Towards a characterisation of the wild legume bitter vetch (*Lathyrus linifolius* L. (Reichard) Bässler): heteromorphic seed germination, root nodule structure and N-fixing rhizobial symbionts.

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Abstract

- *Lathyrus linifolius L.* (Reichard) Bässler (bitter vetch) is a fabaceous nitrogen (N) fixing species. A coloniser of low nutrient (N) soils it supports biodiversity such as key moth and butterfly species and its roots are known for their organoleptic and claimed therapeutic properties. Thus, the species has high potential for restoration, conservation, novel cropping and as model species. The latter owing to its genetic synteny with important pulse crops. However, regeneration and functional attributes of *L. linifolius* remain to be characterised.
- Seeds of *L. linifolius* were characterised using physical, colourimetric and chemical data. Ultrastructural and functional characterisation of the N fixing root nodules included immunolabelling with nifH-protein antibodies (recognising the N fixing enzyme, nitrogenase). Endosymbiotic bacteria were isolated from the root nodules and characterised phylogenetically using *16S* rRNA, *nodA* and *nodD* gene sequences.
- *L. linifolius* yielded hetermorphic seeds of distinct colour classes: green and brown. Seed morphotypes had similar carbon:N ratios and were equally germinable (*ca.* 90 %) after scarification at differing optimal temperatures (16 and 20^{o}C, respectively). Brown seeds were larger and comprised a larger proportion of the seed batch (69%). *L. linifolius* root nodules appeared indeterminate in structure, effective (capable of fixing atmospheric N) and accommodated strains with high similarity to *Rhizobium leguminosarum* biovar *viciae.*
- The findings and rhizobial isolates have potential application for ecological restoration and horticulture using native seeds. Also, the data and rhizobial resources have potential application in comparative and functional studies with related and socio-economically important crops such as *Pisum*, *Lens* and *Vicia*.

Key words:

Lathyrus linifolius L., bitter vetch, seed germination, legume root nodules, *Rhizobium*.

Introduction

The genus *Lathyrus* L. encompasses plants referred to generically as 'Sweet Peas' which belong to the tribe Fabeae, a subdivision of the family Fabaceae, or Leguminosae. The tribe Fabeae is composed of approximately 380 species that evolved in the Eastern Mediterranean in the middle Miocene period, approximately 14 Mya. Species of the tribe then spread to Eurasia, tropical Africa and the Americas. The genus *Lathyrus* is made up of approximately 160 species which are distributed across the northern hemisphere with 52, 30, 78, 24 and 24 species in Europe, North America, Asia, South America and tropical East Africa, respectively. Regions in and around the Mediterranean basin and in North and South America are considered to be the primary and secondary centres of diversity of the genus, respectively. The genus presents both annual and perennial species with a climbing or sprawling habit often assisted by simple or branched tendrils, and pollinator dependant flowers which may vary in colour including yellow, orange, red, purple, violet, blue or white (Asmussen and Liston, 1998; Kenicer et al., 2005).

Lathyrus includes many species which are of agricultural and ecological importance due to their capacity to provide food and animal feed without an in-organic nitrogen (N) input. They can be productive on low nutrient soils as a function of their capacity for biological N fixation (BNF), and their roots can help stabilise the sandy soils of arid environments (Asmussen and Liston, 1998; Kenicer et al., 2005; Lewis et al., 2005; Schaefer et al., 2012). The latter property extends to *L. linifolius* (Reichard) Bässler (National Biodiversity Network, 2013), a mesophyte. The species is also genetically syntenous with other genera which include

domesticated types characterised by common pulse crops such as faba bean (*Vicia faba* L.), pea (*Pisum sativum* L.) and lesser known crops such as Indian-pea (*Lathyrus sativus* L.; Schaefer et al., 2012). *Lathyrus linifolius* has been popularised for its value as an horticultural species being cultivated for aesthetic reasons and for its aromatic flowers. In semi-natural systems and in ecological interactions this species is important for its attraction to oligophagous butterflies and moths, such as *Leucoptera lathyrifoliella form orobi*; *Phyllonorycter nigrescentella*; *Grapholita jungiella*; *Grapholita lunulana*; and *Zygaena lonicerae* (the Narrow-Bordered Five-Spot Burnet moth). Its loss from such habitats has been related to the extinction of specialist butterflies (*Leptidea sinapis,* the wood white butterfly; Nilsson et al., 2008). No records are found regarding its involvement in soil-microorganism interactions.

Lathyrus linifolius is found in extensively grazed and non-grazed semi-natural low altitude (20-350 m) grasslands, with soils of low nutrient status and pH ranging from 4 to 7 (Grime et al., 2014; Rose and O'Reilly, 2006). *L. linifolius* is present in 59% of European territories, though it is absent in the cold climatic extremes of North Europe, and is found throughout Britain though is rarely found in the South East of England (Grime et al., 2014). It is also recorded in distribution records on the National Vegetation Classification (NVC) floristic tables (JNCC, 2009) in *Quercus* (W11) and *Juniper communis* dominated woodland (W19). Rose (1999) considered *L. linifolius* an indicator of ancient woodland. As such, it is perhaps not surprising that *L. linifolius* is scarce in centres of arable crop production such as the UK (Grime et al. (2014).

