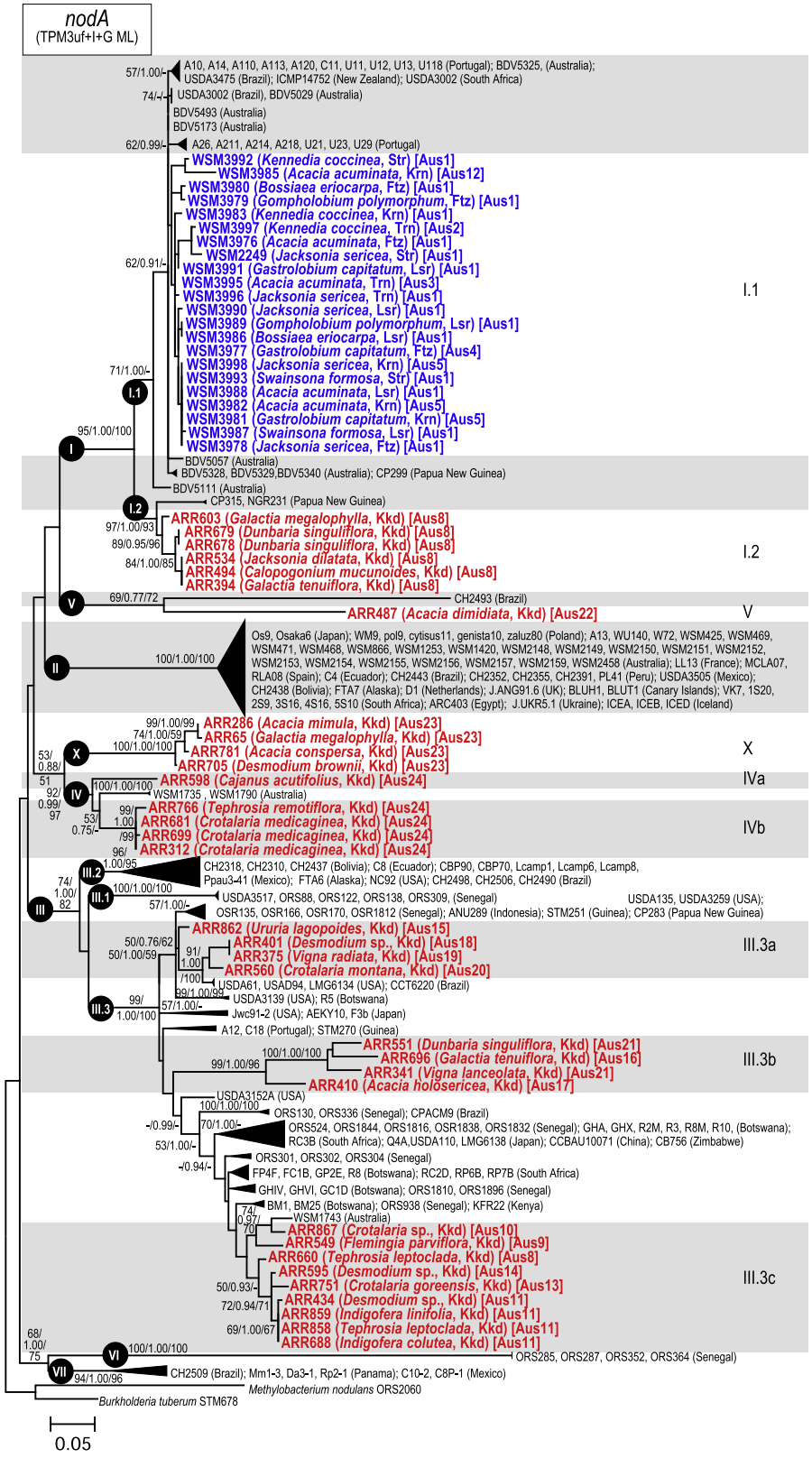

Fig. 3. ML phylogeny of *Bradyrhizobium nodA* using only the first two codon positions of the gene. The major *nodA* clades (Clades I–X) are indicated with black dots at the respective nodes. Host and geographic origin for the isolates from WA (indicated in bold blue) and NT (indicated in bold red) are provided in parentheses (see Fig. 1 for location abbreviations). For each of the isolates examined, the respective clade designations based on the housekeeping sequence information (see Fig. 1) are provided in square brackets. Also, the clades to which these WA and NT isolates belong are indicated on the right. Similar clades were recovered using ML analysis of the inferred amino acid data. ML bootstrap support (>50%) and BI posterior probabilities (>0.65) are indicated at the branches in the order: nucleotide ML/nucleotide BI/amino acid ML. Those branches not receiving support in one or more of the analyses are indicated with "-". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nodulate a broad range of legumes, comprises 11 species – *B. betae*, *B. canariense*, *B. elkanii*, *B. iriomotense*, *B. japonicum*, *B. jicamae*, *B. liaoningense*, *B. pachyrhizi*, and *B. yuanmingense* (Kuykendall et al., 1992; Xu et al., 1995; Yao et al., 2002; Rivas et al., 2004; Vinuesa et al., 2005a; Islam et al., 2008; Ramirez-Bahena et al., 2009), including two recently described spp. *B. cytisi* and *B. lablabi* spp. (Chang et al., 2011; Chahboune et al., 2011). Additionally, many studies reveal a number of lineages that group outside these delineated species, and which due to the lack of sufficient sequence data have not been classified as separate species. Strains of the genus *Bradyrhizobium* have been reported in all parts of the world, essentially in all areas inhabited by Leguminosae spp. (e.g. Doignon-Bourcier et al., 1999; Willems et al., 2001a; Jarabo-Lorenzo et al., 2003; Ormeno-Orillo et al., 2006; Parker, 2008; Cardinale et al., 2008; Steenkamp et al., 2008).

