BIODIVERSITY RESEARCH

Jack-of-all-trades and master of many? How does associated rhizobial diversity influence the colonization success of Australian *Acacia* species?

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ABSTRACT

Aim To evaluate the role of rhizobial diversity, and symbiotic promiscuity, on the invasive ability of Australian acacias (*Acacia* species in subgenus *Phyllodineae* native to Australia).

Location Global.

Methods A bibliographic review of the rhizobial diversity associated with Australian *Acacia* species was performed to assess symbiotic promiscuity for invasive and non-invasive species. The rhizobial diversity associated with *Acacia dealbata* and *A. saligna* in Australia and Portugal and with *A. pycnantha* in Australia and South Africa was assessed by 16S rDNA and intergenic spacer sequencing of bacteria isolated from field-collected nodules.

Results All studied Australian acacias are nodulated by strains in the genus *Bradyrhizobium*, which appears to be the dominant group of acacia symbionts in native and non-native soils. Both literature and experimental data from this study suggest that Australian bradyrhizobia might have been co-introduced with acacias to new geographical regions. The studied *Acacia* species can also harbour other root-nodulating alpha and betaproteobacteria genera, although these are less abundant than *Bradyrhizobium*.

Main conclusions There is no clear difference in the diversity of rhizobial species associated with invasive and non-invasive Australian acacias. All studied invasive acacias nodulate in both native and non-native regions, harbouring predominantly *Bradyrhizobium* strains but showing some degree of symbiotic promiscuity. The co-introduction of compatible root-nodulating bacteria from Australia might explain the establishment of invasive populations, but novel associations with rhizobia from the invaded soils are also possible. Invasive legumes might use both strategies but species with low symbiotic promiscuity would become invasive only if compatible bacteria are co-introduced in the new regions. The progress of invasion and the impacts on the invaded ecosystems might also differ depending on the nodulation strategy.

Keywords
Biological invasions, *Bradyrhizobium, Burkholderia,* mutualism, root nodule, wattles

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INTRODUCTION

One of the most fascinating forms of plant-microbe symbiosis is that between leguminous plants and soil bacteria commonly known as rhizobia (or root-nodulating bacteria). These bacteria are capable of entering the root and inducing the development of root nodules where biological nitrogen fixation takes place. Inside these root nodules, atmospheric nitrogen is reduced into organic forms that can be utilized by the host plant, allowing legumes to grow in nitrogen-poor soils. Legumes, and their

associated rhizobia, are key components of agriculture ecosystems and natural and semi-natural terrestrial ecosystems. While this association has been widely studied in species of economic interest – mainly soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), clovers (*Trifolium* spp.), vetches (*Vicia* spp.) and alfalfa (*Medicago* spp.) – only a small proportion of wild legumes have been screened for nodulation globally (Sprent, 2001). Not all legumes can nodulate and, indeed, nodulation is not uniformly distributed in the three subfamilies within the Leguminosae. The percentage of nodulating species in the subfamilies Mimosoideae and Papilionoidae ranges between 90% and 97%, while only 23% of the studied species in the Caesalpinioideae subfamily nodulate (Sprent, 2001).

Although for the first century of rhizobial research, all known legume symbionts belonged to the class alphaproteobacteria, root-nodulating bacteria are a phylogenetically diverse group (Fig. 1), and this diversity is likely to increase as more legumes are studied for nodulation. Nevertheless, most of the known legume symbionts belong to only five different genera: *Rhizobium, Mesorhizobium, Ensifer* (formerly *Sinorhizobium*) and *Bradyrhizobium* in the alphaproteobacteria, and *Burkholderia* in the betaproteobacteria (Sawada *et al.*, 2003; Bontemps *et al.*, 2010; Weir, 2010).

The specificity of the legume–rhizobia association is controlled by a fine molecular communication between the plant
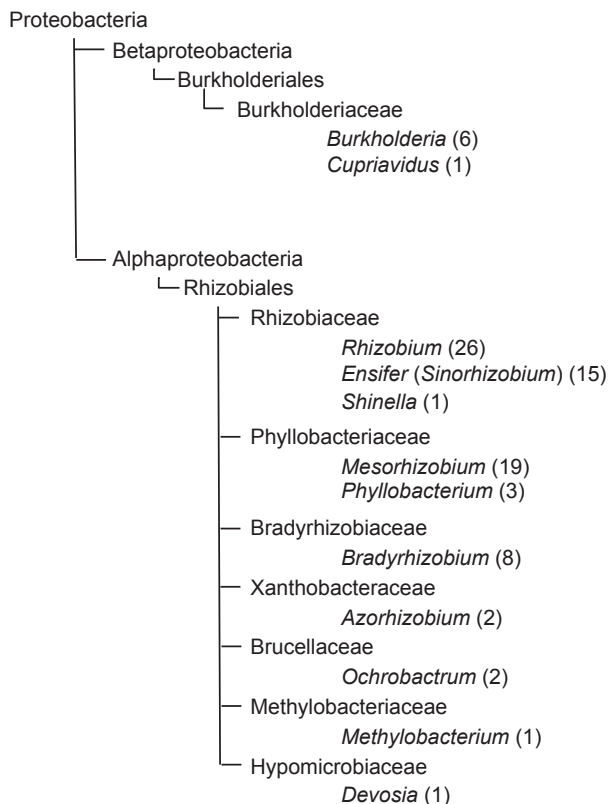
and the bacteria (Perret *et al.*, 2000). Most of the analysed species have a certain degree of promiscuity and can associate with more than one symbiotic partner. However, there are some species with very narrow ranges of specificity, mainly legumes belonging to the tribes Cicereae, Trifolieae and Viceae that associate with *Rhizobium* spp., and others with broad compatibility range, also known as symbiotic promiscuity.