Despite the absence of *L. linifolius* in agricultural ecosystems, ethnobotanical uses of the species have been recorded extensively, but are predominantly from seventeenth century

historical records of the Scottish Highlands (Beith, 1995; Cook, 1995; Hatfield, 2004; Johnston, 2012; Lightfoot, 1777; Moffat et al., 2014; Pennant, 2014; Vickery, 1995). During this period the species was prized for its root tubers which were used for satiation in times of famine, to offset the symptoms of inebriation due to excess alcohol consumption, as a medicine to relieve excessive flatulence, chest ailments, and, as a flavouring, most commonly of beverages. Raw tubers were also sliced and infused with hot water or neutral spirits, the latter sometimes after roasting to make flavoured beverages owing to their liquorice-like flavour, and/or for use as a general tincture. Hence the many vernacular names for *L. linifolius* include liquor-knots, liquory-knots and liquorice vetch (Brenchley, 1920; Henderson and Dickson, 1994; Johnston, 2012; Pratt and Step, 1899). The tubers were also used as a flouring agent, and were fermented to make beer, which on occasions was distilled to produce flavoured neutral spirit. Of the literature sources cited here, there is general agreement that the root tubers were used to promote feelings of satiation. *L. linifolius.* Therefore, this species also has the potential for development as a therapeutic and novel crop and while scientific evidence for its impact in these regards is scant recent research by Woods et al., (2012) revealed that consumption of bitter vetch tubers by rats significantly altered the expression of hypothalamus genes involved in regulating metabolism.

Whether developed for study as an academic model species, exploited *via* commercial native seed suppliers for use in restoration and conservation projects, or developed as a novel crop, maximising the germination of *L. linifolius* seeds is important. However, there is a paucity of scientific peer-reviewed reports dedicated to the species, and hence there are no similar reports which characterise their seeds or which describe optimal methods for seed germination. Our own observations show that *L. linifolius* has heteromorphic seeds, as distinguished by their different seed colours, and so this aspect also remains to be

characterised with respect to their germination capacities. The structure of the N-fixing root nodules of *L. linifolius* also remains to be reported, and there has been only limited characterisation of the rhizobial symbionts from other *Lathyrus* species (*L. pratensis* or meadow vetchling, *L. aphaca* or meadow vetchling and, *L. nissolia* or grass vetchling, Mutch and Young, 2004; also *L. latifolius* or perennial peavine, de Meyer et al., 2011). Therefore, herein we report on a characterisation of *L. linifolius* seeds, and test seed treatments (temperature and scarification) to optimise seed germination; 2) we also report upon N-fixing root-nodule structure; and 3), the isolation and molecular characterisation of the root nodule symbionts using sequences of their core (16S rRNA) and symbiotic (*nodA, nodD*) genomes: in a comparative phylogenetic analysis with the same sequences from rhizobia isolated from related legume genera.

Materials and methods

Seed material and morphometric characterisation

L. linifolius seeds were purchased from Bitter-Vetch Ltd., a commercial seed supplier. Seeds could be characterised as heteromorphic based on seed colours, which were categorised superficially as either green or brown. These colour morphs were characterised using 300 seeds (100 seeds *per* replicate) by assessing the relative abundance of each morphotype, their fresh weight recorded to using a 4 decimal places (ug) (Adventurer® Explorer, Ohaus), and by colourimetric analysis using a high resolution digital image (1200 dpi), and subsequent analysis using the Fiji/ImageJ software. Images were taken using a scanner (Epson Expression 10000XL Pro) and saved as Tiff format. The images were segmented using the FIJI SIOX plugin (Simple Interactive Object Extraction; Friedland et al., 2005), and seed measurements were acquired by using the Analyse particles function on the binary image.

This was also used to produce region of interest (ROI) locating for each seed. For the colorimetric analysis each image was split into each component of a red – green – blue (RGB) scale. The respective ROI were transferred onto each of the split images and the particle analyses process was repeated with "integrated density" included in the measurement. "Integrated density" is the product of the area and the Mean Gray Value.

Seed carbon:nitrogen ratio

C and N content were measured for each seed morph. Three replicates of 4 seeds were weighed and oven dried for 48 h at 75 °C. From each replicate 2 mg were weighed (with Sartorius SE2 Ultra-micro balance) into tin containers for combustion and processed with a CE440™ Elemental Analyser (Exeter Analytical Inc., USA).

Optimal germination temperature of seed heteromorphs and assessment of seed scarification

To investigate optimal germination for each colour morphotype at a range of temperatures, three replicates (comprising 12 seeds each) were used *per* morphotype and temperature combination. Seeds were surface sterilised by immersion with gentle shaking for 3 min in sodium hypochlorite solution diluted to provide 2.5 % [v/v] active chlorine. The seeds were then washed three times with 2 mL sterile distilled water (SDW) and placed on two disks of 3MM Whatmann filter paper (90 mm radius) in 90 mm diameter Petri dishes. After addition of 7 mL of SDW to the petri-dish, each dish was placed onto a tray within a sealed plastic bag (to avoid dehydration of the filter papers/seeds). Each set of replicates were placed into incubators at constant temperatures at either: 5, 10, 15, 20 or 25 °C. After the initial imbibition phase, plates were monitored daily, and germination was scored as radicle protrusion (1-2 mm) until germination ceased. Data were used to determine the proportion of

germinated seeds and the time taken for 50% of seeds to germinate (t_{50}).

The effect of seed scarification on total germination rate and t_{50} was assessed using six replicates from each morphotype (15 seeds *per* replicate). For each morphotype, the seeds of three replicates were scarified manually using a sharp scalpel blade, with the incision being made along the axis transverse to the seed embryo. The seeds were then germinated in Petri dishes as described above in dark conditions at 16 and 20 °C, the respective optimal germination temperatures for the green and the brown morphs.

All germination trials were prepared in sterile containment at the James Hutton Institute, Dundee (56°27'23"N, 3°04'14"W), and within a time frame of six months from January to June 2016. All control and treatment Petri dishes were fully randomised on trays for incubation. Germination tests were performed in an LMS Cooled Incubator 600 (LMS Ltd), without light. Seed germination was monitored as above for optimal germination experiments.