The presence of rhizobia showing the slow-growing phenotype in the nodules of native Australian legumes have been reported since the beginning of rhizobium research in this country (Lange, 1961; Barnet et al., 1985; Barnet and Catt, 1991), and more recently, by Lafay and Burdon (1998, 2001), and Hoque et al. (2011). In the latter studies *Bradyrhizobium* spp. associated with Australian legumes were grouped by amplified ribosomal DNA restriction analysis (ARDRA). Based on ARDRA profiles, Lafay and Burdon (1998, 2001, 2006, 2007) described no fewer than 27 distinct genospecies among the Australian *Bradyrhizobium* isolates. Because ARDRA profiles generally provide low resolution at the subgeneric level, we used a multilocus sequence analysis (MLSA) approach (Gevers et al., 2005; Martens et al., 2008; Rivas et al., 2009). The results of our phylogenetic analyses of housekeeping and nodulation loci clearly showed that the native legumes in the WA and NT regions are nodulated by highly diverse members of the genus *Bradyrhizobium*.

Analysis of the housekeeping gene sequences revealed several deep divergences within the overall *Bradyrhizobium* phylogeny. Indeed, all 24 groups or lineages could be placed in either one of the main groups of this genus that are known as the *B. japonicum* and *B. elkanii* super-groups (Ramirez-Bahena et al., 2009), (Figs. 1 and 2). The *B. japonicum* super-group (lineages Aus1–14) included all WA isolates and some NT strains, together with *B. betae*, *B. canariense*, *B. iriomotense*, *B. japonicum B. liaoningense*, and *B. yuanmingense*. The *B. elkanii* super-group, together with *B. elkanii*, also included lineages Aus15–21, as well as *B. lablabi* (Chang et al., 2011), *B. jicamae* and *B. pachyrhizi* (Ramirez-Bahena et al., 2009). Furthermore, two additional deep lineages represented by Aus22–23 and Aus24, respectively, were observed. The Australian continent thus appears to be home to a significant proportion of the diversity observed in *Bradyrhizobium*, suggesting that it may be linked to the enormous diversity of legume hosts and complexity of edaphic and climatic factors, all of which must have favoured the persistence of many *Bradyrhizobium* taxa.

Although certain isolates examined in this study tended to associate with known species in some of our phylogenetic trees, none of these associations were consistently supported in all of the single and multigene analyses. Therefore, none of the species that are commonly regarded as cosmopolitan were recovered during our study. This is especially true for *B. japonicum*, which is thought to possess adaptations enabling nodulation of a broad range of legume species under various soil and climatic conditions (Stępkowski et al., 2011). Our results are thus in line with earlier reports that also contradicted the cosmopolitan nature of *B. japonicum* (Lafay and Burdon, 1998, 2001; Steenkamp et al., 2008). Therefore, as has been shown for other bacteria (e.g., Papke et al., 2003; Whitaker et al., 2003; Schmidt et al., 2011), the distribution pattern of the *Bradyrhizobium* groups and lineages recovered from WA and NT is not consistent with the idea of microbial cosmopolitism.

Diverse and novel groups were also identified among the isolates from WA and NT when we analyzed nodulation gene *nodA*. This gene has been applied as a primary phylogenetic marker in various studies of the symbiotic component of the rhizobial genome (Moulin et al., 2004; Stępkowski et al., 2005, 2007, 2011; Steenkamp et al., 2008; Cardinale et al., 2008; Rodríguez-Echeverría, 2010; Muñoz et al., 2011). In this study, we detected five major clades (Clades I, III, IV, V and X) of which Clade X is new (Figs. 3 and S3). Among the *Bradyrhizobium* isolates examined, those originating from WA were all restricted to a subclade of Clade I, while the isolates from the NT region formed part of all five clades. Of those clades not recovered in the current study, Clade VI has been reported from photosynthetic *Bradyrhizobium* strains nodulating *Aeschynomene* species (Chaintreuil et al., 2001) and Clade VII from legume species native to the tropical parts of South and Central America (Stępkowski et al., 2007). The fact that Clade II was not detected strongly indicates that *Bradyrhizobium* strains harboring this *nodA* allele in Western Australia are restricted to areas inhabited by introduced lupine and serradella legumes (Stępkowski et al., 2005), or by other Genisteae, some of which like *Cytisus scoparius*, are invasive species worldwide (Lafay and Burdon, 2006).