THE LEGUME–RHIZOBIA SYMBIOSIS IN INVASION ECOLOGY

Access to compatible rhizobia is one of the most critical factors determining the colonizing and invasive ability of symbiotic legumes, making these poorer colonists than flowering plants in general (Parker, 2001 and references therein). Nonetheless, some legume taxa are aggressive invaders, with Richardson & Rejmánek (2011) listing 121 woody legume taxa that qualify as 'invasive' (sensu Pyšek *et al.*, 2004). Furthermore, these species have large impacts on invaded communities mainly through altering nutrient content and cycling (e.g. Yelenik *et al.*, 2007; Liao *et al.*, 2008; Le Maitre *et al.*, 2011). The role of the legume–rhizobia symbiosis in plant invasions is virtually unknown, and only recently have studies addressed the diversity of rhizobia and the relevance of this mutualism for invasiveness in legumes (Richardson *et al.*, 2000; Weir *et al.*, 2004; Lafay & Burdon, 2006; Parker *et al.*, 2006; Rodríguez-Echeverría *et al.*, 2007, 2009; Andam & Parker, 2008; Rodríguez-Echeverría, 2010). The remarkable invasive capacity of some legumes might be related to specific traits that facilitate the access to compatible rhizobia in the new soils (Parker, 2001). Such traits would include a broad symbiotic promiscuity and the ability to nodulate at low rhizobial abundance, a likely situation in soils where a legume species is introduced for the first time.

Symbiotic promiscuity grants an advantage in colonizing new soils by allowing promiscuous legumes to find compatible symbionts more easily than those with a narrow symbiotic specificity. Indeed, highly promiscuous woody legumes such as *Robinia pseudoacacia* L. and *Cytisus scoparius* (L.) Link (Papilionoideae) and *Mimosa* spp. (Mimosoideae), that are globally invasive in a variety of ecosystems have been shown to acquire new symbionts in their introduced ranges (Fogarty & Facelli, 1999; Perez-Fernandez & Lamont, 2003; Lafay & Burdon, 2006; Leary *et al.*, 2006a). Therefore, symbiotic promiscuity has been considered a characteristic trait of invasive legumes (Richardson *et al.*, 2000; Parker, 2001), although few species have been examined in detail to corroborate this assumption.

Invasion by exotic legumes might be favoured in regions with closer phylogenetically related native legumes, because they may be more likely to host rhizobia that can nodulate the exotic species, facilitating invasion by providing compatible rhizobia. This could be the case for Australian acacias (*Acacia* species in subgenus *Phyllodineae* native to Australia, Miller *et al.* 2011, Richardson *et al.*, 2011) in South Africa, European

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Proteobacteria
 ├─ Betaproteobacteria
 │   └─ Burkholderiales
 │       └─ Burkholderiaceae
 │               Burkholderia (6)
 │               Cupriavidus (1)
 │
 └─ Alphaproteobacteria
     └─ Rhizobiales
         ├─ Rhizobiaceae
         │       Rhizobium (26)
         │       Ensifer (Sinorhizobium) (15)
         │       Shinella (1)
         ├─ Phyllobacteriaceae
         │       Mesorhizobium (19)
         │       Phyllobacterium (3)
         ├─ Bradyrhizobiaceae
         │       Bradyrhizobium (8)
         ├─ Xanthobacteraceae
         │       Azorhizobium (2)
         ├─ Brucellaceae
         │       Ochrobactrum (2)
         ├─ Methylobacteriaceae
         │       Methylobacterium (1)
         └─ Hypomicrobiaceae
                 Devosia (1)
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Figure 1 Taxonomy of root-nodulating bacteria associated with legumes. The number between brackets indicates the number of species described for each genus according to Weir (2010).

Lathyrus, Lotus, Trifolium and *Vicia* species in California, and the American *Mimosa pigra* in Australia (Parker, 2001; Parker *et al.*, 2007).

Nonetheless, recent studies have shown that native and exotic legumes are nodulated by different rhizobial communities, thus suggesting that native legumes might not be the main source of rhizobia for the invaders. For example, co-existing exotic and native woody legumes are nodulated by different and unrelated rhizobial groups in New Zealand (Weir *et al.*, 2004). Similarly, the rhizobial communities of invasive *C. scoparius* in Australia are more similar to each other than to their co-occurring native partners, although in this case, there was some overlap between the rhizobia nodulating both species assemblages (Lafay & Burdon, 2006). Interestingly, Lafay & Burdon (2006) also found that five of the twenty-one rhizobial genospecies isolated from *C. scoparius* occurred in both native and invasive range populations. A step further in understanding the source of rhizobia for invasive exotic legumes is provided by recent studies with the invasive *Acacia longifolia* in Portugal. In this case, the rhizobial community associated with the exotic legume is little diverse and is composed mainly by exotic bacteria probably introduced with the invasive plant (Rodríguez-Echeverría *et al.*, 2007; Rodríguez-Echeverría, 2010).

THE LEGUME–RHIZOBIA SYMBIOSIS IN AUSTRALIAN ACACIAS

To date, all screened Australian *Acacia* species nodulate in both their native and non-native regions. The dominance of slow-growing *Bradyrhizobium* species over fast-growing bacteria seems to be characteristic of Australian acacias and has even been suggested to be a taxonomical character differentiating this group from American and African *Acacia* (*senso lato*), which mainly associate with fast-growing rhizobia (Leary *et al.*, 2006b). In spite of this, it is clear that other bacteria, including fast-growing *Rhizobium*, can nodulate Australian *Acacia* species (Tables 1 and 2).