Plant growth for root nodule formation and harvesting

Seeds were treated using a standardised approach developed at the Royal Botanic Garden Edinburgh (Scotland, UK). In April 2016 seeds were soaked overnight in tap water containing detergent (domestic; 0.01 % [v/v]), and placed onto the surface of small pots containing dry sieved, rooting medium that comprised 3:1 Sylvamix® Special (Melcourt, www.melcourt.co.uk) and horticultural sand. After sowing, horticultural grit (2–6 mm) was added to cover ('top-dress') the seeds to a depth of approximately 2 mm. The pots were then placed in an empty container, which was filled with water to just below the level of the internal rooting medium. This allowed water to saturate the compost from the base up, and produces even saturation throughout the pots. The pots were then removed and allowed to

drain, then stratified in an incubator 5 °C for 14 d before transfer to the unheated glasshouse, where they were subject to ambient conditions. Plants were not fed, but only watered when needed, and were allowed to nodulate naturally.

Root nodule ultrastructure

Root nodules were harvested from juvenile plants of around 5–10 cm in height. The plants were gently removed from their rooting medium and the root systems carefully washed in running tap water. Freshly harvested root nodules were gently sliced in half using a sterile scalpel (longitudinally if elongated) and then fixed in 2.5 % glutaraldehyde as described by James et al. (2011). The fixed nodules were then subject to an ethanol-LR White acrylic resin dilution series before polymerisation for 48 h at 60 °C in 100% LR White. The nodules were then sectioned using a glass knife on a Leica UCT ultramicrotome for light and transmission electron microscopy. Light microscope sections were collected on slides, stained with 0.5 % toluidine blue and digital micrographs taken as described by dos Reis et al., (2010). Sections for TEM were treated by immunogold labelling using an antibody against the nitrogenase Fe-(*nifH*) protein according to (James et al., 2002). Digital micrographs were taken using a JEOL JEM 1400 TEM.

Root bacterial isolation and molecular characterisations

Root nodules for bacterial isolation were harvested from juvenile plants, as described above. Three nodules were selected randomly from each of two *L. linifolius* plants. Nodules were washed with tap water and were then surface sterilised by rinsing in 70 % [v/v] ethanol, followed by immersion in 2.5 % sodium hypochlorite (Fisher Chemical S/5042/15) [v/v] for 4 min, and rinsing three times with sterile deionized water. Nodules were then crushed using sterile pellet pestles (Sigma Z359947-100EA) and spread onto 90 mm diameter sterile triple

vent Petri dishes (Sterilin 101VR20) containing 20 mL of yeast mannitol agar (YMA; 54.89 mM mannitol; 2.87 mM K_2HPO_4; 0.81 mM $MgSO_4$; 1.71 mM NaCl; yeast extract, 0.5 g L^{-1}; Fred and Waksman, 1928; Vincent 1970) with filter sterilised congo red (0.025 g L^{-1}). The dishes were incubated at 28 °C for 24–48 h. Single colonies were selected and purified. Single colonies from purified plates were used to inoculate 5 mL of sterile tryptone yeast (TY) broth (tryptone, 5 g L^{-1}; yeast extract, 3 g L^{-1}; 6.05 mM $CaCl_2$-$2H_2O$; pH 6.8; Beringer (1974). Liquid cultures were grown in a rotary shaker (150 rpm) at 28 °C overnight then used to prepare 25 % glycerol stocks for long-term storage and to extract DNA for strain molecular characterisation.

For DNA extraction, bacterial cells were harvested from liquid cultures by centrifugation at 11,000 x g for 10 min. Bacterial pellets were re-suspended in 420 µL of freshly made lysis buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0, 0.5 % w/v sodium dodecyl sulphate and 10 µL of proteinase K (Sigma P4850)) and incubated at 37°C for 1 h. Then, 420 µL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1 [v/v/v]; Sigma P2069) was added to each sample, vortexed and centrifuged at 11,000 x g for 10min. The aqueous phase of the upper layer (~175µL) was recovered, and was first mixed with 0.1 x volume (~17.5 µL) of 3 M sodium acetate (pH 5.2). This was later mixed with approximately 3x volumes (~655 µL) of isopropanol (Sigma I9030). DNA was precipitated by incubating at -80 °C for 15 min (or -20°C overnight) and pelleted by centrifugation at 11,000 x g for 15 min. The DNA pellet was washed with 200 µl 70 % [v/v] ethanol and centrifuged at 11,000 x g for 1 min. Supernatant was removed using an aspirator and residual liquid was air dried. The DNA pellet was re-suspended in 50 µL SDW. Each DNA sample was assessed for its quality and quantity using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, US).

The primers and thermal profiles used to generate gene specific products to identify root nodule bacterial isolates *via* their core (16S rRNA) and symbiotic (*nodA* and *nodD*) genomes are given in Supplementary Table 1. Each 50 µL PCR reaction contained 1 µL DNA template, 1x reaction buffer, 1.25 unit GoTaq® G2 DNA polymerase (Promega M7845), 0.2 mM each dNTP and 0.4 µM of each primer. For all primer combinations initial denaturation was for 95°C for 2 min, followed by the amplification cycles (Supplementary Table 1) for each primer pair. Each amplification cycle comprised 95 °C for 1.5 min, the annealing temperature (shown in Supplementary Table 1), and extension at 72 °C for 1.5 min. There was also a final single extension cycle at 72 °C for 15 min. Purified products (Illustra™ ExoStar™ 1-Step, GE Healthcare Life Sciences) were sequenced using an ABI3730 DNA analyser.

All gene sequences were deposited in GenBank with the accession numbers from MG546080 to MG546091 (16S rRNA), MG546092 to MG546102 (*nodA*) and MG546103 to MG546113 (*nodD*). Sequences were amplified from DNA extracted and purified from isolates cultured from dry nodules of *L. sativus* as a comparison, and these have been deposited in the NCBI database with the accession numbers from MG546114 to MG546116.