4.2. Bradyrhizobium communities in temperate Western Australia significantly differ from their counterparts in the tropical Northern Territory

The presence of multiple unique lineages or groups in the housekeeping and nodulation gene phylogenies suggests that geographic isolation had a significant impact on the evolution of *Bradyrhizobium* spp. in Australia. However, our results indicate that geographic isolation of this landmass is not the only factor responsible for the phylogenetic patterns observed. In both the housekeeping and nodulation gene trees, the WA and NT strains were situated in various, largely, non-overlapping groups (Figs. 1 and 3). This suggests that temperate and tropical Australia are inhabited by highly dissimilar *Bradyrhizobium* communities. This separation was also reflected by various population genetic estimates, which detected highly significant levels of divergence and differentiation between the WA and NT populations (Table 2). These findings are consistent with the report of Lafay and Burdon (2007), in which nine genospecies of *Bradyrhizobium* were identified among 45 isolates from the Kakadu National Park. Of these seven were unique and only two genospecies were shared with a collection of 863 isolates from New South Wales, Victoria and Australian Capital Territory (Lafay and Burdon, 1998, 2001). This relatively small area in the NT is thus inhabited by a highly heterogeneous and unique community of *Bradyrhizobium*, with diversity comparable to those sampled in much larger areas of temperate Australia.

All WA strains cluster in the "*B. japonicum* super-group", with the majority of strains forming part of a single group (Aus1) based on our *glnII + recA + atpD* (Fig. 1). 16S rRNA sequences of our Aus1 isolates are identical to those of strains belonging to *Bradyrhizobium* genospecies A. Interestingly, this genospecies has been described as the dominant lineage in the temperate, south-eastern part of Australia, comprising > 50% (475) of all *Bradyrhizobium* isolates that originated from root nodules of legumes native to the region (Lafay and Burdon, 1998, 2001). Our study thus suggests that this lineage may be widespread throughout temperate Australia. In addition, the 16S rRNA gene sequences for Aus1 isolates are identical to that of *B. cytisi*, which was recently described from *Cytisus triflorus* nodules in Morocco (Chahboune et al., 2011). Aus1 isolates are also closely related to *Bradyrhizobium* strain 1S20 that was isolated from lupine grown in acid soil in the Cape Town area, South Africa. However, unlike our Aus1 isolates that harbor the Clade I allele of *nodA*, the type strain of *B. cytisi* and strain 1S20 harbor the

Clade II allele of *nodA*, which is the dominant form of *nodA* among bradyrhizobia nodulating Genisteae and Loteae legumes in Europe and North Africa (Stępkowski et al., 2005, 2007, 2011; Stępkowski, unpublished). These findings thus imply that Aus1 is a widespread taxon throughout Mediterranean areas, and that the Mediterranean climate and/or acid soil conditions could be important factors determining its occurrence.

All the examined WA isolates and the majority of strains recovered in south-eastern Australia from part of subclade I.1 of the *nodA* Clade I, irrespective of their position in the housekeeping gene trees (Stępkowski et al., 2005). It thus seems that *Bradyrhizobium* strains of the *nodA* Clade I.1 group are prevalent in temperate Australia, *i.e.*, in areas where the endemic tribes Bossiaeeae–Mirbelieae together with *Acacia* species dominate the indigenous legume communities. The dominance of Clade I bradyrhizobia (most of which represent lineage Aus1) may result from the presence of sets of genes enabling effective nodulation of broad range of indigenous legume spp. The *nodA* Clade I.1 is characterized by relatively short internal branches due to limited sequence diversity of its members despite the evolutionary distance which separates the hosts (*i.e.*, Mimosoideae and Papilionoideae) from which they originate. The diversification of *nodA* Clade I.1 is therefore a relatively recent phenomenon that could, presumably, be traced to final stages of aridification during the Pleistocene period (Martin, 2006; Byrne et al., 2008, 2011). This aridification process led to habitat fragmentation thereby contributing not only to extinction of many, mainly mesic taxa, but also to allopatric speciation of sclerophyllus legume taxa (Crisp and Cook, 2009) and, as we believe, the extinction of some and synchronous diversification of other rhizobial symbionts. Consistent with this idea, the youngest and most species-rich lineages of the Bossiaeeae–Mirbelieae tribes and of the *Acacia* genus generally occur in temperate Australia (Crisp and Cook, 2009; Murphy et al., 2010). In contrast, the NT strains are more divergent, with average nucleotide diversities for the respective housekeeping and *nodA* datasets that were generally twice and five times higher than those estimated for the WA isolates. The more pronounced and earlier diversification associated with the NT isolates may be ascribed to the emergence of tropical monsoon climate in northern Australia, which can be traced back to the Middle Miocene (Bowman et al., 2010), thus allowing for a much longer evolutionary period (~15 million years) than that associated with the WA lineages.