An extensive survey of the rhizobial diversity associated with 13 *Acacia* species confirmed *Bradyrhizobium* as the most abundant genus associated with this group in Australia (Table 1). About 97% of the isolated rhizobia belonged to this genus and were mostly related to *B. japonicum,* although strains of *B. elkanii* were found in root nodules of *A. cincinnata* (Lafay & Burdon, 2001). Lafay & Burdon (2001) also isolated *Rhizobium tropici* from three of the 13 studied *Acacia* species (*A. dealbata*, *A. mearnsii* and *A. melanoxylon*). These species were the most thoroughly sampled in this study, which seems to indicate that *Rhizobium* was not a common symbiont (Table 1). There are published reports of eight more species screened for nodulation in Australia. Overall, all studied acacias in Australia are nodulated by *Bradyrhizobium* strains predominantly related to *B. japonicum* and half of them can also be nodulated by other alphaproteobacteria, mainly by *Rhizobium* strains (Table 1). A recent study on the rhizobial diversity of *A. stenophylla* and *A. salicina* questions the

Table 1 Diversity of root-nodulating bacteria isolated from *Acacia* in Australia.

Acacia species	Origin	Root-nodulating bacteria	Reference
A. cangaiensis	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
A. cincinnata	Qld	*Bradyrhizobium elkanii*	Lafay & Burdon, 2001
A. dealbata*	NSW	*Bradyrhizobium japonicum, Rhizobium tropici*	Lafay & Burdon, 2001
	Tas	*Bradyrhizobium japonicum*	
	Vic	*Bradyrhizobium japonicum, Rhizobium tropici*	
A. deanei	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
A. decurrens*	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
A. fulva	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
A. glaucocarpa	Qld	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
A. implexa*	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
	Vic	*Bradyrhizobium japonicum*	
A. irrorata	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
A. longifolia*	NSW	*Bradyrhizobium* and *Rhizobium*†	Barnet *et al.*, 1985
	Vic	*Rhizobium*†	Lawrie, 1983
A. mangium*	Qld	*Bradyrhizobium elkanii*	Galiana *et al.*, 1990
A. mearnsii*	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
	SA	*Bradyrhizobium japonicum*	
	Tas	*Bradyrhizobium japonicum*	
	Vic	*Bradyrhizobium japonicum, Rhizobium tropici*	
A. melanoxylon*	ACT	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
	NSW	*Bradyrhizobium japonicum, Rhizobium tropici*	
	Qld	*Bradyrhizobium japonicum*	
	SA	*Bradyrhizobium japonicum*	
	Tas	*Bradyrhizobium japonicum*	
	Vic	*Bradyrhizobium japonicum, Rhizobium tropici*	

Table 1 (Continued).

Acacia species	Origin	Root-nodulating bacteria	Reference
A. obliquinervia	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 1998
A. parramattensis	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
A. parvipinnula	NSW	*Bradyrhizobium japonicum*	Lafay & Burdon, 2001
*A. salicina**	NSW	*Bradyrhizobium, Rhizobium, Ensifer, Mesorhizobium, Burkholderia, Phyllobacterium* and *Devosia*	Hoque et al., 2011
*A. saligna**	WA	*Bradyrhizobium japonicum, Rhizobium leguminosarum, Rhizobium tropici*	Marsudi et al., 1999, Yates et al., 2004
	NSW	*Bradyrhizobium* and *Rhizobium†*	Barnet et al., 1985
A. stenophylla	NSW	*Bradyrhizobium, Rhizobium, Ensifer, Mesorhizobium, Burkholderia, Phyllobacterium* and *Devosia*	Hoque et al., 2011
A. suaveolens	NSW	*Bradyrhizobium* and *Rhizobium†*	Barnet et al., 1985
A. terminalis	NSW	*Bradyrhizobium†*	Barnet et al., 1985

Qld, Queensland; WA, Western Australia; SA, South Australia; NSW, New South Wales; Tas, Tasmania; ACT, Australian Capital Territory; Vic, Victoria.

*Invasive species (Richardson & Rejmánek, 2011; this issue).

†Described as fast-growing strains, not sequence available.

dominance of *Bradyrhizobium* strains for all Australian acacias (Hoque et al., 2011). These authors found *Rhizobium, Ensifer, Mesorhizobium, Burkholderia, Phyllobacterium* and *Devosia* in the root nodules collected from both *Acacia* species in Australia (Table 1). These new results suggest that the rhizobial diversity associated with Australian acacias in the native range might be underestimated, unless *A. salicina* and *A. stenophylla* are highly distinctive and promiscuous species. Interestingly, these two species are not invaders of significant global importance even though *A. salicina* is naturalized and invasive in the Caribbean Islands and Israel (Richardson & Rejmánek, 2011).

Data available on root-nodulating bacteria of Australian acacias in the native range do not show a clear difference on the symbiotic promiscuity of invasive and non-invasive species. This conclusion is based on data at the genus or species level but could be different at finer levels of taxonomic resolution because some rhizobial species, such as *B. japonicum*, are very heterogeneous and still poorly characterized.

Most studies outside Australia have focused on forestry plantations and nurseries, and little information on the rhizobial diversity for invasive *Acacia* populations is available (Table 2). Nevertheless, these studies also show that *Bradyrhizobium* is the dominant genus of acacia symbionts outside of Australia. Interestingly, some symbionts that were not previously reported from Australia have been found in non-native ranges (Table 2). For example, strains of *Ensifer meliloti* have been isolated from root nodules of *A. melanoxylon* and *A. saligna* in North Africa (Swelim et al., 1997; Amrani et al., 2009), while these have not been reported from Australia (Marsudi et al., 1999; Lafay & Burdon, 2001; Yates et al., 2004). Similarly, a study in South Africa by Joubert (2003) found *B. elkanii* and *R. leguminosarum* nodulating *A. mearnsii*. The same applies for *A. longifolia* although in this case the identity of fast-growing rhizobia from Australia reported by Lawrie (1983) and Barnet et al. (1985) is unclear because no genetic information is available for these strains. These results suggest that Australian *Acacia* species may associate with local bacterial strains from the newly colonized soils, but further work is needed to confirm this.

