

Nodule morphology, symbiotic specificity and association with unusual rhizobia are distinguishing features of the genus *Listia* within the southern African crotalarioid clade *Lotononis s.l.*

Julie K. Ardley^{1,*}, Wayne G. Reeve¹, Graham W. O'Hara¹, Ron J. Yates^{1,2}, Michael J. Dilworth¹ and John G. Howieson¹

¹Centre for Rhizobium Studies, Murdoch University, Murdoch WA 6150, Australia and ²Department of Agriculture and Food Western Australia, Baron Hay Court, South Perth WA 6151, Australia

*For correspondence. E-mail J.Ardley@murdoch.edu.au

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• **Background and Aims** The legume clade *Lotononis sensu lato* (*s.l.*; tribe Crotalariaeae) comprises three genera: *Listia*, *Leobordea* and *Lotononis sensu stricto* (*s.s.*). *Listia* species are symbiotically specific and form lupinoid nodules with rhizobial species of *Methylobacterium* and *Microvirga*. This work investigated whether these symbiotic traits were confined to *Listia* by determining the ability of rhizobial strains isolated from species of *Lotononis s.l.* to nodulate *Listia*, *Leobordea* and *Lotononis s.s.* hosts and by examining the morphology and structure of the resulting nodules.

• **Methods** Rhizobia were characterized by sequencing their 16S rRNA and *nodA* genes. Nodulation and N₂ fixation on eight taxonomically diverse *Lotononis s.l.* species were determined in glasshouse trials. Nodules of all hosts, and the process of infection and nodule initiation in *Listia angolensis* and *Listia bainesii*, were examined by light microscopy.

• **Key Results** Rhizobia associated with *Lotononis s.l.* were phylogenetically diverse. *Leobordea* and *Lotononis s.s.* isolates were most closely related to *Bradyrhizobium* spp., *Ensifer meliloti*, *Mesorhizobium tianshanense* and *Methylobacterium nodulans*. *Listia angolensis* formed effective nodules only with species of *Microvirga*. *Listia bainesii* nodulated only with pigmented *Methylobacterium*. Five lineages of *nodA* were found. *Listia angolensis* and *L. bainesii* formed lupinoid nodules, whereas nodules of *Leobordea* and *Lotononis s.s.* species were indeterminate. All effective nodules contained uniformly infected central tissue. *Listia angolensis* and *L. bainesii* nodule initials occurred on the border of the hypocotyl and along the tap root, and nodule primordia developed in the outer cortical layer. Neither root hair curling nor infection threads were seen.

• **Conclusions** Two specificity groups occur within *Lotononis s.l.*: *Listia* species are symbiotically specific, while species of *Leobordea* and *Lotononis s.s.* are generally promiscuous and interact with rhizobia of diverse chromosomal and symbiotic lineages. The seasonally waterlogged habitat of *Listia* species may favour the development of symbiotic specificity.

Key words: Crotalariaeae, *Lotononis s.l.*, *Listia*, *Leobordea*, nodulation, nodule structure, symbiotic specificity, rhizobia, *Methylobacterium*, *Microvirga*.

INTRODUCTION

The basal legume tribe Crotalariaeae is the largest tribe of papilionoid legumes within Africa and also the largest tribe within the genistoid alliance, comprising some 51 % of this legume group (Boatwright *et al.*, 2008). Although the rhizobial nodulation database in GRIN (<http://www.ars-grin.gov/~sbmljw/cgi-bin/taxnodul.pl>) has reported nearly 550 nodulated species within Crotalariaeae, very few studies have examined their nodule structure or have characterized the root nodule bacteria (rhizobia) associated with this tribe. Indeterminate nodules with uniformly infected central tissue appear to be a characteristic feature of species in tribe Crotalariaeae, and of the genistoid clade within which it is placed (Sprent, 2009). Uniformly infected central tissue is also a feature of the determinate nodules found in the dalbergioid clade (Lavin *et al.*, 2001). In species belonging to these more basal legume clades, rhizobial infection occurs via

epidermal entry rather than by root hair curling, and infection threads are not formed (Sprent and James, 2007).

Previous reports suggest that Crotalariaeae are nodulated by a wide diversity of rhizobia. Several species of *Crotalaria* growing in Senegal are specifically nodulated by strains of *Methylobacterium nodulans* (Sy *et al.*, 2001), whereas some other *Crotalaria* species are nodulated by bradyrhizobia (Moulin *et al.*, 2004; Renier *et al.*, 2008). More recently, novel rhizobial taxa of *Burkholderia* have been isolated from root nodules of the crotalarioid legume *Lebeckia ambigua* collected from the South African Cape fynbos region (Howieson *et al.*, 2013). The acid, infertile soils of the fynbos appear to support a diverse population of Papilionoideae-nodulating *Burkholderia* rhizobia (Elliott *et al.*, 2007; Garau *et al.*, 2009; Gyaneshwar *et al.*, 2011). *Lebeckia* has recently been divided into three genera: *Lebeckia sensu stricto* (*s.s.*), *Calobota* and *Wiborgiella* (Boatwright *et al.*, 2009). While *Burkholderia*

rhizobia nodulate and fix nitrogen with the acicular-leaved fynbos species *L. ambigua* and *L. sepiaria* (Howieson *et al.*, 2013), strains of *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* (syn. *Ensifer*) are reported to nodulate other species within *Lebeckia sensu lato* (s.l.; Phalane *et al.*, 2008).

The other major Crotalariaeae group which has been studied for nitrogen-fixing symbiotic associations is *Lotononis* s.l. This clade has a centre of origin in South Africa and consists of approx. 150 species, originally divided into 15 taxonomic sections (van Wyk, 1991; Boatwright *et al.*, 2011). In a recent taxonomic revision, the three distinct clades within *Lotononis* s.l. are now recognized at the generic level as *Listia*, *Leobordea* and *Lotononis* s.s. (Boatwright *et al.*, 2011).

The genus *Listia* consists of seven species of herbaceous perennials: *L. angolensis*, *L. bainesii*, *L. heterophylla* (previously *Lotononis listii*), *L. marlothii*, *L. minima*, *L. solitudinis* and *L. subulata* (Boatwright *et al.*, 2011). *Listia angolensis* has a tropical distribution (in the uplands encircling the Zaire basin), while *L. heterophylla* extends from South Africa into southern Central Africa, *L. bainesii* is native to Botswana, Mozambique (south), Namibia and South Africa, and the remaining species are endemic to South Africa (van Wyk, 1991). *Listia* species are distinguished by a stoloniferous habit, which is thought to be associated with their seasonally wet habitats (van Wyk, 1991; Boatwright *et al.*, 2011) (although they are also drought tolerant), and the formation of lupinoid root nodules (Yates *et al.*, 2007). In contrast, field observations have indicated that *Leobordea* and *Lotononis* s.s. species have indeterminate nodules (J. Howieson and R. Yates, unpubl. data). Species of these two genera are annuals or perennials and are mostly endemic to South Africa, although a few species have a wider distribution in Africa, or extend into the Mediterranean and to Asia (van Wyk, 1991).

Renewed interest in the potential of *Listia* (and other *Lotononis* s.l. species) as perennial pasture plants in southern Australian agricultural systems has prompted recent research into their associated rhizobia (Yates *et al.*, 2007; Howieson *et al.*, 2008). Most previous studies have been conducted on *L. bainesii*, which is recognized as a model of strict symbiotic specificity (Pueppke and Broughton, 1999), able to nodulate only with red-pigmented, narrow host range rhizobia (Norris, 1958), although Norris did not test *L. bainesii* with any rhizobial strains derived from other *Lotononis* s.l. hosts. The pigmented rhizobia isolated from *L. bainesii* and other *Listia* hosts have since been identified as strains of *Methylobacterium* sp. (Jaftha *et al.*, 2002; Yates *et al.*, 2007) that are unable to utilize methanol (Ardley *et al.*, 2009).

These pigmented methylobacteria have now been isolated from *L. bainesii*, *L. heterophylla*, *L. marlothii*, *L. solitudinis* and *L. subulata*, and are effective for nitrogen fixation on all studied *Listia* species except *L. angolensis*, which forms ineffective (i.e. non-fixing) nodules with these rhizobia (Yates *et al.*, 2007; R. Yates, unpubl. data). Instead, *L. angolensis* is effectively nodulated by species of *Microvirga* (Ardley *et al.*, 2012). There has as yet been no detailed characterization of the rhizobia associated with *Leobordea* and *Lotononis* s.s., although studies conducted on various *Lotononis* s.l. hosts by the CSIRO in Queensland, Australia indicate that their rhizobial isolates were phenotypically diverse (Eagles and Date, 1999).