Disparate evolutionary histories and ecological requirements of legume hosts are likely to be among the primary causes of the differences observed in housekeeping and *nodA* gene phylogenies between NT and WA isolates. Accordingly, some NT legume hosts, e.g. *Crotalaria*, *Indigofera*, *Tephrosia*, *Uraria* spp. are native to the tropical part of Australia or its Eremean zone, and only sporadically inhabit the south-western part of Western Australia (http://www.chah.gov.au/avh/). Moreover, in contrast to the WA hosts, 13 out of the 15 NT legume genera have their centers of diversification outside Australia, and most of them colonized this landmass during the last several million years (Sniderman and Jordan, 2011). For instance, based on phylogenetic analyses, there were at least three independent colonization events from Africa to Australia by *Indigofera* spp. (Schrire et al., 2009). It is thus conceivable that continental drift towards Asia, which facilitated multiple colonization by legume taxa, also contributed to colonization of this landmass by their rhizobial symbionts. For example, this could have been the case for *B. elkanii*, *B. liaoningense*, and *B. yuanmingense*, all of which have been reported as important groups on other continents (Ormeno-Orillo et al., 2006; Appunu et al., 2008; Steenkamp et al., 2008; Zhang et al., 2008; Gueye et al., 2009). Consistent with this view, the isolates in the "*B. elkanii* super-group" (*i.e.*, lineages Aus15–21; Figs. 1 and 2) and the deeply divergent groups Aus22–23 and Aus24 mostly originated from the pantropical legume genera, *Crotalaria*, *Dunbaria*, *Uraria*, and *Vigna*. Notably, with the exception of lineage Aus24, their *nodA* sequences cluster within the large pantropical *nodA* Clade III, which is widespread in sub-Saharan Africa, in the Americas, and in southern and eastern Asia (Moulin et al., 2004; Stępkowski et al., 2007). However, the fact that the same legumes also host *Bradyrhizobium* strains that cluster in clades regarded as "Australian" (e.g., *nodA* Clades IV and X) may thus reflect host swaps of indigenous Australian *Bradyrhizobium* lineages to the new "exotic" hosts, a phenomenon that is probably common in this rhizobium genus (Rodríguez-Echeverría, 2010).

We conclude that the differences in climate characteristics in WA and the NT, favoring different legume taxa, contributed to the formation of distinct *Bradyrhizobium* communities which in both areas are composed of multiple lineages. However, the *Bradyrhizobium* communities in Australia are likely to have been shaped, not only by the dynamic events related to evolution and migration of their legumes hosts, but also by the complex and interlinked edaphic, geographic and climatic processes that underpin the history of this continent. Given the limited number of strains and locations we investigated, it remains to be determined whether the pattern we detected in this study is indeed common throughout Australia. Elucidation of this issue will require a broad-scale sampling combined with application of metagenomic approaches targeting selected symbiotic and housekeeping genes (Suenaga, 2012).

Acknowledgments

This work was financed by Grant N303 333736 (TS) from Polish Ministry of Science and Education. EW was supported by the Murdoch University Special Research Grant, and ETS by the South African National Department of Science and Technology (DST) and the National Research Foundation (NRF). The ARR isolates were made available courtesy of the Supervising Scientist Division, NT, Australian Government Department of Sustainability, Environment, Water, Population and Communities.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.12.020.

References

Abascal, F., Zardoya, R., Posada, D., 2005. ProtTest: selection of bestfit models of protein evolution. Bioinformatics 21, 2104–2105.
Andam, C.P., Parker, M.A., 2008. Origins of *Bradyrhizobium* nodule symbionts from two legume trees in the Philippines. J. Biogeogr. 35, 1030–1039.
Appunu, C., N'Zoue, A., Laguerre, G., 2008. Genetic diversity of native bradyrhizobia isolated from soybeans (*Glycine max* L.) in different agricultural-ecological-climatic regions of India. Appl. Environ. Microbiol 74, 5991–5996.
Barnet, Y.M., Catt, P.C., 1991. Distribution and characteristics of root-nodule bacteria isolated from Australia *Acacia* spp. Plant Soil 135, 109–120.
Barnet, Y.M., Catt, P.C., Hearne, D.H., 1985. Biological nitrogen fixation and root-nodule bacteria (*Rhizobium* sp. and *Bradyrhizobium* sp.) in two rehabilitating sand dune areas planted with *Acacia* spp. Aust. J. Bot. 33, 595–610.
Bontemps, C., Elliott, G.N., Simon, M.F., Dos Reis, F.B.D., Gross, E., Lawton, R.C., Neto, N.E., Loureiro, M.D., De Faria, S.M., Sprent, J.I., James, E.K., Young, J.P.W., 2010. *Burkholderia* species are ancient symbionts of legumes. Mol. Ecol. 19, 44–52.
Bowman, D.M.J.S., Brown, G.K., Braby, M.F., Brown, J.R., Cook, L.G., Crisp, M.D., Ford, F., Haberle, S., Hughes, J., Isagi, Y., Joseph, L., McBride, J., Nelson, G., Ladiges, P.Y., 2010. Biogeography of the Australian monsoon tropics. J. Biogeogr. 37, 201–216.
Brennan, K., 1992. Annotated Checklist of Vascular Plants of the Alligator Rivers Region. Open File Record 62. Office of the Supervising Scientist, Alligator Rivers Region Research Institute, Jabiru, NT, Australia.
Byrne, M., Yeates, D.K., Joseph, L., Kearney, M., Bowler, J., Williams, M.A.J., Cooper, S., Donnellan, S.C., Keogh, J.S., Leys, R., Melville, J., Murphy, D.J., Porch, N., Wyrwoll, K.H., 2008. Birth of a biome: insights into the assembly and maintenance of the Australian arid zone biota. Mol. Ecol. 17, 4398–4417.
Byrne, M., Steane, D.A., Joseph, L., Yeates, D.K., Jordan, G.J., Crayn, D., Aplin, K., Cantrill, D.J., Cook, L.G., Crisp, M.D., Keogh, J.S., Melville, J., Moritz, C., Porch, N.,