On the other hand, recent studies on invasive populations of *A. longifolia* in Europe have shown that exotic symbiotic bacteria might have been co-introduced with the invasive leguminous species facilitating its establishment and spread (Rodríguez-Echeverría, 2010). All *A. longifolia* symbionts in the invaded area belonged to *Bradyrhizobium,* and the analysis of the *nifD* and *nodA* genes indicated that they were related to Australian rather than European bacteria. How these bacteria got introduced into Europe remains unclear. Either they were intentionally co-introduced during plantation, as for other acacias (Prin et al., 2003), or accidentally introduced attached to seed coats (Perez-Ramirez et al., 1998). Although Australian bradyrhizobia are the predominant symbionts of invasive *A. longifolia* in Portugal, this species can also nodulate with other bradyrhizobia present in the invaded soils (Rodríguez-Echeverría et al., 2007, 2009). The relative contribution(s) of exotic *vs.* native rhizobia to the successful invasion of this species in particular, and of acacias in general, remains unknown.

A challenge to understand the relationship between rhizobial diversity and *Acacia* invasiveness is the lack of data from the native range for many invasive species. A literature survey shows that only seven *Acacia* species (*A. dealbata, A. decurrens, A. longifolia, A. mangium, A. mearnsii, A. melanoxylon* and *A. saligna*) have been analysed in the native and non-native ranges (Tables 1 and 2). Even for these species, conclusions about invasiveness and the rhizobial diversity in both ranges should be drawn with extreme caution because sampling designs are very different and could lead to inaccurate interpretations. Clearly, studies that use standardized methods to compare the rhizobial diversity of the same species in native and invaded areas are warranted.

Table 2 Diversity of root-nodulating bacteria isolated from Australian *Acacia* in introduced areas. Invasive status follows the author of the cited paper.

Species	Origin	Invasive?	Root-nodulating bacteria	Reference
A. auriculiformis	Malaysia, French Guiana, Côte d'Ivore, Hawaii	No	*Bradyrhizobium elkanii*	Le Roux *et al.*, 2009
	Indonesia	No	*Bradyrhizobium elkanii, Rhizobium* spp., *Ensifer (Sinorhizobium)* spp.	Nuswantara *et al.*, 1997
	Indonesia	No	*Bradyrhizobium elkanii, B. japonicum, Mesorhizobium loti*	Clapp *et al.*, 2001
	Philippinnes	No	*Rhizobium tropici*	Ngom *et al.*, 2004
	Thailand	No	*Bradyrhizobium elkanii, Ochrobactrum* spp.	Ngom *et al.*, 2004
A. cyclops	Libya	No	*Bradyrhizobium and Rhizobium*	Mohamed *et al.*, 2000
A. dealbata	South Africa	Yes	*Bradyrhizobium japonicum, Rhizobium leguminosarum, Mesorhizobium* spp., *Rhizobium* spp.	Joubert, 2003
A. decurrens	South Africa	Yes	*Bradyrhizobium japonicum*	Joubert, 2003
A. longifolia	New Zealand	Yes	*Bradyrhizobium japonicum*	Weir *et al.*, 2004
	Portugal	Yes	*Bradyrhizobium japonicum, B. elkanii*	Rodríguez-Echeverría *et al.*, 2007, Rodríguez-Echeverría, 2010
	Algeria	No	*Bradyrhizobium japonicum, Ensifer (Sinorhizobium) meliloti, Rhizobium gallicum*	Amrani *et al.*, 2009
A. mangium	Malaysia, French Guiana, Côte d'Ivore	No	*Bradyrhizobium elkanii*	Le Roux *et al.*, 2009
A. mangium × A. auriculiformis	Malaysia	No	*Bradyrhizobium japonicum*	Le Roux *et al.*, 2009
A. mearnsii	South Africa	Yes	*Bradyrhizobium japonicum, B. elkanii, Rhizobium leguminosarum, R. tropici*	Joubert, 2003
A. melanoxylon	Algeria	No	*Bradyrhizobium japonicum, Ensifer (Sinorhizobium) meliloti, Rhizobium gallicum*	Amrani *et al.*, 2009
A. saligna	Libya	No	*Bradyrhizobium* and Rhizobium**	Mohamed *et al.*, 2000
	Algeria	No	*Bradyrhizobium japonicum, Ensifer (Sinorhizobium) meliloti, Rhizobium gallicum*	Amrani *et al.*, 2009
	Egypt	No	*Bradyrhizobium* and Rhizobium**	Swelim *et al.*, 1997

*Not sequence available.

CURRENT RESEARCH

Research on the role of rhizobia in the legume invasion process in general, and for Australian acacias in particular, has developed only over the last decade. The currently available information on Australian acacias is limited to ten of the 23 species considered invasive world-wide (Richardson & Rejmánek, 2011). Not only is information on basic rhizobial ecology in the native range generally scarce, but also studies from non-native regions have mainly focused on rhizobial diversity associated with nursery plants only. A common research framework that uses comparative approaches in native and non-native regions for different acacias is essential to assess the role of *Acacia*–rhizobia symbiosis in the invasion process. The first data from two studies following this rationale are presented later and represent an important contribution to fully understand the global experiment of human-mediated introductions of Australian acacias (Richardson *et al.*, 2011). These studies were performed in two distant regions – Portugal and South Africa – where Australian acacias have become significant components of their floras. The data obtained from these studies constitute a new piece to the puzzle of how these species establish and grow as introduced species in novel ecosystems around the world.

Invasive Australian acacias in Portugal

Several Australian acacias were introduced into Portugal during the 19th and 20th centuries for dune stabilization, gardening and forestry purposes. Nowadays, three species, *A. longifolia, A. dealbata* and *A. saligna,* are widespread invaders with important impacts on the structure and functioning of native ecosystems. To date, the role of rhizobia in invasion has been investigated only for *A. longifolia* with no information available for the other species. Currently ongoing work is focused on the rhizobial diversity associated with *A. dealbata* and *A. saligna* and its role in the invasion process.