Rhizobial *Methylobacterium* and *Microvirga* species are uncommon microsymbionts, as the majority of legumes nodulate with strains of *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Lindström *et al.*, 2010). Do *Listia* species form symbioses only with these unusual rhizobia or can they be nodulated by the rhizobial species that are associated with the other genera that comprise *Lotononis* s.l.? Has the evolution and diversification of *Lotononis* s.l. species within their centre of origin resulted in their recruitment of different microsymbiont lineages and, if so, what factors might influence the patterns of symbiotic association found in this legume group?

As part of the development of novel perennial pasture legumes and associated rhizobia that are adapted to the arid climate and acid, infertile soils found in Western Australian agricultural systems, the Centre for *Rhizobium* Studies (CRS) undertook collections of nodules and seeds from a range of *Lotononis* s.l. species growing in diverse sites in South Africa. This collection of legume germplasm and rhizobia, together with *Microvirga* strains isolated from *L. angolensis* hosts, was used to examine symbiotic relationships and specificity within *Lotononis* s.l. In addition, we present details of nodule morphology and structure in *Listia*, *Leobordea* and *Lotononis* s.s. species, and the processes of infection and nodule initiation in *L. angolensis* and *L. bainesii*.

MATERIALS AND METHODS

Rhizobial strains and plant material

The *Lotononis* s.l. rhizobia used in this study are listed in Table 1, together with details of their host plant and the collection site. The host species are described according to their position within the *Lotononis* s.l. taxonomic groupings given by van Wyk (1991) and Boatwright *et al.* (2011), where this was known. The bioregion of the South African collection sites was described according to Mucina and Rutherford (2006). As the important research question for us was to determine symbiotic relationships and specificities within *Lotononis* s.l., rather than to perform a comprehensive survey of the rhizobia that nodulate this group of legumes, all strains used were authenticated sub-sets of a wider collection of nodule isolates obtained from South African or Zambian *Lotononis* s.l. species. Strain WSM2598 was selected from 67 pink-pigmented methylobacterial isolates obtained from nodules of South African *Listia bainesii*, *L. heterophylla* and *L. solitudinis*, which were all authenticated as effective for N₂ fixation on *L. bainesii* (R. Yates, unpubl. data). Strain WSM3557^T is the type strain of the novel rhizobial species *Microvirga lotononidis* (Ardley *et al.*, 2012). It was selected for this study from seven *L. angolensis* strains, derived from strains housed in the CB Strain Collection (Eagles and Date, 1999), due to its high symbiotic effectiveness on *L. angolensis* (Ardley, 2012). The remaining seven strains were isolated from diverse South African *Leobordea* and *Lotononis* s.s. species. All had been authenticated as root nodule bacteria, but did not form nodules on *L. bainesii* or *L. heterophylla* (Ardley, 2012; R. Yates, unpubl. data).

Additional rhizobial strains inoculated onto *L. angolensis*, *L. bainesii* and *L. heterophylla* are listed in Supplementary Data Table S1.

TABLE 1. *Rhizobial strains used in this study*

Strain (synonym)	Identification from 16S rRNA gene sequence	Host* (<i>Lotononis s.l.</i> section)	Geographical location, site and bioregion [†]	Site pH [‡]	Latitude	Longitude	Reference (collector)
WSM2596	<i>Bradyrhizobium</i> sp.	<i>Leobordea pulchra</i> (Lipozygis)	South Africa SA34 Drakensberg Grassland	5.5	S29 04 414	E29 36 491	This study (Yates, Real and Law)
WSM2598	<i>Methylobacterium</i> sp.	<i>Listia bainesii</i> (Listia)	South Africa SA35 Drakensberg Grassland	6.0	S29 01 540	E29 52 165	Yates et al. (2007) (Yates, Real and Law)
WSM2624	<i>Mesorhizobium tianshanense</i>	<i>Lotononis s.l.</i> sp. (ND)	South Africa SA20 Upper Karoo	7.0	S32 14 082	E22 55 081	This study (Yates, Real and Law)
WSM2632	<i>Bradyrhizobium</i> sp.	<i>Lotononis s.l.</i> sp. (ND)	South Africa SA41 Sub-escarpment Savannah	5.5	S28 38 902	E31 55 793	This study (Yates, Real and Law)
WSM2653	<i>Ensifer meliloti</i>	<i>Lotononis sparsiflora</i> (Oxydium)	South Africa SA22 Upper Karoo	8.0	S32 21 109	E22 36 127	This study (Yates, Real and Law)
WSM2667	<i>Methylobacterium nodulans</i>	<i>Leobordea calycina</i> (Leptis)	South Africa SA53 Central Bushveld Savannah	6.0	S25 35 432	E28 22 665	This study (Yates, Real and Law)
WSM2783	<i>Bradyrhizobium</i> sp.	<i>Leobordea carinata</i> (Leptis)	South Africa SA49 Mesic Highveld Grassland	6.5	S27 12 979	E31 10 635	This study (Yates, Real and Law)
WSM3040	<i>Ensifer meliloti</i>	<i>Lotononis laxa</i> (Oxydium)	South Africa SA14 Upper Karoo	6.5	S32 11 075	E23 55 631	This study (Yates, Real and Law)
WSM3557 ^T (CB1322)	<i>Microvirga lotononidis</i>	<i>Listia angolensis</i> (Listia)	Chipata [§] , Zambia ND [¶]	ND	S 13-65	E 32-63	Eagles and Date (1999), Ardley et al. (2012) (Verboom)

*According to the taxonomy of van Wyk (1991) and Boatwright et al. (2011).

[†]Bioregion according to Mucina and Rutherford (2006).

[‡]pH measured with a kit.

[§]Formerly known as Fort Jameson.

[¶]Likely to have been woodland savannah, consisting of a grass cover 1–2 m high and hardwood trees and/or shrubs, interspersed with grassy wetlands, according to vegetation maps of the area.

ND, not determined.

Root nodules were collected from field sites in South Africa and desiccated *in situ* for transport back to the CRS laboratories in Australia (Yates et al., 2004). The desiccated nodules were then re-imbibed in distilled water, surface sterilized and rhizobia were subsequently isolated (Yates et al., 2004). Strains were routinely sub-cultured on modified $\frac{1}{2}$ lupin agar ($\frac{1}{2}$ LA) (Yates et al., 2007), or on $\frac{1}{2}$ LA with succinate replacing glucose and mannitol as a carbon source, or on TY agar (Beringer, 1974). All strains were stored at -80°C in media supplemented with 12% (v/v) glycerol.

The *Lotononis s.l.* species used in this study are listed in Table 2, along with details of their taxonomy and distribution. Seeds of *L. angolensis*, *L. bainesii* and *L. heterophylla* were obtained from the Department of Agriculture and Food Western Australia and the CRS. Cultivar names or line numbers for these species are also given in Table 2. Seed for all other *Lotononis s.l.* species was collected from wild plants growing at various sites in South Africa.

General glasshouse procedures

Rhizobial strains were assessed for their capacity to nodulate and fix N_2 with legume hosts using an axenic sand culture system (Howieson et al., 1995; Yates et al., 2004). Briefly, free-draining pots (1 kg) were lined with absorbent paper, filled with a 3:2 mix of yellow sand and washed river sand, moistened with deionized (DI) water and then sterilized by steam treatment or autoclaving. Each pot was flushed twice with hot, sterile DI water to remove inorganic nitrogen. Sterile polyvinyl chloride

tubes (25 mm diameter) with lids were inserted into the sand mix for supply of water and nutrients. To maintain sterility, pots were covered with plastic film until inoculation and, post-inoculation, the soil surface was covered with sterile alkathene beads. Pots were watered as required with sterile DI water. Sterile nitrogen-free nutrient solution (20 mL) (Howieson et al., 1995) was supplied weekly to each pot. N^+ controls received 5 mL of 0.1 M KNO_3 per pot weekly. To evaluate *Listia* symbiotic specificity further, a closed vial system was used, with screw-topped polycarbonate vials (500 mL) that contained sterile mixed sand medium (400 g) and DI water (50 mL) (Yates et al., 2004). Each vial had a single dose of nutrient solution (20 mL) added at the time of planting. Seeds were surface sterilized and germinated as described by Yates et al. (2007), and aseptically sown into pots or vials when the radicles were 1–3 mm in length. Plants were inoculated with rhizobial cell suspensions (1 mL per plant, containing approx. 3.0×10^7 – 1.0×10^9 live cells mL^{-1}) (Yates et al., 2007) and were grown in a naturally lit, controlled-temperature (maximum 24°C) glasshouse.