Sniderman, Sunnucks, P., Weston, P.H., 2011. Decline of a biome: evolution, contraction, fragmentation, extinction and invasion of the Australian mesic zone biota. J. Biogeogr. 38, 1635–1656.
Cardinale, M., Lanza, A., Bonnì, M.L., Marsala, S., Puglia, A.M., Quatrini, P., 2008. Diversity of rhizobia nodulating wild shrubs of Sicily and some neighbouring islands. Arch. Microbiol. 190, 461–470.
Chahboune, R., Carro, L., Peix, A., Barrijal, S., Velázquez, E., Bedmar, E.J., 2011. *Bradyrhizobium cytisi* sp. nov. isolated from effective nodules of *Cytisus villosus* in Morocco. Int. J. Syst. Evol. Microbiol. 61, 2922–2927.
Chaintreuil, C., Boivin, C., Dreyfus, B., Giraud, E., 2001. Characterization of the common nodulation genes of the photosynthetic *Bradyrhizobium* sp. ORS285 reveals the presence of a new insertion sequence upstream of *nodA*. FEMS Microbiol. Lett. 194, 83–86.
Chang, Y.L., Wang, J.Y., Wang, E.T., Liu, H.C., Sui, X.H., Chen, W.X., 2011. *Bradyrhizobium lablabi* sp. nov., isolated from effective nodules of *Lablab purpureus* and *Arachis hypogaea* grown in Southern China. Int. J. Syst. Evol. Microbiol. 61, 2496–2502.
Chen, W.M., Moulin, L., Bontemps, C., Vandamme, P., Bena, G., Boivin-Masson, C., 2003. Legume symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. J. Bacteriol. 185, 7266–7272.
Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res. 16, 10881–10890.
Crisp, M.D., Cook, L.G., 2003. Phylogeny and embryo sac evolution in the endemic Australasian papilionoid tribes Mirbelieae and Bossiaeeae. In: Klitgaard B.B., Bruneau, A. (Eds.), Advances in Legume Systematics, Part 10. Higher Level Systematics. The Royal Botanic Gardens, Kew, pp. 253–268.
Crisp, M.D., Cook, L.G., 2009. Explosive radiation or cryptic mass extinction? Interpreting signatures in molecular phylogenies. Evolution 63, 2257–2265.
Crisp, M.D., Laffan, S., Linder, H.P., Monro, A., 2001. Endemism in the Australian flora. J. Biogeogr. 28, 183–198.
Crisp, M., Cook, L., Steane, D., 2004. Radiation of the Australian flora: what can comparisons of molecular phylogenies across multiple taxa tell us about the evolution of diversity in present-day communities? Philos. Trans. Roy. Soc. B. 359, 1551–1571.
Dalton, H., 1980. The cultivation of diazotrohic organisms. In: Bergerson, F.J. (Ed.), Methods for Evaluating Biological Nitrogen Fixation. Wiley and Sons, Chichester, pp. 13–64.
DeConto, R.M., Pollard, D., 2003. Rapid Cenozoic glaciation of Antarctica induced by declining atmospheric CO_2. Nature 421, 245–249.
Dénarié, J., Debellé, F., Promé, J.C., 1996. Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65, 503–535.
Doignon-Bourcier, F., Sy, A., Willems, A., Torck, U., Dreyfus, B., Gillis, M., de Lajudie, P., 1999. Diversity of bradyrhizobia from 27 tropical leguminosae species native of Senegal. Syst. Appl. Microbiol. 22, 647–661.
Doolittle, W.F., Papke, R.T., 2006. Genomics and the bacterial species problem. Genome Biol. 7, 116.
Doyle, J.J., 1998. Phylogenetic perspectives on nodulation: evolving views of plants and symbiotic bacteria. Trends Plant Sci. 3, 473–478.
Florindo, F., Cooper, A.K., O'Brien, P.E., 2003. Introduction to 'Antarctic Cenozoic palaeoenvironments: geologic record and models'. Palaeogeogr. Palaeoclimat. 198, 1–9.
Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F.L., Swings, J., 2005. Opinion: re-evaluating prokaryotic species. Nat. Rev. Microbiol. 3, 733–739.
Green, J.L., Bohannan, B.J.M., Whitaker, R.J., 2008. Microbial biogeography: from taxonomy to traits. Science 320, 1039–1043.
Gu, C.T., Wang, E.T., Sui, X.H., Chen, W.F., Chen, W.X., 2007. Diversity and geographical distribution of rhizobia associated with *Lespedeza* spp. in temperate and subtropical regions of China. Arch. Microbiol. 188, 355–365.
Gueye, F., Moulin, L., Sylla, S., Ndoye, I., Béna, G., 2009. Genetic diversity and distribution of *Bradyrhizobium* and *Azorhizobium* strains associated with the herb legume *Zornia glochidiata* sampled from across Senegal. Syst. Appl. Microbiol. 32, 387–399.
Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.
Han, T.X., Tian, C.F., Wang, E.T., Chen, W.X., 2010. Associations among rhizobial chromosomal background, *nod* genes, and host plants based on the analysis of symbiosis of indigenous rhizobia and wild legumes native to Xinjiang. Microb. Ecol. 59, 311–323.
Hill, R.S., 2004. Origins of the southeastern Australian vegetation. Philos. Trans. Roy. Soc. B 359, 1537–1549.
Hopper, S.D., Gioia, P., 2004. The Southwest Australian Floristic Region: evolution and conservation of a global hot spot of biodiversity. Ann. Rev. Ecol. Evol. Syst. 35, 623–650.
Hoque, M.S., Broadhurst, L.M., Thrall, P.H., 2011. Genetic characterisation of root nodule bacteria associated with *Acacia salicina* and *A. stenophylla* (*Mimosaceae*) across southeastern Australia. Int. J. Evol. Syst. Microbiol. 61, 299–309.
Hudson, R.R., Boos, D.D., Kaplan, N.L., 1992a. A statistical test for detecting population subdivision. Mol. Biol. Evol. 9, 138–151.