Phylogenetic relationships were calculated using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) and were conducted in MEGA7 (Kumar et al., 2016), using the following parameters: Clustal Ω for alignment, complete deletion for gaps or missing data, 1000 bootstrap replicates and uniform rates among sites. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees were drawn to scale, with branch lengths measured in the number of

substitutions *per* site. Accession numbers for reference species and strains used in all phylogenetic analyses are included in Supplementary Table 2.

Statistical analysis

Results were analysed with Microsoft excel and RStudio (RStudio Version 1.1.383 and R version 3.1.1; Team, 2015). Data obtained from the morphological characterisation and the C and N analysis were compared between seed morphs through analysis of variance (ANOVA). Data obtained from the final germination percentage were analysed using a generalised linear model (GLM), as described in Crawley (2012). Germination rate was used to calculate the optimal growth temperature for each morph. The germination rate was calculated as $1/t_{50}$; with the t_{50} calculated as the inflection point of a Boltzmann sigmodal curve that was fitted to the curve of germinated proportion against time (fitted using the model type 'Non-Linear-least Squares', or NLS). Two approaches were adopted for the analysis of the germination rate ($1/t_{50}$) related to temperature. In the first case it was regressed using a linear model as a function of temperature according to the procedure described in Garcia-Huidobro et al. (1982) for the calculation of species cardinal temperatures using Excel 2010 (Microsoft). Briefly, the data for each morphotype was separated into sub-optimal and supra-optimal temperature range and a linear regression was fitted as to find T_b and T_c. Both T_b and T_c were identified as the x-axis intercept with each regression line. T_b (base temperature for germination) is linked to the sub-optimal temperature ranges and T_c (maximum temperature for germination) to the supra-optimal ranges. Optimal temperature was calculated for both morphs as the intercept of sub and supra-optimal temperature response functions. The second approach involves the use of a polynomial regression fitted to the t_{50} data plotted against the five constant temperatures (analysis for this second approach was performed within Excel and R, using a Generalized Linear Mixed Model, GLMM). According to this approach, the optimal germination

temperature lies on the maximum point identified in the polynomial relationship between t_{50} against temperature. The maximum of the regression was calculated by using the following: considering $y = ax^2 + bx + c$ as the general equation of the polynomial regression, the formula for the maximum regression point is – b/2a. In R prediction were made from the polynomial model and the position of the maximum prediction was selected.

Results

Morphological characterisation and carbon:nitrogen ratio

Lathyrus linifolius seed batches included two seed heteromorphs which could be distinguished visually by apparent differences in their relative size and colour, as either 'smaller green' or 'larger brown' (Fig. 1). This was confirmed by weight, colour discrimination and germination characterisation. Green seeds represented 31 % of the seeds in the seed batch (Table 1), with the average fresh weight of individual green seeds being slightly less than that of the larger brown seeds. The green seed averages for length, width, area, surface area (SA) and volume (V) were all significantly less than those values recorded for brown seeds. Brown seeds were also more elongated, with green seeds demonstrating significantly greater circularity and higher SA:V ratio. After drying, the weight of the seed morphotypes was still significantly different indicating that the greater weight of brown seeds cannot be attributed only to their higher moisture content.

Seed colour analysis confirmed that the visible colour morphs can be discriminated on the basis of their red, green and blue integrated colour densities and average relative pixel number (Table 1). It may be argued that the average red, green and blue colour content *per* pixel may have more reliable discriminatory power than integrated density. The assessment of carbon

330 (C) and N content, however, did not show any differences between the two morphotypes.

331

332 **Optimal germination temperature and effect of scarification**

333 Trials determining percentage of total germination of non-scarified seeds across a temperature
334 series (Figure 2) showed that seed germination varied with seed incubation temperature.
335 Specifically, the green morphotype showed germination values between 70–80 % over a
336 temperature range from 5 to 20 °C, whereas for the brown morphotype the trend was similar
337 with an exception at 5°C, where the green morphotype showed significantly higher
338 germination (72 %), than the brown type (41%; Fig. 2A). At 25 °C seed germination dropped
339 to 40 % for both morphotypes. A generalised linear modelling (GLM) showed that there is a
340 statistically significant relationship between temperature and percentage of seed germination
341 ($P = 0.8574$; $F = 0.2546$; $R^2 = -0.08356$; $y = - 0.9467 x + 1.7676619$; Fig. 2A). Similarly, a
342 significant relationship was found between percentage of germination and seed morphotype
343 using the GLM ($P = 0.8574$; $F = 0.2546$; $R^2 = -0.08356$, $y = -0.7772632 x + 1.7676619$).
344 According to the cardinal temperature model the optimal germination temperatures were
345 identified at 16.3 and 20.3 °C for green and brown colour morphotypes, respectively (Table 1;
346 model not shown).

347

348 Assessments of the seed germination rate ($1/t_{50}$; Fig. 2B) by fitting polynomial models to
349 average data for green- ($P < 0.001$; $R^2 = 0.7534$; $y = - 0.0003x^2 + 0.0085x - 0.0158$), and
350 brown-coloured seed types ($P < 0.001$; $R^2 = 0.6063$; $y = -0.0002x^2 + 0.0054x - 0.0045$) gave
351 optimal germination temperatures estimated at 14.1 and 13.5 °C for the green and brown
352 morph, respectively. Polynomial fitting to full dataset gave estimates of optimal germination
353 of 16.1 and 17.5 °C. Therefore, estimates for the optimal temperature for the green
354 morphotype varied between 14.1 and 16.3 °C, whereas estimates for the brown morphotype

355 varied between 13.5 and 20.3 °C.

356

357 When incubated at the temperature for optimum germination rate for each seed batch,
358 scarification significantly improved seed germination by 10 or almost 17 % for green and
359 brown seed morphotypes, respectively (Table 1). Germination rate (t_{50}), varied significantly
360 between scarified green and brown seeds, achieving 50 % in 16 or 9 d faster, respectively
361 representing a time reduction of 1/3 in each case.