Glasshouse experiment 1: nodulation studies

Before being included in a large-scale experiment to determine nodulation and N_2 fixation with *Lotononis s.l.* hosts, the seven *Leobordea* and *Lotononis s.s.* isolates were assessed for their ability to nodulate a range of *Leobordea* and *Lotononis s.s.* species. Pots containing up to two seedlings of a given species were inoculated with a single rhizobial strain, and an uninoculated nitrogen-free control was included in the

TABLE 2. List of host plants used in this study and their distribution in southern Africa

Genus (<i>Lotononis</i> s.l. section)	Species (accession no./cultivar)	Distribution [†]	Glasshouse experiment			
			1	2	3	4
<i>Listia</i> (<i>Listia</i>)	<i>Listia angolensis</i> (8363)	Tropical central Africa		*	*	*
	<i>Listia bainesii</i> (Miles)	Eastern and central southern Africa		*	*	*
	<i>Listia heterophylla</i> (2004 CRSL69)	Eastern and central southern Africa			*	
<i>Leobordea</i> (<i>Digitata</i>)	<i>Leobordea longiflora</i>	Dry mountainous regions of the north-west Cape		*		
<i>Leobordea</i> (<i>Leptis</i>)	<i>Leobordea mollis</i>	Western Cape of South Africa	*			
<i>Leobordea</i> (<i>Leobordea</i>)	<i>Leobordea platycarpa</i>	Drier areas of southern Africa		*		
	<i>Leobordea stipulosa</i>	North-eastern interior of southern Africa		*		
<i>Leobordea</i> (<i>Synclistus</i>)	<i>Leobordea bolusii</i>	Western and central South Africa		*		
	<i>Leobordea polycephala</i>	Western and central South Africa	*			
	<i>Lotononis</i> s.s. (<i>Oxydium</i>)	<i>Lotononis crumanina</i>	Limestone or lime-rich soils in central southern Africa		*	
<i>Lotononis</i> s.s. (<i>Oxydium</i>)	<i>Lotononis delicata</i>	ND	*			
	<i>Lotononis falcata</i>	Widespread in southern Africa		*		
	<i>Lotononis laxa</i>	Widespread along the east coast of southern Africa	*			
	<i>Lotononis</i> s.s. (<i>Cleistogama</i>)	<i>Lotononis pungens</i>	Central South Africa	*		

Accession numbers or cultivars, where relevant, are given in parentheses.

[†]Species distribution is according to van Wyk (1991).

ND, not determined.

treatments. Because of the scarcity of host germplasm, only 1–4 plants of each species were included in the trial. Plants were assessed for nodulation, which was deemed to be effective if the plants were greener than the uninoculated control and the nodules were pink in colour.

Glasshouse experiment 2: nodulation and N₂ fixation tests

Nodulation and N₂-fixing capabilities of the nine *Lotononis* s.l. rhizobia on eight *Listia*, *Leobordea* or *Lotononis* s.s. species were determined using three replicate pots per treatment, with uninoculated nitrogen-free and supplied nitrogen (N+) treatments as controls. Each pot was sown with up to six germinated seeds and then thinned to four seedlings 3 weeks after planting (except for *L. stipulosa*, which produced insufficient germinated seeds). Because seed of *L. bolusii* was scarce, tip cuttings were taken from pot-grown plants maintained in the glasshouse. The cuttings (approx. 6 cm long) were stripped of their lower leaves, placed in DI water, then transferred to sodium hypochlorite (1% w/v; 1 min) and rinsed twice in sterile DI water. The cut end was then dipped in striking powder [active ingredient 4-indol-3-yl butyric acid (0.3% w/w)] and aseptically planted, with four cuttings per pot. Pots with cuttings were supplied as required with sterile DI water for the first 3 weeks, and then given a single dose (20 mL) of nutrient solution containing 0.25 g l⁻¹ KNO₃, to aid establishment. The rooted cuttings were subsequently supplied with sterile DI water and weekly doses of sterile nitrogen-free nutrient solution (20 mL). Pots containing germinated seeds were inoculated after the seedlings had emerged (7–10 d after sowing, depending on the growth rates of the different *Lotononis* s.l. species). Cuttings were inoculated 6 weeks after planting.

Glasshouse experiment 3: further evaluation of symbiotic specificity within *Listia*

Seedlings of *L. angolensis*, *L. bainesii* and *L. heterophylla* were grown in closed vials and inoculated separately with the

rhizobia shown in Supplementary Data Table S1. Treatments were duplicated and an uninoculated control was used.

Glasshouse experiment 4: infection and nodule initiation in *Listia angolensis* and *L. bainesii*

Listia angolensis and *L. bainesii* were inoculated with their respective microsymbiont strains WSM3557^T and WSM2598. Plants were grown in the glasshouse, either in pots (using the axenic sand culture system described above) or in gnotobiotic growth pouches (CYG Seed Germination Pouch, Mega International, West St. Paul, MN, USA) (Journet et al., 2001). Three-day-old seedlings (50–60) were incubated with 5 mL of late log phase ½ LA broth cultures (OD_{600nm} = 0.5) for 1 h in sterile Petri dishes and then aseptically planted into growth pouches or pots.

Harvesting and light microscopy

Pot-grown plants were harvested 10 weeks post-inoculation and those in vials 6 weeks post-inoculation. The effectiveness of N₂ fixation was determined by measuring the increase in above-ground plant biomass, which was excised, dried at 60 °C and then weighed. Nodules were assessed for colour, number and morphology. Selected nodules were prepared for nodule sectioning and light microscopy (Yates et al., 2007).

For infection and nodule initiation studies, both pot- and pouch-grown plants were harvested at regular intervals. Selected plants were cleared and stained with Brilliant Green to highlight root and nodule initials (O'Hara et al., 1988). Sections of fresh plants that included the hypocotyl and the upper portion of the main root were excised and prepared for sectioning and light microscopy (Yates et al., 2007).

Amplification and sequencing of 16S rRNA and nodA genes

The primers used for DNA amplification and sequencing are described in Table 3. PCR amplification of the nearly full-length

TABLE 3. Primers used in this study

Primer	Sequence	Reference
16S rRNA		
FGPS6	5'-GGAGAGTTAGATCTTGGCTCAG-3'	Normand <i>et al.</i> (1992)
FGPS1509	5'-AAGGAGGGGATCCAGCCGCA-3'	Normand <i>et al.</i> (1992)
420F	5'-GATGAAGGCCTTAGGGTTGT-3'	Yanagi and Yamasato (1993)
800F	5'-GTAGTCCACGCCGTAAACGA-3'	Yanagi and Yamasato (1993)
1100F	5'-AAGTCCCAGCACGAGCGCAA-3'	Yanagi and Yamasato (1993)
520R	5'-GCGGCTGCTGGCACGAAGTT-3'	Yanagi and Yamasato (1993)
920R	5'-CCCCGTCAATTCCTTTGAGT-3'	Yanagi and Yamasato (1993)
1190R	5'-GACGTCATCCCACCTTCCT-3'	Yanagi and Yamasato (1993)
16S-1924r	5'-GGCACGAAGTTAGCCGGGGC-3'	Sy <i>et al.</i> (2001)
16S-1080r	5'-GGGACTTAACCCAACATCT-3'	Sy <i>et al.</i> (2001)
<i>nodA</i>		
M4-46 nodD 89-109f*	5'-ATTGCGGGCTGGCTTAGGTTG-3'	This study
M4-46 nodB 29-48r2*	5'-CGCGCAATGACACAGAACG-3'	This study
nodA-1	5'-TGCRGTGGAARNTRNNCTGGGAAA-3'	Haukka <i>et al.</i> (1998)
nodA-2	5'-GGNCCGTCRTCRAAWGTCARGTA-3'	Haukka <i>et al.</i> (1998)

R, A, G; W, A, T; N, all.

*The position of the primer in the corresponding sequence of the *Methylobacterium sp.* 4-46 target gene.

16S rRNA gene was performed using the universal eubacterial primers FGPS6 and FGPS1509 (Normand *et al.*, 1992). DNA template was obtained from whole cells, using fresh plate culture resuspended in 0.89 % (w/v) NaCl to an OD_{600nm} of 2.0. The reaction mixture contained 1 µL of bacterial cells, 5 µL of 5 × PCR Polymerisation Buffer (Fisher Biotech), 1.5 mM MgCl₂, 1.0 µM of each of the two primers FGPS6 and FGPS1509 and 1 U of *Taq* DNA polymerase (Invitrogen) in a final volume of 25 µL. Thermal cycling conditions were 5 min at 94 °C, then 35 cycles of 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 1 min at 70 °C, with a final 7 min extension at 72 °C. Amplicons were purified and sequenced as described by Yates *et al.* (2007), using FGPS6 and FGPS1509 and the internal primers described by Yanagi and Yamasato (1993).