Hudson, R.R., Slatkin, M., Maddison, W.P., 1992b. Estimation of levels of gene flow from DNA sequence data. Genetics 132, 583–589.
Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.
Islam, M.S., Kawasaki, H., Muramatsu, Y., Nakagawa, Y., Seki, T., 2008. *Bradyrhizobium iriomotense* sp. nov., isolated from a tumor-like root of the legume *Entada koshunensis* from Iriomote Island in Japan. Biosci. Biotechnol. Biochem. 72, 1416–1429.
Jarabo-Lorenzo, A., Perez-Galdona, R., Donate-Correa, J., Rivas, R., Velazquez, E., Hernandez, M., Temprano, F., Martinez-Molina, E., Ruiz-Argueso, T., Leon-Barrios, M., 2003. Genetic diversity of bradyrhizobial populations from diverse geographic origins that nodulate *Lupinus* spp. and *Ornithopus* spp. Syst. Appl. Microbiol. 26, 611–623.
Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.
Jordan, J.C., 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow growing root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. 32, 136–139.
Kellogg, C.A., Griffin, D.W., 2006. Aerobiology and the global transport of desert dust. Trends Ecol. Evol. 21, 638–644.
Kuykendall, L.M., Saxena, B., Devine, T.E., Udell, S.E., 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. 38, 501–503.
Lafay, B., Burdon, J.J., 1998. Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. Appl. Environ. Microbiol. 64, 3989–3997.
Lafay, B., Burdon, J.J., 2001. Small-subunit rRNA genotyping of rhizobia nodulating Australian *Acacia* spp. Appl. Environ. Microbiol. 67, 396–402.
Lafay, B., Burdon, J.J., 2006. Molecular diversity of rhizobia nodulating the invasive legume *Cytisus scoparius* in Australia. J. Appl. Microbiol. 100, 1228–1238.
Lafay, B., Burdon, J.J., 2007. Molecular diversity of legume root-nodule bacteria in Kakadu National Park, Northern Territory, Australia. PLoS ONE 7;2(3): e277.
Lafay, B., Bullier, E., Burdon, J.J., 2006. Bradyrhizobia isolated from root nodules of *Parasponia* (Ulmaceae) do not constitute a separate coherent lineage. Int. J. Syst. Evol. Microbiol. 56, 1013–1018.
Lange, R.T., 1961. Nodule bacteria associated with the indigenous Leguminosae of south-western Australia. J. Gen. Microbiol. 61, 351–359.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.
Lavin, M., Herendeen, P.S., Wojciechowski, M.F., 2005. Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the tertiary. Syst. Biol. 54, 575–594.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.
Lin, D.X., Man, C.X., Wang, E.T., Chen, W.X., 2007. Diverse rhizobia that nodulate two species of *Kummerowia* in China. Arch. Microbiol. 188, 495–507.
Marsudi, N.D.S., Glenn, A.R., Dilworth, M.J., 1999. Identification and characterization of fast-and slow-growing root nodule bacteria from South-Western Australian soils able to nodulate *Acacia saligna*. Soil Biol. Biochem. 31, 1229–1238.
Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P., Willems, A., 2008. Advantages of Multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer*. Int. J. Syst. Evol. Microbiol. 58, 200–214.
Martin, H.A., 2006. Cenozoic climatic change and the development of the arid vegetation in Australia. J. Arid Environ. 66, 533–563.
Martiny, J.B.H., Bohannan, B.J.M., Brown, J.H., Colwell, R.K., Fuhrman, J.A., Green, J.L., Horner-Devine, M.C., Kane, M., Krumins, J.A., Kuske, C.R., Morin, P.J., Naeem, S., Ovreås, L., Reysenbach, A.L., Smith, V.H., Staley, J.T., 2006. Microbial biogeography: putting microorganisms on the map. Nat. Rev. Microbiol. 4, 102–112.
Masson-Boivin, C., Giraud, E., Perret, X., Batut, J., 2009. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends Microbiol. 17, 458–466.
McInnes, A., Ashwath, N., 1992. Techniques for the Collection, Isolation, Authentication and Maintenance of Rhizobia. Internal Report 84. Supervising Scientist for the Alligator Rivers Region, Jabiru, NT, Australia.
McInnes, A., Date, R.A., 2005. Improving the survival of rhizobia on *Stylosanthes* and *Desmanthus* seed at high temperature. Aust. J. Exp. Agricult. 45, 171–182.
Moulin, L., Munive, A., Dreyfus, B., Boivin-Masson, C., 2001. Nodulation of legumes by members of the Beta-subclass of Proteobacteria. Nature 411, 948–950.
Moulin, L., Béna, G., Boivin-Masson, C., Stępkowski, T., 2004. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. Mol. Phyl. Evol. 30, 720–732.
Muñoz, V., Ibañez, F., Tonelli, M.L., Valetti, L., Anzuay, M.S., Fabra, A., 2011. Phenotypic and phylogenetic characterization of native peanut *Bradyrhizobium* isolates obtained from Córdoba, Argentina. Syst. Appl. Microbiol. 34, 446–452.
Murphy, D.J., Brown, G.K., Miller, J.T., Ladiges, P.Y., 2010. Molecular phylogeny of *Acacia* Mill. (Mimosoideae: Leguminosae): evidence for major clades and informal classification. Taxon 59, 7–19.
Nei, M., 1987. Molecular Evolutionary Genetics. Columbia Univ. Press, New York.
Ngom, A., Nakagawa, Y., Sawada, H., Tsukahara, J., Wakabayashi, S., Uchiumi, T., Nuntagij, A., Kotepong, S., Suzuki, A., Higashi, S., Abe, M., 2004. A novel symbiotic nitrogen-fixing member of the *Ochrobactrum* clade isolated from root nodules of *Acacia mangium*. J. Gen. Appl. Microbiol. 50, 17–27.