362

363 **Nodulation Characterisation**

364 *L. linifolius* nodules were indeterminate, and this nodule type is typical of the genetically
365 syntenous *Pisum*, *Vicia* and *Lathyrus* genera(Schaefer et al., 2012). These genera also belong
366 to the 'inverse repeat-lacking monophyletic clade' (IRLC) of the sub-family Faboideae
367 (Papilionaceae) which include the majority of cropped legume species (Sprent et al. 2017).
368 Light micrographs of mature *L. linifolius* nodules show the typical arrangement which from
369 tip to base comprise a continuum of meristem; invasion, biological N fixing, and senescent-
370 zones (Fig. 3A and B). At harvest, the nodules appeared pink (due to leghaemoglobin
371 content), indicative of active BNF, and TEM showed rhizobial infected cells in the BNF zone
372 contained apparently functional bacteroids (Fig. 3C) which were immunogold labelled with
373 an antibody against the Fe-(nifH) protein of the nitrogenase enzyme, which catalyses
374 atmospheric di-nitrogen gas to biologically useful N forms.

375

376 Root nodule bacteria were isolated from the *L. linifolius* root nodules and their 16S rRNA,
377 *nodA* and *nodD* genes were sequenced (Fig. 1A) to discern their identity as potentially
378 nodulating rhizobia. To perform the phylogenetic analysis, sequences from 16S rRNA, *nodA*
379 and *nodD* were compared using BLAST against those held in the NCBI nucleotide data base

(Altschul et al., 1990) and non-nodulating genera were excluded from further analysis. Our phylogenetic analysis of 16S rRNA sequence data showed that of the 12 potentially nodulating isolates tested, 11 segregated with R. *leguminosarum* and one isolate grouped with the *R. etli*, *R. pisi, R. phaseoli, R. esperanzae, R. ecuadorense, R. binae, R. lentis, R. bangladeshense, R. aegyptiacum* and *R. sophoriradicis* species complex. Comparative phylogenetic characterisation using *nodA* gene sequence (Fig. 4) showed that all the rhizobia isolated form *L. linifolius* segregated with *R. pisi*, and alongside *R. leguminosarum, R. lentis, R. bangladeshense, R. binae* and *R. laguerreae* and a strain from *L. sativus* L. (grass pea).

Discussion

The presence of an impermeable seed coat is a seed characteristic of the many genera in the family *Fabaceae*, including *Lathyrus*; the existence of a hard seed coat usually involves physical dormancy, which implies the development of mechanisms and/or structures that allow the passage of water that can, therefore, trigger seed imbibition and germination processes. The approach used in the present study is designed to characterise these aspects and develop an effective strategy to improve seed germination through pre-sowing techniques.

The distinction in two colour morphs (green and brown) is confirmed through the image colour analysis and also by the differences in their fresh and dry weight. However, this heteromorphism does not influence germination traits. For both morphotypes natural (without treatments) optimal temperature covers a range from 14.1–16.3 (green morphotype) to 13.5–20.3 °C (brown morphotype), and scarification treatment generally improved germination performance (in terms of final germination % and germination rate, t_{50}) regardless of colour morph. Two methods were used to for data analyses to estimate optimal temperatures. The

cardinal temperature approach (Garcia-Huidobro et al., 1982) identified 16.2 for green and 20.3 ºC for green and brown seed morphs, respectively. This approach was found to be limited with the optimal regression for both morphotypes comprising only two data points (at least one side of the intersection), which are not statistically sufficient to define the real trend. Thus, this method is more appropriate when a broader range of temperatures are applied than were used here. The alternative method of fitting a curve to the $1/T_{50}$ *versus* temperature data was more suitable to the data points available here (16.1 and 17.5 ºC for green– and brown seed morphotypes, respectively). However, the fit to the data was lower in terms of R^2 (0.68 and 0.64 using the 1/T50 curve fit, *versus* 0.87 and 0.97 for the cardinal-fit lines: green and brown seed morphotypes, respectively). Whichever method is used to model the temperature x germination data they are in agreement, since the temperatures all indicate a preference for the species to germinate and develop its growth during the warm season. This reflects the adaptation of *L. linifolius* to temperate climates. Also, the increased germination percentage and faster germination after scarification confirms the presence of physical dormancy in these seeds.

Chemical (sulphuric acid) and mechanical based scarification appear to be the most effective and frequently used treatment on *Lathyrus* (Basaran et al., 2012; Justice and Marks, 1943; Walmsley and Davy, 1997). In the present study only the latter was applied in order to assess the economic feasibility of the lower-priced method for possible application in large-scale production. This form of seed treatment may also improve seed vigour and seedling survival. Scarification may also be combined with other seed pre-germination techniques, such as seed priming and rhizobia inoculation, in order to improve establishment.

There are very few published peer-reviewed reports on nodulation and BNF fixation by *L.*