Root nodules were collected from nine sites in Portugal across the invasive distributions of *A. dealbata* and *A. saligna*

(see Table S1 in Supporting Information). For comparison, root nodules were also collected from two different locations in Australia (see Table S1). In addition, root nodules from co-occurring *A. melanoxylon*, *A. longifolia* and *A. pycnantha* were also collected.

Bacteria were isolated and cultured from surface-disinfected nodules following standard procedures (Vincent, 1970), and DNA was extracted at 95°C from single colonies as described previously (Rodríguez-Echeverría *et al.*, 2003). Twenty-nine isolates were identified as different strains by PCR-BOXA1R and used for further analysis (Rodríguez-Echeverría *et al.*, 2003). The 16S rRNA gene was amplified with the primers 63f and 1387r (Marchesi *et al.*, 1998) with the following PCR program: initial denaturation at 94°C for 2 min; 35 cycles of (94°C for 1 min, 55°C for 1 min and 72°C for 1 min) and final elongation of 10 min. All reactions were carried out in a volume of 50 µL which contained: 2 µL template DNA, 0.8 µM of each primer, 200 µM of each dNTP (Bioron GmbH, Ludwigshafen, Germany), 1.5 mM MgCl$_2$, 1 U of Taq DNA polymerase (Bioron GmbH) in Taq DNA polymerase reaction buffer (Bioron GmbH). The sequencing reactions were carried out using an ABI PRISM Dye Terminator Cycle Sequence Reaction Kit (Perkin Elmer, Waltham, CA, USA). A BLAST search was conducted for the different sequences obtained in this study and those that retrieved the highest similarity values were included in the phylogenetic analysis. We conducted a maximum likelihood analysis using the HKY model of evolution, which was selected as the best-fit model using the Akaike information criterion (AIC) in Modeltest v3.7 (Posada & Crandall, 1998). The phylogenetic analysis was carried out using PhyML (Guindon & Gascuel, 2003) in Phylemon (Tárraga *et al.*, 2007). Branch support was assessed using bootstrapping (1000 resamplings).

Irrespective of the host plant species, all isolates from the invasive studied acacias in Portugal belonged to the genus *Bradyrhizobium*. Most isolates clustered with *B. japonicum*, with only one isolate from *A. dealbata* related to *B. elkanii* (Fig. 2). Five isolates from *A. dealbata*, *A. saligna* and *A. pycnantha* in Portugal were related to *B. canariense*, while none of the isolates from Australia clustered with this species. A higher phylogenetic diversity was found for the isolates from Australia because half of the isolates were *Bradyrhizobium*, and the remaining isolates were closely related to *R. lusitanum* and *M. amorphae* (Fig. 2).

Our results support the predominance of *Bradyrhizobium* among root-nodulating bacteria associated with Australian acacias (Lafay & Burdon, 2001). The lack of a distinction between the bacteria nodulating different *Acacia* species indicates a low specificity of the plants and of their associated bacteria, although this statement should be taken with caution because of the different sample size used for the different species and the low 16S sequence variation in the *Bradyrhizobium* genus. Even though all the *Acacia* species used in this study can establish symbiosis with nitrogen-fixing bacteria in Portugal, the diversity of bacteria associated with *A. dealbata* and *A. saligna* was higher in Australia than
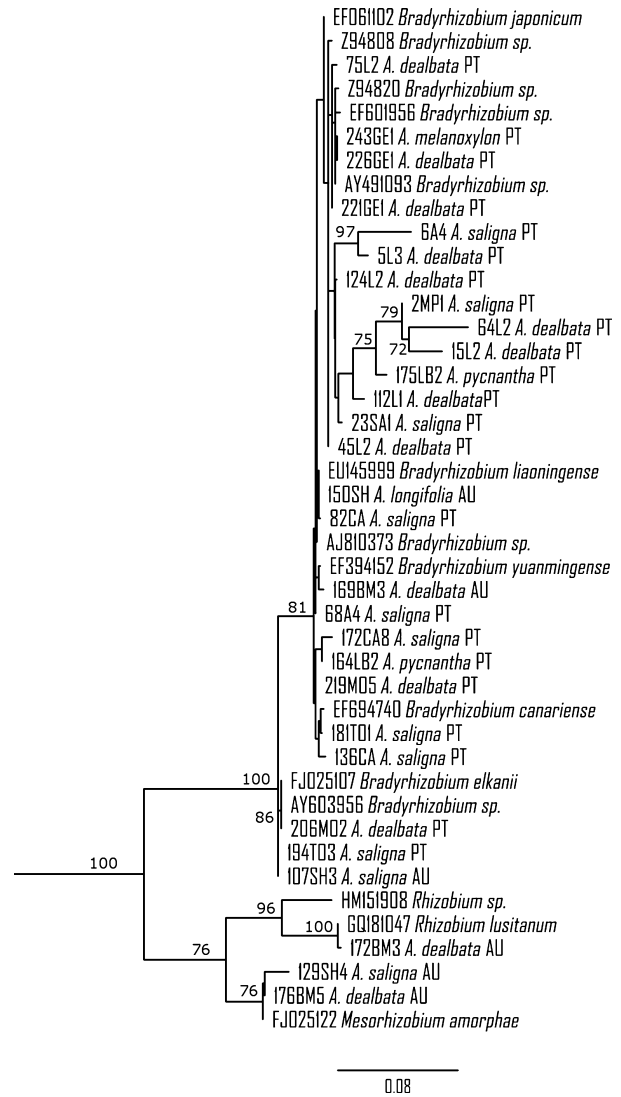
Figure 2 Maximum likelihood tree based on 16S rRNA gene sequences of rhizobial isolates obtained from *Acacia dealbata*, *A. longifolia*, *A. melanoxylon*, *A. pycnantha* and *A. saligna* in this study. Reference-related sequences obtained from Genbank are included with their accession numbers. New isolates from this study are named with the isolation number, host plant species and country of isolation (AU, Australia; PT, Portugal). Numbers at the nodes are bootstrap percentages (1000 resamplings); values below 50% are not shown. The scale bar indicates the number of substitutions per site.