Ormeno-Orillo, E., Vinuesa, P., Zuniga-Davila, D., Martınez-Romero, E., 2006. Molecular diversity of native bradyrhizobia isolated from Lima bean (*Phaseolus lunatus* L.) in Peru. Syst. Appl. Microbiol. 29, 253–262.

Papke, R.T., Ramsing, N.B., Bateson, M.M., Ward, D.M., 2003. Geographical isolation in spring cyanobacteria. Environ. Microbiol. 5, 650–659.

Parker, M.A., 2008. Symbiotic relationships of legumes and nodule bacteria on Barro Colorado Island, Panama: a review. Microb. Ecol. 55, 662–672.

Parker, M.A., Lafay, B., Burdon, J.J., Van Berkum, P., 2002. Conflicting phylogeographic patterns in rRNA and *nifD* indicate regionally restricted gene transfer in *Bradyrhizobium*. Microbiology 148, 2557–2565.

Parker, M.A., Wurtz, A.K., Paynter, Q., 2007. Nodule symbiosis of invasive *Mimosa pigra* in Australia and in ancestral habitats: a comparative analysis. Biol. Invasions 9, 127–138.

Posada, D., 2008. JModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.

Radutoiu, S., Madsen, L.H., Madsen, E.B., Felle, H.H., Umehara, Y., Gronlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., Stougaard, J., 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature 425, 585–592.

Ramirez-Bahena, M.H., Peix, A., Rivas, R., Camacho, M., Rodriguez-Navarro, D.N., Mateos, P.F., Martinez-Molina, E., Willems, A., Velazquez, E., 2009. *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov. isolated from effective nodules of *Pachyrhizus erosus*. Int. J. Syst. Evol. Microbiol. 59, 1929–1934.

Rivas, R., Velazquez, E., Willems, A., Vizcaino, N., Subba-Rao, N.S., Mateos, P.F., Gillis, M., Dazzo, F.B., Martinez-Molina, E., 2002. A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) druce. Appl. Environ. Microbiol. 68, 5217–5222.

Rivas, R., Willems, A., Palomo, J.L., García-Benavides, P., Mateos, P.F., Martínez-Molina, E., Gillis, M., Velázquez, E., 2004. *Bradyrhizobium betae* sp. nov., isolated from roots of *Beta vulgaris* affected by tumour-like deformations. Int. J. Syst. Evol. Microbiol. 54, 1271–1275.

Rivas, R., Martens, M., de Lajudie, P., Willems, A., 2009. Multilocus sequence analysis of the genus *Bradyrhizobium*. Syst. Appl. Microbiol. 32, 101–110.

Rodríguez-Echeverría, S., 2010. Rhizobial hitchhikers from Down Under: invasional meltdown in a plant–bacteria mutualism? J. Biogeogr. 37, 1611–1622.

Sawada, H., Kuykendall, L.D., Young, J.M., 2003. Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J. Gen. Appl. Microbiol. 49, 155–179.

Schmidt, S.K., Lynch, R.C., King, A.J., Karki, D., Robeson, M.S., Nagy, L., Williams, M.W., Mitter, M.S., Freeman, K.R., 2011. Phylogeography of microbial phototrophs in the dry valleys of the high Himalayas and Antarctica. Proc. Roy. Soc. B 278, 702–708.

Schrire, B.D., Lewis, G.P., Lavin, M., 2005. Biogeography of Leguminosae. In: Lewis, G., Schrire, B., MacKinder, B., Lock, M. (Eds.), Legumes of World. The Royal Botanic Gardens, Kew, pp. 21–56.