430 *linifolius*, though James et al. (2011), has reported that it does not appear to nodulate well,
431 even in environments where it is found. Therefore, and since the species occurs mainly in
432 improved grasslands and/or woodlands, we may speculate that *L. linifolius* may be more soil
433 N dependant than other related wild species such as some *Vicia* species (*V. lutea*, *V. salvatica*
434 and *V. sativa*) which typically occupy soils with 3- or more fold less soil N (at 0.02–0.10 %)
435 (James et al., 2011). On the other hand, the nodules reported here were clearly effective, as
436 indicated by their pink internal colouration (due to legheamoglobin), their general structure
437 and the fact that their bacteroids contained nitrogenase protein. Nevertheless, actual
438 quantification of BNF using techniques such as the ^{15}N natural abundance approach
439 (Unkovich et al., 2008) remain to be carried out for the species in natural systems, and
440 controlled environments.
441
442 Rhizobia inoculation can be applied to (mostly) crop species to improve seed germination
443 performance and yield production. Positive effects on seedling growth and later development
444 stages have been found, especially when species-specific rhizobia strains were used for the
445 treatment (Cassan et al., 2009; de Souza et al., 2016; Kumar et al., 2016; Schlindwein et al.,
446 2008; Shcherbakova et al., 2017; Sorty et al., 2016). The identification of specific species–
447 rhizobia interactions are, therefore, fundamental for the application of this novel methodology
448 in crop production enhancement. In the present study, the characterisation of apparently
449 effective *L. linifolius*-nodulating bacteria is included in this context. Further investigation on
450 the effectiveness of this association in the improvement of *L. linifolius* germination and
451 production is needed.
452
453 The sequences of both *nodA* and *nodD* were used here to assess rhizobial isolate diversity
454 (Chen et al., 2005; Cummings et al., 2009; Fonseca et al., 2012; Gehlot et al., 2013; Mutch

and Young, 2004). In particular, the "canonical" nod genes *nodABC,* have been identified as major determinants of rhizobial-host specificity (Sprent et al. 2017). This is because they are involved in the biosynthesis of lipochitooligosaccharide (Adu et al. 2014) "nod factors" (NF), which determines rhizobial host specificity (Atkinson et al., 1994; Röhrig et al., 1994), whereas the *nodD* gene, is involved in rhizobia-host recognition (Fisher and Long, 1992; Van Rhijn and Vanderleyden, 1995). Nodulating bacteria of the *Pisum*, *Vicia* and *Lathyrus* cross-inoculation group span a wide range of species, but the vast majority are in the genus *Rhizobium* (Drouin et al., 1996; James et al., 2011; Villadas et al., 2017). This also proved to be the case with the *L. linifolius* rhizobial isolates which all segregated with *nodA* sequences for *R. leguminosarum* biovar *viciae*, including the sequenced type strain 3841 (Fig. 4; Young et al., 2006). This segregation was even stronger with the *nodD* sequence data, with all the *L. linifolius* isolates aligning with those of *R. leguminosarum* biovar *viciae* (Fig. 5B). The *nodD* and *nodA* phylogenies show a high level of consistency and confirm that the genetic synteny shared by *Pisum, Vicia* and *Lathrus* is reflected in the diversity of their nodulating bacteria.

In summary, *L. linifolius* has the potential to be developed as an alternative crop with several potential consumers and end uses. In order to fulfil the requirements of large scale production knowledge is required on germination traits and how germination and production can be improved. The approach adopted here is to characterise seed morphology, germination traits and fundamental soil microbial interactions of the wild legume species, using techniques that demonstrate good potential for use in the large scale production of *L. linifolius*. Further investigations are now needed to consider the effect of seed priming, bacteria (rhizobia) inoculation and the interactions between the treatments to select the most effective one for functional applications.

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References

Adu, M.O., Chatot, A., Wiesel, L., Bennett, M.J., Broadley, M.R., White, P.J., Dupuy, L.X. (2014) A scanner system for high-resolution quantification of variation in root growth dynamics of *Brassica rapa* genotypes. Journal of Experimental Botany, eru048.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. Journal of Molecular Biology, **215**(3), 403–410.

Asmussen, C. and Liston, A. (1998) Chloroplast DNA characters, phylogeny, and classification of Lathyrus (Fabaceae). American Journal of Botany, **85**(3), 387–387.

Atkinson, E.M., Palcic, M.M., Hindsgaul, O., Long, S.R. (1994). Biosynthesis of *Rhizobium meliloti* lipooligosaccharide Nod factors: NodA is required for an N-acyltransferase activity. Proceedings of the National Academy of Sciences, **91**(18), 8418-8422.

Basaran, U., Mut, H., Asci, O.O., Ayan, I., Acar, Z. (2012) Germination pattern of naturally grown *Lathyrus* and *Vicia* species to different methods and seedbeds. International Journal of Plant Production, **6**(3), 325–335.

Beith, M. (1995) Healing threads. Traditional medicines of the highlands and islands. Edinburgh: Polygon 294pp. ISBN, **74866199**(9).

Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. Journal of General Microbiology, **84**(1), 188-198.

505 Brenchley, W.E. (1920) *Weeds of farm land* Longmans, Green and Company.
506 Cassan, F., Perrig, D., Sgroy, V., Masciarelli, O., Penna, C., Luna, V. (2009) *Azospirillum*
507 *brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in
508 combination, promote seed germination and early seedling growth in corn (*Zea mays*
509 L.) and soybean (*Glycine max* L.). European Journal of Soil Biology, **45**, 28–35.
510 Chen, W.M., James, E.K., Chou, J.H., Sheu, S.Y., Yang, S.Z., Sprent, J.I. (2005) β-Rhizobia
511 from *Mimosa pigra*, a newly discovered invasive plant in Taiwan. New Phytologist,
512 **168**, 661–675.
513 Cook, F.E.M. (1995) *Economic botany data collection standard* Royal Botanic Gardens
514 (Kew).
515 Crawley, M.J. (2012) *The R book* John Wiley & Sons.
516 Cummings, S.P., Gyaneshwar, P., Vinuesa, P., Farruggia, F.T., Andrews, M., Humphry, D.,
517 Elliott, G.N., Nelson, A., Orr, C., Pettitt, D., Shah, G.R., Santos, S.R., Krishnan, H.B.,
518 Odee, D., Moreira, F.M., Sprent, J.I., Young, J.P., James, E.K. (2009) Nodulation of
519 *Sesbania* species by *Rhizobium (Agrobacterium)* strain IRBG74 and other rhizobia.
520 Environmental Microbiology, **11**(10), 2510–2525.
521 de Souza, E.M., Bassani, V.L., Sperotto, R.A., Granada, C.E. (2016) Inoculation of new
522 rhizobial isolates improve nutrient uptake and growth of bean (*Phaseolus vulgaris*)
523 and arugula (*Eruca sativa*). Journal of the Science of Food and Agriculture, **96**(10),
524 3446-3453.
525 De Meyer, S.E., Van Hoorde, K., Vekeman, B., Braeckman, T., Willems, A. (2011) Genetic
526 diversity of rhizobia associated with indigenous legumes in different regions of
527 Flanders (Belgium). Soil Biology and Biochemistry, 43(12), 2384-2396.
528 dos Reis, F.B., Jr., Simon, M.F., Gross, E., Boddey, R.M., Elliott, G.N., Neto, N.E., Loureiro
529 Mde, F., de Queiroz, L.P., Scotti, M.R., Chen, W.M., Noren, A., Rubio, M.C., de Faria,