PCR amplification of the *nodA* gene of *Lotononis s.l.* rhizobia was performed using the primers M4-46 nodD 89-109f and M4-46 nodB 29-48r2 (this study) and the *nodA* primers developed by Haukka *et al.* (1998). M4-46 nodD 89-109f and M4-46 nodB 29-48r2 were designed based on conserved regions of the *nodD* and *nodB* genes in the sequenced genome of the *L. bainesii* micro-symbiont *Methylobacterium sp.* 4-46. The *nodA* PCR reaction components were the same as those used for PCR amplification of the 16S rRNA gene, but used *nodA* primers and 1.0 mM MgCl₂. Thermal cycling conditions using the M4-46 *nodA* primers were 4 min at 94 °C, then 35 cycles of 30 s at 94 °C, annealing for 30 s at 52 °C and extension for 30 s at 70 °C, with a final 5 min extension at 72 °C. The optimized cycling conditions for the *nodA*-1 and *nodA*-2 primers were modified slightly from the original conditions cited by Haukka *et al.* (1998) and consisted of 4 min at 94 °C, then 35 cycles of 45 s at 94 °C, annealing for 45 s at 55 °C and extension for 2 min at 68 °C, with a final 5 min extension at 70 °C. Amplicons were purified and sequenced as for the 16S rRNA gene.

Phylogenetic analyses

Sequences were manually edited using Genetool Lite (version 1.0; Double Twist Inc., Oakland, CA, USA). Searches for

sequences with high sequence similarity were conducted using BLASTN (Altschul *et al.*, 1990) against sequences deposited in the National Centre for Biotechnology Information GenBank database. Phylogenetic trees and molecular analyses were determined using MEGA version 5.0 (Tamura *et al.*, 2011). 16S rRNA sequences were trimmed to the same length and aligned. The *nodA* sequence alignments were based on full-length, or nearly full-length gene sequences.

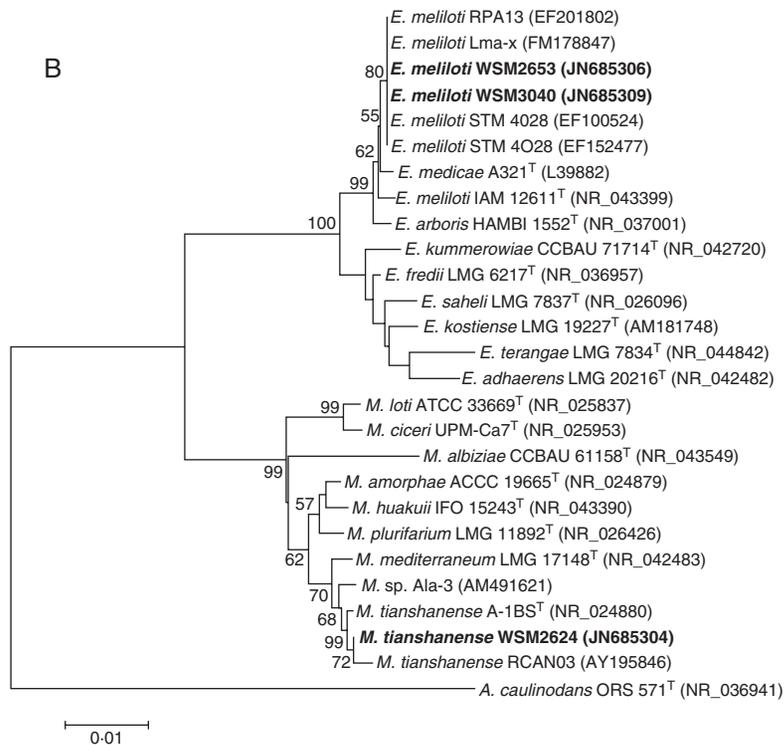
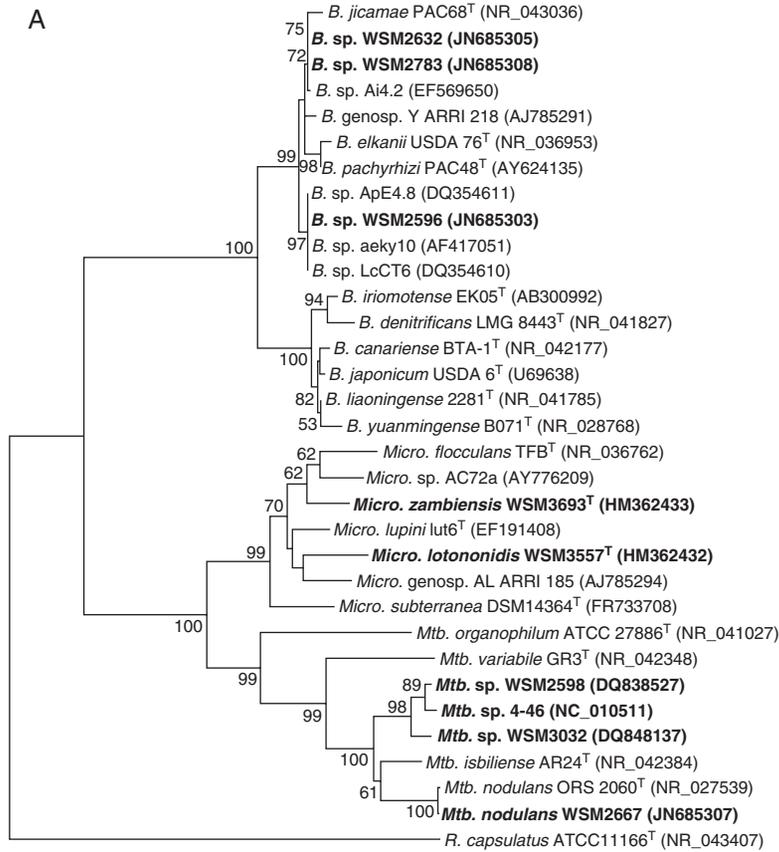
Phylogenetic trees were generated by Neighbor-Joining (NJ; Saitou and Nei, 1987) maximum likelihood (ML) and parsimony methods, and bootstrapped with 1000 replicates.

RESULTS

Identification of rhizobia associated with *Lotononis s.l.*

The 16S rRNA gene sequences of WSM2598 and WSM3557^T have been described previously (Yates *et al.*, 2007; Ardley *et al.*, 2012). Nearly full-length portions of the 16S rRNA gene were amplified and sequenced for the remaining seven *Lotononis s.l.* strains. The resulting NJ phylogenetic trees were based on a 1388 bp alignment of the nine *Lotononis s.l.* rhizobia, closely related strains and reference strains (Fig. 1A, B). Phylogenetic trees reconstructed using maximum-parsimony (MP) or minimal-evolution (ME) methods gave similar topologies.

The rhizobia associated with *Lotononis s.l.* are remarkably diverse. WSM2598 is a pigmented *Methylobacterium sp.*, most closely related to other pigmented *Listia* methylobacteria and to *Methylobacterium nodulans* (Yates *et al.*, 2007). Analysis of the nearly full-length (1416 bp) 16S rRNA gene sequence of WSM2667 also identified this strain as a species of *Methylobacterium*, with 99.9 % sequence identity with the type strain of *M. nodulans* ORS 2060^T, isolated from *Crotalaria podocarpa* in Senegal (Sy *et al.*, 2001). WSM3557^T is the type strain of *Microvirga lotononidis* (Methylobacteriaceae) (Ardley *et al.*, 2012). In addition to grouping with the rhizobial species *Microvirga lupini* (strain Lut6^T) and *Microvirga zambiensis* (strain WSM3693^T) and described non-symbiotic species of *Microvirga*, WSM3557^T



was also closely related to two authenticated rhizobial strains that grouped within the *Microvirga* clade (Fig. 1A). These were AC72a, isolated from nodules of *Phaseolus vulgaris* growing in Ethiopia (Wolde-Meskel *et al.*, 2005), and ARRI 185 (genospecies AL), from northern Australian *Indigofera linifolia* (Lafay and Burdon, 2007).

Strains WSM2596, WSM2632 and WSM2783 were identified as bradyrhizobia that grouped, with high bootstrap support, within a cluster containing *Bradyrhizobium elkanii*, *B. jicamiae* and *B. pachyrhizae* (Fig. 1A), rather than with the cluster of *B. japonicum*, *B. canariense*, *B. liaoningense* and *B. yuanmingense* strains [Group II and Group I, respectively, according to the phylogeny of Menna and Hungria (2011)]. Within Group II, strain WSM2596 was placed in a lineage that included strains ApE4-8, LcCT6 and aeky10 and was separate from the *B. elkanii*, *B. jicamiae* and *B. pachyrhizae* type strains. Strains WSM2632 and WSM2783 had 100 % sequence identity and grouped with strains Ai4-2, ARRI 218 and PAC68^T.