in Portugal (Fig. 2). Future research would include determining the origin of the *Bradyrhizobium* found in Portugal. If these root-nodulating bacteria are proven to be of Australian origin, as it is the case for *A. longifolia* in Portugal (Rodríguez-Echeverría, 2010), the invasion of Australian acacias might be leading to the homogenization of rhizobial communities in different habitats in Portugal. These results also suggest that symbiotic promiscuity is not important for the invasive success of *A. dealbata* and *A. saligna* in Portugal.

Invasive Australian acacias in South Africa

Australian acacias features prominently in the invasive flora of South Africa (Nel et al., 2004; Richardson et al., 2011), yet very little is known about nitrogen fixation and the diversity of rhizobia associated with these species. Joubert (2003) characterized the root-nodulating bacteria associated with invasive bipinnate acacias (A. dealbata, A. decurrens and A. mearnsii) in South Africa and found these to be mainly strains of the slow-growing genus Bradyrhizobium. Here, we analyse the diversity of rhizobia associated with Acacia pycnantha in South Africa and Australia. Specifically, we are interested in whether the invasive populations in South Africa are promiscuous species or have a narrow symbiotic specificity.

Root nodules were collected from ten localities in Australia and seven localities in South Africa throughout A. pycnantha's ranges (see Table S1). Rhizobia isolation and DNA extraction were performed as earlier (Vincent, 1970). Thirty isolates were chosen as different from the original 144 bacterial isolates based on growth rate and morphology of colonies. An 800–1200 bp fragment of the 16S-23S intergenic spacer region (IGS) was sequenced using primers FGPS 1490-72 (5′-TGC GGC TGG ATC CCC TCC TT-3′) and FGPL 132′-38 (5′-CCG GGT TTC CCC ATT CGG-3′) (Romdhane et al., 2005). PCRs were carried out in 50 μL reactions containing 5 pmol of the forward and reverse primers, 0.5 μL of 20 mM dNTPs, 10× buffer, 1.5 mM $MgCl_2$ and 0.5 μL Taq DNApolymerase (5 U/μL). The following thermocycle was used: initial denaturation at 94°C for 5 min followed by 30 cycles of (94°C for 30 s, 58°C for 30 s and 72°C for 60 s) followed by a final extension at 72°C for 7 min. A BLAST search was conducted for the different sequences obtained and those sequences that retrieved the highest similarity values were included in the phylogenetic analysis. Two groups of highly divergent sequences were retrieved in this study, and the two resulting data sets were thus analysed separately. Maximum likelihood analysis was performed for the sequences with similarity to Bradyrhizobium using PAUP* v4.0. The best-fit model of evolution was HKY + I + G, according to the Akaike information criterion (AIC) implemented in Modeltest v3.7 (Posada & Crandall, 1998). Branch support for ML trees was assessed using bootstrapping (100 replicates). The second dataset contained sequences with similarity to Burkholderia isolates. Because of the lack of comparable sequences from identified reference Burkholderia species, the phylogenetic analysis was not performed for this dataset.

The isolates obtained from A. pycnantha were identified as both slow-growing B. japonicum and fast-growing Burkholderia spp. Bradyrhizobium japonicum was highly represented compared with Burkholderia and was found in all populations sampled. There was no clear distinction between the isolates from South Africa and Australia for either Bradyrhizobium or Burkholderia strains (Fig. 3 and Table 3). The obtained Bradyrhizobium isolates were separated into two main clades (Fig. 3). Clade 1 contained closely related strains from the Western Cape in South Africa (Caledon) and South Australia
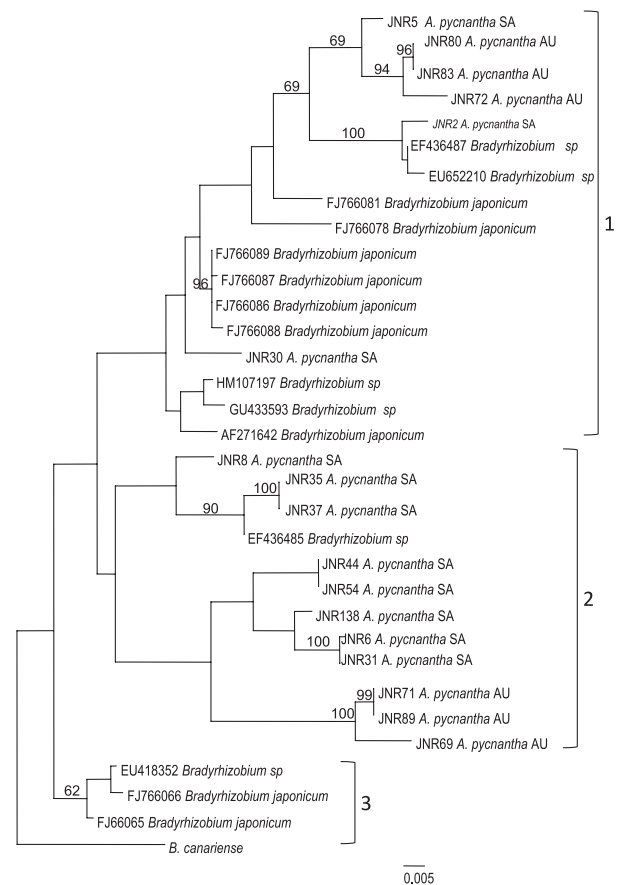
Figure 3 Maximum likelihood tree based on 16S-23S rRNA sequences of Bradyrhizobium strains isolated from Acacia pycnantha in this study. Reference-related sequences obtained from Genbank are included with their accession numbers. New isolates from this study are named with the isolation number, host plant species and country of isolation (AU, Australia; SA, South Africa). Numbers at the nodes are bootstrap percentages (100 resamplings); values below 50% are not shown. The scale bar indicates the number of substitutions per site.