S.M., Bontemps, C., Goi, S.R., Young, J.P., Sprent, J.I., James, E.K. (2010) Nodulation and nitrogen fixation by *Mimosa spp*. in the Cerrado and Caatinga biomes of Brazil. New Phytologist, **186**(4), 934–946.

Drouin, P., Prevost, D., Antoun, H. (1996) Classification of bacteria nodulating *Lathyrus japonicus* and *Lathyrus pratensis* in northern Quebec as strains of *Rhizobium leguminosarum* biovar *viciae*. International Journal of Systematic Bacteriology, **46**(4), 1016–1024.

Fisher, R.F., Long, S.R. (1992). Rhizobium–plant signal exchange. Nature, **357**(6380), 655-660.

Fonseca, M.B., Peix, A., de Faria, S.M., Mateos, P.F., Rivera, L.P., Simoes-Araujo, J.L., Costa Franca, M.G., dos Santos Isaias, R.M., Cruz, C., Velazquez, E., Scotti, M.R., Sprent, J.I., James, E.K. (2012) Nodulation in *Dimorphandra wilsonii* Rizz. (Caesalpinioideae), a Threatened Species Native to the Brazilian Cerrado. Plos One, **7**(11), e49520.

Fred, E. B., Waksman, S.A. (1928). *Laboratory manual of general microbiology-with special reference to the microorganisms of the soil*. Mcgraw-Hill Book Company, Inc; New York; London.

Friedland, G., Jantz, K., Rojas, R., Soc, I.C. (2005) *SIOX: Simple interactive object extraction in still images*: pp.253-259.

Garcia-Huidobro, J., Monteith, J.L., Squire, G.R. (1982) Time, temperature and germination of pearl millet (*Pennisetum typhoides* S. & H.) I. Constant temperature. Journal of Experimental Botany, **33**(2), 288–296.

Gehlot, H.S., Tak, N., Kaushik, M., Mitra, S., Chen, W.M., Poweleit, N., Panwar, D., Poonar, N., Parihar, R., Tak, A., Sankhla, I.S., Ojha, A., Rao, S.R., Simon, M.F., Reis Junior, F.B., Perigolo, N., Tripathi, A.K., Sprent, J.I., Young, J.P., James, E.K., Gyaneshwar, P.

555 (2013) An invasive *Mimosa* in India does not adopt the symbionts of its native
556 relatives. Annals of Botany, **112**(1), 179–196.
557 Grime, J.P., Hodgson, J.G., Hunt, R. (2014) *Comparative plant ecology: a functional*
558 *approach to common British species,* Springer.
559 Hatfield, G. (2004) *Encyclopedia of folk medicine: old world and new world traditions* ABC-
560 CLIO.
561 Henderson, D.M. and Dickson, J.H. (1994) *A naturalist in the Highlands: James Robertson,*
562 *his life and travels in Scotland 1767-1771* Scottish Acadademic Press.
563 Haukka, K., Lindström, K., Young, J. P.W. (1998) Three phylogenetic groups of *nodA* and
564 *nifH* Genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees
565 growing in Africa and Latin America. Applied and Environmental Microbiology,
566 **64**(2), 419-426.
567 James, E.K., Gyaneshwar, P., Mathan, N., Barraquio, W.L., Reddy, P.M., Iannetta, P.P.M.,
568 Olivares, F.L., Ladha, J.K. (2002) Infection and colonization of rice seedlings by the
569 plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Molecular Plant-
570 Microbe Interactions, **15**(9), 894-906.
571 James, E.K., Iannetta, P.P.M., Kenicer, G., Sprent, J.I., Squire, G.R. (2011) Nodulation of
572 *Lathyrus* and *Vicia* spp. in non-agricultural soils in East Scotland. Aspects of Applied
573 Biology **109**, 119–124.
574 Johnston, G. (2012) *Botany of the Eastern Borders* Rarebooksclub Com.(JNCC) Joint Nature
575 Conservation Committee (2009) Description of NVC types & floristic tables.
576 Available online: http://jncc.defra.gov.uk/page-4265 [Accessed 28th March 2018].
577
578 Justice, O.L. and Marks, R.W. (1943) Germination of unscarified and scarified seed of
579 *Lathyrus hirsutus* L., under laboratory, greenhouse and field conditions. In