Strains WSM2653 and WSM3040 had identical 16S rRNA gene sequences and were closely related to *Ensifer* (syn. *Sinorhizobium*) *meliloti* strains, with 99.7 % sequence identity to the type strain (Fig. 1B). The sequences contained the specific primer sequence that differentiates *E. meliloti* from *E. medicae* (Garau *et al.*, 2005). WSM2624 was most closely related to strains of *Mesorhizobium tianshanense* (Fig. 1B). A 1452 bp fragment of the 16S rRNA gene had sequence identity of 99.8 % with the *M. tianshanense* type strain A-1BS^T (Chen *et al.*, 1995; Jarvis *et al.*, 1997).

Amplification and sequencing of *nodA*

PCR amplification and sequencing using the primers M4-46 *nodD* 89-109f and M4-46 *nodB* 29-48r2 yielded sequences of 937 and 965 bp for WSM2598 and WSM2667, respectively. These contained a portion of *nodD*, the NodD *nod* box-binding region, the complete *nodA* gene and a portion of *nodB*. Both strains had 645 bp *nodA* genes, which is the same length as the published *nodA* sequences of the rhizobial methylobacteria strains *Methylobacterium* sp. 4-46, which nodulates *Listia bainesii*, and *M. nodulans* ORS 2060^T. The sequence identity of the *nodA* gene of WSM2598 was <80 % of the WSM2667 *nodA* gene. The WSM2598 *nodA* gene had the highest sequence identity to strain 4-46 (99.4 %), while the WSM2667 *nodA* gene was most closely related to that of *M. nodulans* ORS 2060^T (99.5 %). The proteins deduced from the *Methylobacterium nodA* gene sequences contained 214 amino acids.

Amplification and sequencing of *M. lotononidis* WSM3557^T *nodA* has been described previously and gave 100 % sequence identity with a 562 bp portion of *nodA* of *M. zambiensis* WSM3693^T, which is also a microsymbiont of *L. angolensis* (Ardley *et al.*, 2012). A partial sequence of the *nodA* and *nodB* genes was obtained for WSM2632, WSM2653, WSM2783 and

WSM3040 using the primers *nodA*-1 and *nodA*-2. No amplification products could be obtained for WSM2596 or WSM2624. The 638 bp sequences of the bradyrhizobial strains WSM2632 and WSM2783 were identical. The 631 bp sequences obtained from the *E. meliloti* strains WSM2653 and WSM3040 also had 100 % sequence identity. Alignment of a 565 bp *nodA* fragment showed that the *Bradyrhizobium*, *Ensifer*, *M. nodulans*, pigmented *Methylobacterium* and *Microvirga* strains isolated from *Lotononis s.l.* hosts all possessed distinct *nodA* sequences, with ≤84.9 % sequence identity between each of these groups. *Microvirga lotononidis* WSM3557^T and the *Bradyrhizobium* sp. strains WSM2632 and WSM2783 were characterized by the deletion of a nucleotide triplet at positions 460–462 of the bradyrhizobia *nodA* sequence. This deletion was also a feature of the *nodA* sequences of the *Azorhizobium*, *Ensifer*, *Mesorhizobium*, *Microvirga lupini* and *Rhizobium* reference strains used in the alignment.

The high-quality draft genome sequences of WSM3557^T and WSM2783 have recently become available on the Joint Genome Institute (JGI) website (<http://img.jgi.doe.gov/cgi-bin/gebra/main.cgi>), allowing complete *nodA* sequences to be obtained for these strains. Both had *nodA* genes of 630 bp, with deduced proteins of 210 amino acids. This length is characteristic of the *nodA* genes found in the majority of *Bradyrhizobium* strains and of *Burkholderia* sp. strain STM678, rather than the shorter length *nodA* genes of *Azorhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*, and is due to the presence of codons for additional amino acids at the N-terminal part of the deduced NodA protein (Moulin *et al.*, 2004).

Phylogenetic analyses of the complete or nearly complete *nodA* sequences of the *Lotononis s.l.* strains were performed by distance, parsimony and ML methods. Rhizobial strains with high BLASTN *nodA* sequence similarity and several reference strains were included in the analysis. The resulting trees all had similar topology. The ML phylogenetic tree is shown in Fig. 2. The *Lotononis s.l.* strains formed five clearly defined clusters that were well supported by high bootstrap values. However, the low values obtained for higher nodes indicated that the position of the clusters within the tree was not well supported and the *nodA* phylogenetic relationships therefore could not be determined with confidence.

Analysis of *nodA* of the *E. meliloti* strains WSM2653 and WSM3040 revealed that they formed a sister group to a cluster of African *Ensifer* strains that nodulate *Acacia* [syn. *Senegalia* (Seigler *et al.*, 2006)] and *Prosopis* (Nick *et al.*, 1999; Ba *et al.*, 2002), with sequence identities ranging from 76 to 78 %. The *Lotononis s.l.* bradyrhizobia WSM2632 and WSM2783 were in a lineage that was well separated from *Bradyrhizobium* strains isolated from Australian native legumes (WSM1735 and WSM1790) and from the clade of remaining bradyrhizobia, which included a number of strains isolated from native African legumes. The *Microvirga* strains WSM3557^T and WSM3693^T

FIG. 1. NJ phylogenetic tree, bootstrapped with 1000 replicates, showing the relationships of (A) *Bradyrhizobium*, *Methylobacterium* and *Microvirga* and (B) *Ensifer* and *Mesorhizobium* strains isolated from *Lotononis s.l.* species (in bold type), with related strains and type strains of *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, *Methylobacterium* and *Microvirga*, based on a 1388 bp alignment of the 16S rRNA genes. GenBank accession numbers are in parentheses. Bootstrap values are indicated on branches only when higher than 50 %. Scale bars: (A) = 2 % sequence divergence (two substitutions per 100 nucleotides); (B) = 1 % sequence divergence (one substitution per 100 nucleotides). A., *Azorhizobium*; B., *Bradyrhizobium*; E., *Ensifer*; M., *Mesorhizobium*; Mtb., *Methylobacterium*; Micro., *Microvirga*; R., *Rhodobacter*; ^T = type strain.