(York Peninsula) together with isolates from other geographical regions. Clade 2 grouped Australian and South African isolates from the remaining sampled regions, with a well-supported subclade containing the Australia isolates. Interestingly, our BLAST searches indicated that A. pycnantha isolates from South Africa were very similar to some Bradyrhizobium isolates from the invasive A. longifolia in Portugal (EF436487, EF436485). The Burkholderia spp. isolated form A. pycnantha had a low similarity with other Burkholderia sequences from GenBank (Table 3), which could indicate that these isolates are new species. However, because of the low number of available IGS sequences from reference root-nodulating Burkholderia species, more extensive analyses, including other genes and nodulation experiments, are needed to confirm this.

Our results indicate that A. pycnantha is a generalist species associating with extremely divergent bacteria belonging to the genera Burkholderia and Bradyrhizobium. Acacia pycnantha

Table 3 Database matches of 16S-23S rRNA intergenic spacer region sequences obtained from fast growing isolates *of A. pycnantha* root nodules from Australia and South Africa.

Isolate	Locality	Best Blast hit	% similarity/bp
JNR27	Humansdorp SA	HQ213349 *Burkholderia* sp.	89/686
JNR28	Humansdorp SA	CP002218 *Burkholderia* sp.	90/700
JNR58	Stellenrust SA	CP001053 *Burkholderia phytofirmans*	98/748
JNR86	Frances AU	CP002218 *Burkholderia* sp.	83/744
JNR104	Natimuk AU	CP002218 *Burkholderia* sp.	90/700
JNR106	Natimuk AU	CP001053 *Burkholderia phytofirmans*	90/774
JNR107	Natimuk AU	CP001053 *Burkholderia phytofirmans*	89/784
JNR126	Wagawaga AU	CP001053 *Burkholderia phytofirmans*	91/652
JNR129	Lockhart AU	CP002218 *Burkholderia* sp.	93/790
JNR133	Lockhart AU	CP002217 *Burkholderia* sp.	92/724

SA, South Africa; AU, Australia.

also potentially conforms to both the co-introduction and the novel mutualism hypothesis, although this needs further confirmation using appropriate biogeographical DNA markers. Like most Australian *Acacia* species, *A. pycnantha* is predominantly nodulated by *B. japonicum* strains. The *Burkholderia* strains obtained from root nodules of *A. pycnantha* (this study) and *A. salicina,* and *A. stenophylla* (Hoque *et al.*, 2011) opens new horizons on the biogeography and specificity of legume root-nodulating bacteria and the specificity of potentially root-nodulating *Burkholderia*.

CONCLUSIONS AND PRIORITIES FOR FUTURE WORK

The source of rhizobial symbionts for invasive legumes is ecologically relevant not only to understand the process of invasion but also to assess the impact on the native plant and soil communities. Intuitively, highly promiscuous legumes should be better colonizers than those with a narrow range of symbiotic specificity, but they still depend on the presence and abundance of compatible rhizobia to spread. Nevertheless, the globally important and highly invasive genus *Acacia* appears to be nodulated predominantly by the genus *Bradyrhizobium*. This bacterial genus is geographically extremely widespread, having been isolated from numerous continents and climatic regions around the globe with some strains having been extensively moved around the globe as agricultural inoculations (Stepkowski *et al.*, 2005; Vinuesa *et al.*, 2008). However, because many legume invasions derive from plants introduced for forestry or ornamental purposes, we cannot exclude the possibility that compatible rhizobial symbionts were co-introduced with their legume–hosts. Several studies have demonstrated that rhizobial inoculation can increase the yield of cultivated legumes (Bagyaraj *et al.*, 1979; Young & Mytton, 1983) and seedling survival and growth in revegetation projects (Herrera *et al.*, 1993; Rodríguez-Echeverría & Pérez-Fernández, 2005).

A similar mechanism has been described for the establishment of introduced woody ectomycorrhizal species into new areas. The co-introduction of specific ectomycorrhizal fungi is essential for the establishment of exotic Pinaceae species in the Southern Hemisphere (Nuñez *et al.*, 2009), of *Eucalyptus* spp. in the Northern Hemisphere (Díez, 2005) and of the Australian *Acacia holosericea* and *A. mangium* in Africa (Duponnois *et al.*, 2007). Large numbers of *Pinus* and *Eucalyptus* species were introduced into new geographical areas, mainly in forestry programs, and inoculation with specific fungi was necessary for the establishment of the plantations. In both cases, it has been proved that the exotic fungi are essential for the naturalization of the exotic plants because they are unable to use fungi from the new soils (Díez, 2005; Nuñez *et al.*, 2009). This dependence on the spread and population build-up of the fungal partner could explain the long lag time from the plantation of these species to their invasive spread far from the planted areas (Díez, 2005). A similar mechanism might operate for invasive legumes and rhizobial inoculants, which might be readily dispersed by wind or soil invertebrates. Although introduced inoculants might have difficulties to establish in the new soils because of competition with the resident microbiota, a process that usually leads to the disappearance of the introduced legumes (Marshall *et al.*, 1963; Lowendorf, 1980), the successful introduction of inoculants could contribute to the invasion by exotic legumes and disrupt native legume–rhizobia associations (Rodríguez-Echeverría, 2010). Whether ectomycorrhizal fungi play any role on the invasiveness of Australian acacias has not yet been explored.