Schrire, B.D., Lavin, M., Barker, N.P., Forest, F., 2009. Phylogeny of the tribe Indigofereae (Leguminosae–Papilionoideae): geographically structured more in succulent-rich and temperate settings than in grass-rich environments. Am. J. Bot. 96, 816–852.

Short, P.S., Albrecht, D.E., Cowie, I.D., Lewis, D.L., Stuckey, B.M., 2011. Checklist of the Vascular Plants of the Northern Territory. Northern Territory Herbarium, Department of Natural Resources, Environment, the Arts and Sport, NT.

Sjöström, A., Gross, C.L., 2006. Life-history characters and phylogeny are correlated with extinction risk in the Australian angiosperms. J. Biogeogr. 33, 271–290.

Sniderman, J.M.K., Jordan, G.J., 2011. Extent and timing of floristic exchange between Australian and Asian rain forests. J. Biogeogr. 38, 1445–1455.

Sprent, J.I., 2007. Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. New Phytol. 174, 11–25.

Steenkamp, E.T., Stepkowski, T., Przymusiak, A., Botha, W.J., Law, I.J., 2008. Cowpea and peanut in southern Africa are nodulated by diverse *Bradyrhizobium* strains harboring nodulation genes that belong to the large pantropical clade common in Africa. Mol. Phyl. Evol. 48, 1131–1144.

Stępkowski, T., Moulin, L., Krzyżańska, A., McInnes, A., Law, I.J., Howieson, J., 2005. European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of Western Australia and South Africa. Appl. Environ. Microbiol. 71, 7041–7052.

Stępkowski, T., Hughes, C.E., Law, I.J., Markiewicz, L., Gurda, D., Chlebicka, A., Moulin, L., 2007. Diversification of lupin *Bradyrhizobium* strains: evidence from nodulation gene trees. Appl. Environ. Microbiol. 73, 3254–3264.

Stępkowski, T., Żak, M., Moulin, L., Króliczak, J., Golińska, B., Narożna, D., Safronova, V.I., Mądrzak, C.J., 2011. *Bradyrhizobium canariense* and *Bradyrhizobium japonicum* are the two dominant rhizobium species in root nodules of lupin and serradella plants growing in Europe. Syst. Appl. Microbiol. 34, 368–375.

Suenaga, H., 2012. Targeted metagenomics: a high-resolution metagenomics approach for specific gene clusters in complex microbial communities. Environ. Microbiol. 14, 13–22.

Sullivan, J.T., Ronson, C.W., 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. USA 95, 5145–5149.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., Dreyfus, B., 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol. 183, 214–220.

Tavaré, S., 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura, R.M. (Ed.), Some Mathematical Questions in Biology – DNA Sequence Analysis. Amer. Math. Soc, Providence, RI, pp. 57–86.

Valverde, A., Velazquez, E., Fernandez-Santos, F., Vizcaino, N., Rivas, R., Mateos, P.F., Martinez-Molina, E., Igual, J.M., Willems, A., 2005. *Phyllobacterium trifolii* sp nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. Int. J. Syst. Evol. Microbiol. 55, 1985–1989.

Vincent, J.M., 1970. A Manual for the Practical Study of Root-Nodule Bacteria. Blackwell Scientific Publications, Ltd., Oxford.

Vinuesa, P., Leon-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Perez-Galdona, R., Werner, D., Martinez-Romero, E., 2005a. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies α and *Bradyrhizobium* genospecies β. Int. J. Syst. Evol. Microbiol. 55, 569–575.

Vinuesa, P., Silva, C., Werner, D., Martinez-Romero, E., 2005b. Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. Mol. Phyl. Evol. 34, 29–54.

Vinuesa, P., Rojas-Jiménez, K., Contreras-Moreira, B., Mahnam, S.K., Prasad, B.N., Moe, H., Selvaraju, S.B., Thierfelder, H., Werner, D., 2008. Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the asiatic continent. Appl. Environ. Microbiol. 74, 6987–6996.

Whitaker, R.J., Grogan, D.W., Taylor, J.W., 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301, 976–978.

Willems, A., Coopman, R., Gillis, M., 2001a. Comparison of sequence analysis of 16S–23S rDNA spacer regions, AFLP analysis and DNA–DNA hybridizations in *Bradyrhizobium*. Syst. Appl. Microbiol. 51, 623–632.

Willems, A., Coopman, R., Gillis, M., 2001b. Phylogenetic and DNA–DNA hybridization analyses of *Bradyrhizobium* species. Int. J. Syst. Evol. Microbiol. 51, 111–117.

Xu, L.M., Ge, C., Cui, Z., Li, J., Fan, H., 1995. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybean. Int. J. Syst. Bacteriol. 45, 706–711.

Yao, Z.Y., Kan, F.L., Wang, E.T., Wei, G.H., Chen, W.X., 2002. Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. Int. J. Syst. Evol. Microbiol. 52, 2219–2230.

Zhang, Y.F., Wang, E.T., Tian, C.F., Wang, F.Q., Han, L.L., Chen, W.F., Chen, W.X., 2008. *Bradyrhizobium elkanii*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium japonicum* are the main rhizobia associated with *Vigna unguiculata* and *Vigna radiata* in the subtropical region of China. FEMS Microbiol. Lett. 285, 146–154.