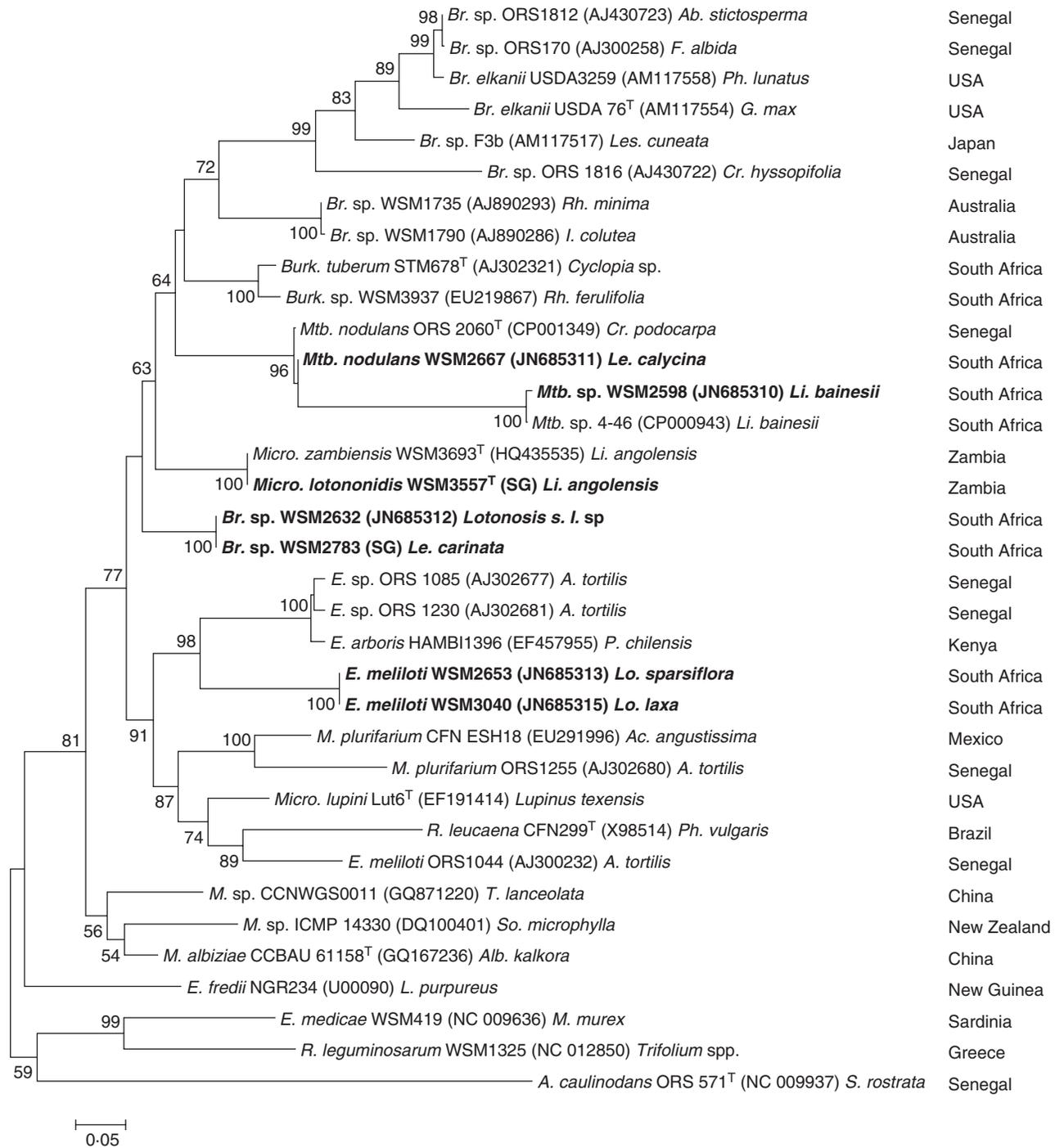


FIG. 2. ML *nodA* phylogenetic tree, bootstrapped with 1000 replicates, showing the relationships of the *Lotononis* s.l.-associated rhizobia (in bold type) and other rhizobia. GenBank accession numbers are in parentheses. Complete *nodA* gene sequences were used for all strains except WSM2632, WSM2653 and WSM3040. For WSM2783 and WSM3557, complete *nodA* gene sequences obtained from high-quality draft genome sequences (available on the JGI website, <http://img.jgi.doe.gov/cgi-bin/w/main.cgi>) were used instead of the *nodA* sequences deposited in GenBank (accession nos JN685314 and HQ435534, respectively). The original host and the geographical location of the strain are also shown. Scale bar = 5% sequence divergence (five substitutions per 100 nucleotides). A., *Azorhizobium*; Br., *Bradyrhizobium*; Burk., *Burkholderia*; E., *Ensifer*; M., *Mesorhizobium*; Mtb., *Methylobacterium*; Micro., *Microvirga*; R., *Rhizobium*. A., *Acacia*; Ab., *Abrus*; Ac., *Acaciella*; Alb., *Albizia*; Cr., *Crotalaria*; F., *Faidherbia*; G., *Glycine*; I., *Indigofera*; L., *Lablab*; Le., *Leobordea*; Les., *Lespedeza*; Li., *Listia*; Lo., *Lotononis*; Lu., *Lupinus*; M., *Medicago*; P., *Prosopis*; Ph., *Phaseolus*; Rh., *Rhynchosia*; S., *Sesbania*; So., *Sophora*; T., *Thermopsis*; Tr., *Trifolium*; ^T = type strain.

formed a sister group to the *Lotononis* s.l. bradyrhizobia. The methylobacterial WSM2598 and WSM2667 were included in a clade that contained the other rhizobial *Methylobacterium*

strains ORS 2060^T and 4-46, but there was a large divergence between the *M. nodulans* strains (ORS 2060^T and WSM2667) and the strains isolated from *Listia bainesii* (4-46 and WSM2598).

TABLE 4. Summary of nodulation and N₂ fixation of *Lotononis s.l.* hosts by rhizobia isolated from *Lotononis s.l.* species

Host		Inoculant strain								
		<i>Bradyrhizobium</i> sp.			<i>Ensifer meliloti</i>		<i>Mesorhizobium tianshanense</i>	<i>Methylobacterium</i>		<i>Microvirga lotononidis</i>
		WSM 2596	WSM 2632	WSM 2783	WSM2653	WSM 3040	WSM 2624	<i>M. sp.</i>	<i>M. nodulans</i>	WSM 3557 ^T
Listia	<i>Listia angolensis</i>	N–	N–	N–	N–	N–	N–	N + F–	N ± F–	N + F +
	<i>Listia bainesii</i>	N–	N–	N–	N–	N–	N–	N + F +	N–	N–
Digitata	<i>Leobordea longiflora</i>	N ± F–	N ± F–	N + F–	N ± F–	N–	N ± F–	N + F–	N + F–	N + F–
Leobordea	<i>Leobordea platycarpa</i>	N–	N–	N–	N + F ±	N + F–	N–	N–	N–	N ± F ±
	<i>Leobordea stipulosa</i>	N + F–	N + F–	N + F–	N–	N–	N + F–	N + F–	N + F–	N + F–
Synclistus	<i>Leobordea bolusii</i>	N ± F ±	N + F +	N + F +	N–	N ± F–	N ± F–	N + F–	N ± F–	N ± F ±
Oxydium	<i>Lotononis crumanina</i>	N ± F ±	N ± F–	N ± F–	N + F +	N + F +	N ± F–	N–	N–	N + F ±
	<i>Lotononis falcata</i>	N + F–	N ± F–	N ± F–	N–	N ± F–	N–	N–	N–	N + F–

N–, no nodulation; N ± F–, some plants nodulated, but no N₂ fixation; N + F–, all plants nodulated, but no N₂ fixation; N ± F ±, some plants nodulated and some nodulated plants were partially effective for N₂ fixation; N + F ±, all plants nodulated and some nodulated plants were effective or partially effective for N₂ fixation; N + F +, all plants nodulated and all were effective for N₂ fixation.

Glasshouse experiment 1: nodulation studies

Rhizobial strains isolated from *Leobordea* and *Lotononis s.s.* hosts were assessed for their ability to nodulate a range of *Leobordea* and *Lotononis s.s.* species. All strains were able to nodulate at least one species of either *Leobordea* or *Lotononis s.s.*, and several were effective for N₂ fixation on some species (Supplementary Data Table S2).

Glasshouse experiment 2: nodulation and N₂ fixation tests

The symbiotic relationships between *Lotononis s.l.* species and their associated rhizobia were assessed by measurement of nodulation and N₂ fixation of nine strains on eight taxonomically diverse *Lotononis s.l.* species (Table 4). Quantification of nodulation and N₂ fixation of rhizobial strains on the individual *Lotononis s.l.* species is given in Supplementary Data Fig. S1a–h. All strains were able to nodulate several *Lotononis s.l.* species. Moreover, all except WSM2624 and WSM2667 were at least partially effective for N₂ fixation with one or more of these hosts, i.e. plants were green and larger than the uninoculated control, although shoot biomass was noticeably less than that of N+ control plants. WSM2667, however, is able to form partially effective nodules on *Leobordea mollis* (Supplementary Data Table S2).

A notable feature of these symbiotic relationships was the extreme specificity exhibited by *L. bainesii* (Table 4; Supplementary Data Fig. S1b), which was nodulated only by the pigmented *Methylobacterium* strain WSM2598. The symbiosis was highly effective, producing plant biomass equivalent to that obtained for the N+ control. *Listia angolensis* was less

specific, as host plants formed ineffective nodules with the methylobacterial strains WSM2598 and WSM2667. Effective nodulation was observed only with *M. lotononidis* WSM3557^T (Table 4; Supplementary Data Fig. S1a).

In contrast, many of the *Leobordea* and *Lotononis s.s.* species could be considered promiscuous, as they were nodulated by a range of *Lotononis s.l.* rhizobia, with no obvious taxonomically based patterns of symbiotic specificity. *Leobordea platycarpa*, for example, was comparatively specific, being nodulated only by WSM3557^T and the *Ensifer* strains WSM2653 and WSM3040, yet *L. stipulosa*, in the same taxonomic section (*Leobordea*) as *L. platycarpa*, was nodulated by all inoculants except the two *Ensifer* strains. Nodulation of the *Leobordea* and *Lotononis s.s.* species was seldom effective. Moreover, the effectiveness was in most cases only partial, as plants were green but biomass was much smaller than for the N+ control. In several instances, the response to the inoculant strain was variable, as within a treatment some individual plants were effectively nodulated, while in others nodulation was only partially effective or ineffective.

Glasshouse experiment 3: further evaluation of symbiotic specificity within *Listia*

Inoculation of *L. angolensis*, *L. bainesii* and *L. heterophylla* with phylogenetically diverse rhizobia (Supplementary Data Table S1) confirmed the symbiotic specificity of these species. *Listia bainesii* and *L. heterophylla* were nodulated only by WSM2598, and nodulation was always effective. *Listia angolensis* was effectively nodulated only by WSM3557^T, but

consistently, or occasionally, formed ineffective nodules with WSM2598 and *M. nodulans* ORS 2060^T, respectively.