Both theoretical (Parker, 2001) and empirical studies (Prin *et al.*, 2003; Rodríguez-Echeverría *et al.*, 2007; Rodríguez-Echeverría, 2010; this study) suggest that the introduction of inoculants with Australian acacias has been a key factor for the establishment of these species in new areas. Nevertheless, the different degree of invasiveness of Australian acacias might still be related to the symbiotic specificity. Even if inoculation is needed for the establishment in new soils, more promiscuous species are more likely to become invasive using resident rhizobia. The data presented here support that invasive acacia associate preferentially with introduced *Bradyrhizobium*; however, the differences detected in the rhizobial diversity between both invaded regions suggest that they can also associate with resident bacteria independently of the co-introduction of root-nodulating bacteria. It remains unknown whether African *Acacia* (*sensu lato*) can be a source of *Burkholderia,* or *Bradyrhizobium*, for Australian acacias.
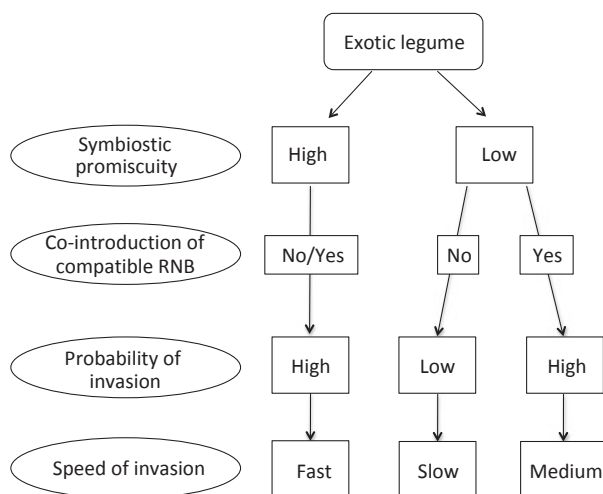
Current knowledge indicates two different strategies for rhizobial acquisition in new areas by Australian acacias, which can be extended to exotic legumes in general (Fig. 4). The first strategy is the establishment of novel mutualisms in the new regions. In this case, symbiotic promiscuity and the diversity of resident rhizobia are important determinants of nodulation for
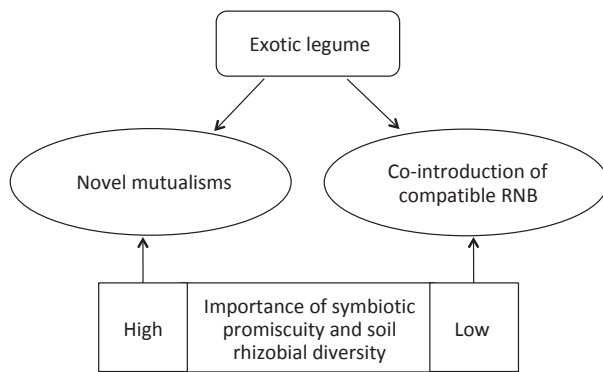
Figure 4 Strategies of exotic legumes for nodulating in new regions. RNB = root-nodulating bacteria.

the exotic legume. The establishment of novel mutualisms in the invaded area might be considered a special case of the Enhanced Mutualism Hypothesis (Reinhart & Callaway, 2006). Alternatively, the establishment of exotic legumes in new areas might be determined by the co-introduction, intentional or unintentional, of compatible root-nodulating bacteria (Fig. 4). These two strategies are not mutually exclusive but their relative importance would depend on specific plant traits and on the availability of a diverse and promiscuous rhizobial community in the areas of introduction. Two factors related with the legume–rhizobia symbiosis are clearly relevant for the probability of the exotic legume becoming invasive and for the speed of invasion (Fig. 5). Promiscuous legumes would have a higher probability of becoming invasive than less promiscuous legumes even in the absence of co-introduction of compatible root-nodulating bacteria. In fact, the co-introduction of compatible root-nodulating bacteria is the only mechanism by which legumes with a narrow symbiotic specificity would become invasive (Fig. 5). The progression of invasion is predicted to be faster for promiscuous species because the invasion of legumes with a higher specificity would be limited by the build-up and dispersion of compatible rhizobia populations (Fig. 5).

Comparative studies in invaded regions around the globe are essential because rhizobial diversity and abundance might change depending on soil conditions, climate and resident vegetation. A more exhaustive analysis of taxa introduced in new regions, but with different invasive status might also help elucidate the role of rhizobial diversity and symbiotic promiscuity in the invasive ability of Australian acacias. An important consideration is the facilitation effect that some *Acacia* species might have on others through the built-up of soil rhizobial populations. Invasive acacias can develop 5–10 times more nodules than native species growing in the same soils (Rodríguez-Echeverría *et al.*, 2009) and could have a great impact on the composition of the soil rhizobial communities. The different invasive ability of the same species in different areas might well depend on the co-introduction of other acacias that boost compatible rhizobial populations.

In summary, invasive Australian acacias develop nodules in non-native areas, although the origin and symbiotic effectiveness of their microbial partners is not clear in most cases. The role of rhizobial diversity, symbiotic promiscuity and the putative introduction of exotic rhizobia need further consideration before fully understanding the invasive process of Australian acacias. These mechanisms could be species specific but might also be modulated by local soil and climatic conditions and the co-occurring vegetation. Many questions remain unanswered and open the opportunity for exciting and much-needed research projects to better understand how rhizobial diversity affects the invasive ability of Australian acacias and legumes in general. Improved insights in this regard will complement advances in the understanding of how other factors mediate invasibility (e.g. Castro-Díez *et al.*, 2011; Gibson *et al.*, 2011; Hui *et al.*, 2011), facilitating more effective management of existing invasions and assisting in the prevention of new invasions.

Figure 5 Importance of symbiotic promiscuity and of the co-introduction of root-nodulating bacteria (RNB) for the invasion of exotic legumes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Genbank accession numbers and nodule collection locations.

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BIOSKETCH

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