Glasshouse experiment 4: infection and nodule initiation in Listia angolensis and L. bainesii

Nodule initials were clearly visible 16–17 days after inoculation (dai) on *L. angolensis* and *L. bainesii* plants grown in both pots and growth pouches, most frequently on the border of the hypocotyl just above the root hair zone (Fig. 3), but also

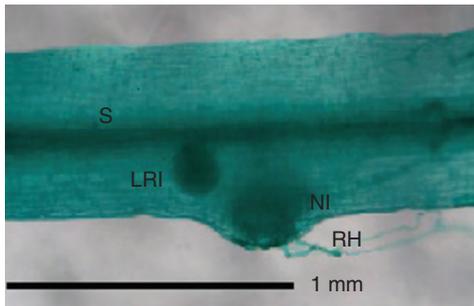


FIG. 3. Light micrograph of a cleared and stained *Listia bainesii* plant, 16 d after inoculation, showing lateral root and nodule initials. Abbreviations: H, hypocotyl; LRI, lateral root initial; NI, nodule initial; RH, root hair; S, stele.

further down the taproot. Nodule initials arose in the outer cortex. Lateral root initials could also be seen along the hypocotyl and taproot, but could be distinguished from nodule initials by their connection to the plant stele (Fig. 3). Curled or deformed root hairs were not observed.

Transverse sections of the hypocotyl of *L. angolensis* seedlings showed root hairs, although these were not curled or deformed and infection threads were not observed (Fig. 4A). The nodule primordium developed in the outer cortical layer. At 6 dai, there was a proliferation of cells in the outer cortex directly under an enlarged epidermal cell (Fig. 4A, B). Both anticlinal and periclinal cell division was observed, and cells in the infection zone had prominent nuclei and dense cytoplasm. There was no simultaneous division of pericycle cells; rather, cell division appeared to spread from the outer to the inner cortex. A similar pattern of nodule development occurred in *L. bainesii*. At 10 dai, the central tissue of the nodule primordium was infected with bacteria (Fig. 4C). Infection pockets, caused by the collapse and death of cortical cells, were not seen in either *L. angolensis* or *L. bainesii*.

Nodule morphology and structure

Nodule morphology clearly differentiated *Listia* species from the other *Listia* s.l. taxa. Both *L. angolensis* and *L. bainesii* formed lupinoid nodules, primarily on the border of the hypo-

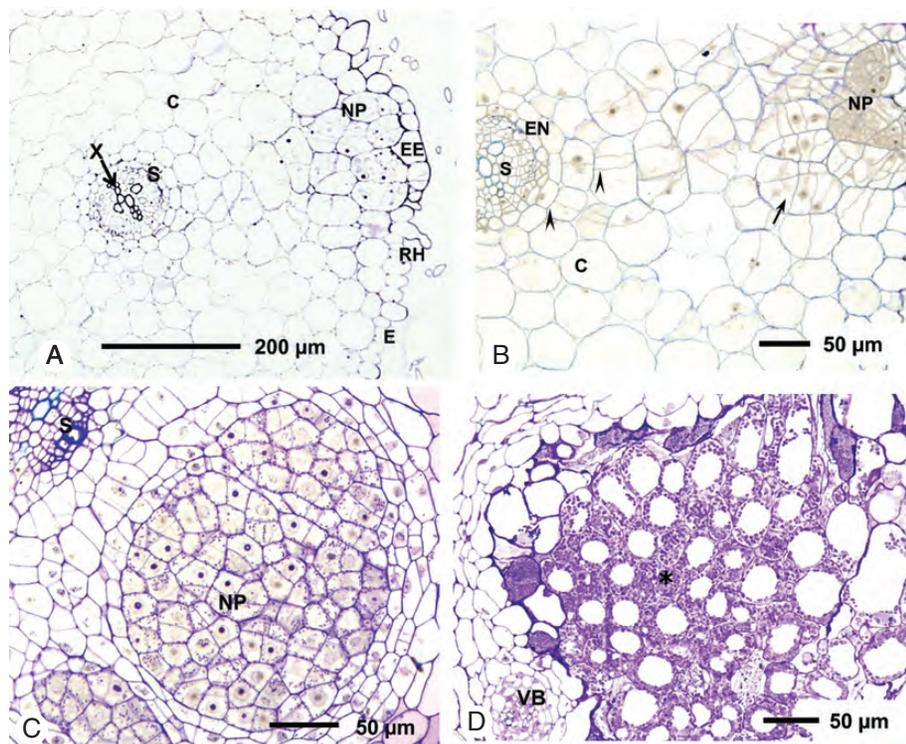


FIG. 4. Nodule development in *Listia* species. (A–C) Light micrographs of *Listia angolensis* and *Listia bainesii* hypocotyl sections (transverse to the primary root axis) showing development of nodule primordia after inoculation with WSM3557^T and WSM2598, respectively. (A) Whole section of *L. angolensis*, 6 days after inoculation (dai). (B) Section of the same nodule at higher magnification. Cells in the nodule primordium have divided repeatedly and show cytoplasmic staining and nuclei (arrow). Cell division can also be seen in the inner cortex (arrowheads). (C) Section of *L. bainesii* at 10 dai; cells in the nodule primordium are infected with bacteria. Another locus of infection is visible in the bottom left-hand corner. (D) Light micrograph of a section of a 10-week-old N_2 -fixing nodule of *L. bainesii*. The central tissue (*) contains infected cells only. Abbreviations: C, cortex; E, epidermis; EE, enlarged epidermal cell; EN, endodermis; NP, nodule primordium; RH, root hair; S, stele; VB, vascular bundle; X, xylem.

cotyl, just above the root hair zone and on the taproot, whereas nodules of *Leobordea* and *Lotononis* s.s. species were indeterminate and distributed throughout the root system. Sections of nodules taken from 10-week-old inoculated *Listia*, *Leobordea* and *Lotononis* s.s. host species were examined by light microscopy to compare their internal structure. All effective nodules contained uniformly infected central tissue with no uninfected interstitial cells, whether from the lupinoid nodules of *L. angolensis* and *L. bainesii* (Fig. 4D) or from the indeterminate nodules of *Leobordea* and *Lotononis* s.s. species. Infected cells of *L. bainesii* nodules were highly vacuolated.

DISCUSSION

Nodule morphology and structure as taxonomic markers

Within *Lotononis* s.l., the distinct differences in nodule morphology suggest that the lupinoid nodule is synapomorphic for the genus *Listia*. Nodule sections of all the *Lotononis* s.l. host species had a central mass of infected tissue with no uninfected interstitial cells, as found in nodules of other genistoid legumes (Sprent, 2009).

Infection and nodule initiation in Listia angolensis and L. bainesii

Infection and nodule organogenesis in *L. angolensis* and *L. bainesii* appear to be similar to the process observed in the genistoid legumes *Lupinus albus* and *Lupinus angustifolius*, where rhizobia penetrate at the junction between epidermal cells and subsequently invade a cortical cell immediately beneath the epidermis (Tang et al., 1992; González-Sama et al., 2004). Unlike the nodules of the dalbergioid legumes *Aeschynomene* and *Stylosanthes* or (under waterlogged conditions) the aquatic robinoid legume *Sesbania rostrata* (Chandler et al., 1982; Alazard and Duhoux, 1990; Lavin et al., 2001; D'Haese et al., 2003), nodulation in *L. angolensis* and *L. bainesii* was not associated with lateral roots, nor did intercellular infection involve the collapse and death of cortical root cells. Neither root hair curling nor infection threads were seen during *L. angolensis* and *L. bainesii* nodulation, similar to the pattern observed in rhizobial infection of *Lupinus albus* (González-Sama et al., 2004) and *Crotalaria podocarpa* (Renier et al., 2011). The salient features of infection and nodule organogenesis in *Listia* species – epidermal infection, a lack of infection threads and a central mass of uniformly infected tissue – appear to be characteristic of the basal genistoid and dalbergioid clades (Sprent and James, 2007) and support the proposed taxonomic value and evolutionary significance of infection pathways and nodule structure in legumes (Sprent, 2007).

Rhizobia associated with Lotononis s.l. species are taxonomically diverse

This is the first report and description of N₂-fixing microsymbionts of *Leobordea* and *Lotononis* s.s. species. Moreover, it demonstrates that *Lotononis* s.l. species are nodulated by and can form effective N₂-fixing symbioses with a remarkable diversity of rhizobia. Some variability in the microsymbiont genotype would be expected, given that South Africa is the centre of diversity for *Lotononis* s.l. and centres of rhizobial diversity are thought to coincide with those of their legume hosts

(Lie et al., 1987; Andronov et al., 2003). Other studies have also reported genotypic diversity in the natural populations of rhizobia that nodulate indigenous legumes (Sylla et al., 2002; Rasolomampianina et al., 2005; Rincón et al., 2008; Lorite et al., 2010; Zhao et al., 2010). What is unusual about the *Lotononis* s.l. symbiotic associations, however, is first, the wide taxonomic diversity of the rhizobia (belonging to five different genera of Alphaproteobacteria) and, secondly, the very different levels of symbiotic specificity seen within these associations.

Lotononis s.l. appears to have two specificity groups: *Listia* species are specifically nodulated by unusual *Methylobacterium* and *Microvirga* rhizobia, while species of *Leobordea* and *Lotononis* s.s. are generally promiscuous and nodulated by species of the classic legume symbionts *Bradyrhizobium*, *Ensifer* and *Mesorhizobium*, in addition to *Methylobacterium* and *Microvirga* strains. The small number of isolates from *Leobordea* and *Lotononis* s.s. hosts prevents a full examination of the possible biogeographical and symbiotic patterns of these rhizobia. However, the *Bradyrhizobium* strains isolated from *Lotononis* s.l. hosts are not closely related to bradyrhizobia from other African legumes (Fig. 1A) (Sylla et al., 2002; Steenkamp et al., 2008; Boulila et al., 2009; Garau et al., 2009), but instead group with isolates from host plants that are phylogenetically and geographically distant from *Lotononis* s.l. species (Qian et al., 2003; Parker and Kennedy, 2006; Parker, 2008; Ramírez-Bahena et al., 2009). Similarly, *M. tianshanense*, the species with the highest 16S rRNA sequence identity to WSM2624, was originally described from strains that nodulate diverse Chinese legume hosts (Chen et al., 1995) and was considered to be endemic to the Xinjiang region (Han et al., 2010). It has not previously been reported as associated with African legumes. In contrast, effective strains of *E. meliloti* have been common isolates of diverse African host species (Ba et al., 2002; Ben Romdhane et al., 2007; Mnasri et al., 2007; León-Barrios et al., 2009; Mnasri et al., 2009; Zurdo-Piñeiro et al., 2009; Fterich et al., 2011; Ourarhi et al., 2011).

Unlike *Bradyrhizobium*, *Ensifer* and *Mesorhizobium* strains, which have been isolated from a wide diversity of legume hosts, rhizobial methylobacteria appear to be associated exclusively with African crotalarioid legumes. Only two lineages have so far been discovered: the pigmented strains from *Listia* spp. (Jaftha et al., 2002; Yates et al., 2007) and the non-pigmented *M. nodulans*, which specifically nodulates species of Senegalese *Crotalaria* (Sy et al., 2001). The isolation of WSM2667 from *Leobordea calycina* and the demonstration of its ability to nodulate other *Lotononis* s.l. species is the first report of the extension of the *M. nodulans* host range to species outside the genus *Crotalaria*. The *Microvirga* strain WSM3557^T appears to have a broad host range within *Lotononis* s.l., nodulating all hosts except *L. bainesii* and fixing N₂ with three of these species in addition to its original host.

Rhizobia associated with Lotononis s.l. species have diverse nodA sequences

Several previous studies of legume–rhizobia symbiotic relationships have found a correlation between *nod* gene phylogeny and host range (Dobert et al., 1994; Haukka et al., 1998; Laguerre

et al., 2001; Suominen et al., 2001; Ba et al., 2002; Lu et al., 2009). In other studies, however, this association is not as strong, and *nod* gene phylogeny is more closely aligned with the rhizobial chromosomal background than with the host (Han et al., 2010; Lorite et al., 2010; Zhao et al., 2010). In the *Lotononis* s.l. rhizobia, there are five different *nodA* lineages, each associated with a specific rhizobial chromosomal background and interspersed with *nodA* from rhizobia isolated from taxonomically diverse hosts (Fig. 2). This indicates, first, that the symbiotic genes of *Lotononis* s.l. rhizobia were derived from different sources or have evolved divergently and, secondly, that the promiscuous species of *Leobordea* and *Lotononis* s.s. do not have stringent requirements for a particular rhizobial *nodA* genotype. In contrast, the long branch length of the pigmented *Methylobacterium nodA* gene (Fig. 2) suggests an ancient origin of this highly specific symbiosis and possible co-evolution of the two partners.

The 100% *nodA* sequence similarity found within strains of similar chromosomal background (*Bradyrhizobium* strains WSM2632 and WSM2783; *Ensifer* strains WSM2653 and WSM3040; and *Microvirga* strains WSM3557^T and WSM3693^T) suggests horizontal gene transfer of symbiotic loci between closely related rhizobia. The *nodA* genes of WSM2783 and WSM3557^T display features characteristic of both bradyrhizobial *nodA* [the signature length of the N-terminal segment (Moulin et al., 2004)] and of fast-growing rhizobia (nucleotide triplet deletion).

A model for the development of symbiotic specificity?

Leobordea and *Lotononis* s.s. species appear to be able to interact (often ineffectively) with rhizobia of diverse chromosomal and symbiotic lineages. (The variable response of individual plants to inoculation may be due to the genetic variability of the wild seed stocks, rather than cultivars, that were used in the experiments.) In contrast, specificity appears to have developed within the genus *Listia*, whose species associate exclusively with strains of *Methylobacterium* or *Microvirga*. The present study has confirmed the extreme symbiotic specificity of *L. bainesii*, which probably extends to the other host species in the *Listia*–*Methylobacterium* cross-inoculation group, and supports Sprent's (2008) association of specificity with effectiveness.

The *Lotononis* s.l. isolates in this study were collected from a diversity of bioregions (Table 1). The genotypic diversity of these rhizobia may be due to topo-edaphic heterogeneity, seen for example in South Africa's Cape Floristic Region, and proposed as a driver of the diversification and speciation of the flora of this region (Cowling et al., 2009). Studies on legumes nodulated by taxonomically diverse rhizobia have found a correlation between eco-regions and/or edaphic factors and the micro-symbiont genotype (Garau et al., 2005; Bala and Giller, 2006; Diouf et al., 2007; Han et al., 2009; Lu et al., 2009). For *Listia*, the adaptation of this genus to seasonally waterlogged habitats (van Wyk, 1991) may have resulted in the selection of microsymbionts that are also adapted to these environments. Members of the genus *Methylobacterium* are commonly found in water (Green, 1992; Hiraishi et al., 1995; Gallego et al., 2005, 2006) as well as soil and air environments. *Microvirga* species have similarly been isolated from aquatic or potentially waterlogged environments; additionally, the tolerance of described

Microvirga species to relatively high temperatures correlates well with the isolation of non-symbiotic species from thermal waters or sub-tropical regions (Kanso and Patel, 2003; Takeda et al., 2004; Zhang et al., 2009; Weon et al., 2010) and rhizobial *Microvirga* strains from tropically or sub-tropically distributed hosts (Wolde-Meskel et al., 2005; Lafay and Burdon, 2007; Ardley et al., 2012).

The particular habitat favoured by *Listia* species may promote the development of symbiotic specificity. In mutualisms, factors that are suggested to align the interests of the symbiont and the host are: vertical transmission of symbionts, genotypic uniformity of symbionts within individual hosts, spatial structure of populations leading to repeated interactions between would-be mutualists, and restricted options outside the relationship for both partners. Conversely, horizontal transmission, multiple symbiont genotypes and varied options decrease symbiotic stability (Herre et al., 1999). The legume–rhizobia symbiosis falls into the latter category (Kiers et al., 2008). The specialized *Listia* habitat may both reduce the diversity of rhizobial partners available for selection by the host plant and restrict the opportunity for horizontal gene transfer of symbiotic loci (Papke and Ward, 2004), thus contributing to symbiotic isolation. Thrall et al. (2000) have noted that *Acacia* species with more limited distributions or tighter ecological requirements have a greater degree of specificity than widespread species. In such cases, the selection pressures may favour the co-evolution of the host and microsymbiont towards a more effective and specific symbiosis.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: list of taxonomically diverse rhizobial strains used to inoculate *Listia angolensis*, *Listia bainesii* and *Listia heterophylla*. Table S2: ability of rhizobial strains isolated from *Leobordea* and *Lotononis* s.s. species to nodulate a range of *Lotononis* s.l. hosts. Figure S1: symbiotic ability of rhizobia associated with *Lotononis* s.l. on taxonomically diverse *Lotononis* s.l. hosts, assessed by nodule number and dry weight of shoots of plants harvested after 10 weeks growth.

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