580 Proceedings of the Association of Official Seed Analysts, JSTOR: pp 104–115.
581 Kenicer, G.J., Kajita, T., Pennington, R.T., Murata, J. (2005) Systematics and biogeography of
582 *Lathyrus* (Leguminosae) based on internal transcribed spacer and cpDNA sequence
583 data. American Journal of Botany, **92**(7), 1199–1209.
584 Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: Molecular evolutionary genetics analysis
585 version 7.0 for bigger datasets. Molecular Biology and Evolution, **33**(7), 1870–1874.
586 Lane, D.J. (1991) 16S/23S rRNA sequencing, p. 115-175. In, Nucleic acid techniques in
587 bacterial systematics, E. Stackebrandt and M. Goodfellow (ed.), John Wiley & Sons,
588 New York.
589 Lewis, G., Schrire, B., MacKinder, B., Lock, M. (2005) *Legumes of the world*. Royal
590 Botanical Gardens. Kew, UK.
591 Lightfoot, J. (1777) *Flora Scotica: or, a systematic arrangement, in the Linnaean method, of*
592 *the native plants of Scotland and the Hebrides* White.
593 Moffat, B., Francia, S., Stobart, A. (2014) Archaeological Sources for the History of Herbal
594 Medicine Practice: The case study of St John's wort with valerian at Soutra medieval
595 hospital. Critical Approaches to the History of Western Herbal Medicine: From
596 Classical Antiquity to the Early Modern Period. Bloomsbury, London, UK, 253–270.
597 Mutch, L.A. and Young, J.P. (2004) Diversity and specificity of *Rhizobium leguminosarum*
598 biovar viciae on wild and cultivated legumes. Molecular Ecology, **13**(8), 2435–2444.
599 National Biodiversity Network (2013). Grid map for Lathyrus linifolius (Reichard) Bässler
600 [Bitter-vetch]. [image online] Available on line:
601 https://data.nbn.org.uk/Taxa/NHMSYS0000460181/Grid_Map [Accessed 28th March
602 2018].
603 Nilsson, S.G., Franzén, M., Jönsson, E. (2008) Long term landuse changes and extinction of
604 specialised butterflies. Insect Conservation and Diversity, **1**(4), 197–207.

605 Pennant, T. (2014) *A tour in Scotland, and voyage to the hebrides, 1772* Cambridge
606 University Press.
607 Pratt, A. and Step, E. (1899) *Flowering plants, grasses, sedges & ferns of Great Britain*
608 Warne.
609 Röhrig, H., Schmidt, J., Wieneke, U., Kondorosi, E., Barlier, I., Schell, J., John, M. (1994).
610 Biosynthesis of lipooligosaccharide nodulation factors: Rhizobium NodA protein is
611 involved in N-acylation of the chitooligosaccharide backbone. Proceedings of the
612 National Academy of Sciences, **91**(8), 3122-3126.
613 Rose, H. (1999) Wytham Wood. A deer management project. Deer, Journal of the British Deer
614 Society, **11**(3), 119-123.
615 Rose, F. and O'Reilly, C. (2006) *The wild flower key: how to identify wild flowers, trees and*
616 *shrubs in Britain and Ireland* Frederick Warne Books.
617 van Rhijn, P., Vanderleyden, J. (1995). The Rhizobium-plant symbiosis. Microbiological
618 reviews, **59**(1), 124-142.
619 Schaefer, H., Hechenleitner, P., Santos-Guerra, A., de Sequeira, M.M., Pennington, R.T.,
620 Kenicer, G., Carine, M.A. (2012) Systematics, biogeography, and character evolution
621 of the legume tribe Fabeae with special focus on the middle-Atlantic island lineages.
622 BMC Evolutionary Biology, **12**(1), 250.
623 Schlindwein, G., Vargas, L.K., Lisboa, B.B., Azambuja, A.C., Granada, C.E., Gabiatti, N.C.,
624 Prates, F., Stumpf, A. (2008) Influence of rhizobial inoculation on seedling vigor and
625 germination of lettuce. Ciência Rural, **38**(3).
626 Shcherbakova, E.N., Shcherbakov, A.V., Andronov, E.E., Gonchar, L.N., Kalenskaya, S.M.,
627 Chebotar, V.K. (2017) Combined pre-seed treatment with microbial inoculants and Mo
628 nanoparticles changes composition of root exudates and rhizosphere microbiome
629 structure of chickpea (*Cicer arietinum* L.) plants. Symbiosis, **73**(1), 57–69.

630 Sorty, A.M., Meena, K.K., Choudhary, K., Bitla, U.M., Minhas, P.S., Krishnani, K.K. (2016)
631 Effect of plant growth promoting bacteria associated with halophytic weed (*Psoralea*
632 *corylifolia* L) on germination and seedling growth of wheat under saline conditions.
633 Applied biochemistry and biotechnology, **180**(5), 872-882.
634 Sprent, J.I., Ardley, J., James, E.K. (2017) Biogeography of nodulated legumes and their
635 nitrogen-fixing symbionts. New Phytologist, **215**(1), 40-56.
636 Tamura, K. and Nei, M. (1993) Estimation of the number of nucleotide substitutions in the
637 control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology
638 and Evolution, **10**, 512–526.
639 Team, R. (2015) RStudio: Integrated development for R, RStudio, Inc., Boston, MA, , Boston.
640 Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., Alves, B.,
641 Chalk, P. (2008) *Measuring plant-associated nitrogen fixation in agricultural systems*
642 Australian Centre for International Agricultural Research
643 Vickery, R. (1995) *A dictionary of plant lore* Oxford University Press.
644 Villadas, P.J., Lasa, A.V., Martinez-Hidalgo, P., Flores-Felix, J.D., Martinez-Molina, E., Toro,
645 N., Velazquez, E., Fernandez-Lopez, M. (2017) Analysis of rhizobial endosymbionts
646 of *Vicia*, *Lathyrus* and *Trifolium* species used to maintain mountain firewalls in Sierra
647 Nevada National Park (South Spain). Systematic and Applied Microbiology, **40**(2),
648 92–101.
649 Vincent, J.M. (1970). A manual for the practical study of root-nodule bacteria., International
650 Biological Programme (IBP) Handbook No. 15. Blackwell Scientific Publications,
651 Oxford, UK.
652 Walmsley, C.A. and Davy, A.J. (1997) Germination characteristics of shingle beach species,
653 effects of seed ageing and their implications for vegetation restoration. Journal of
654 Applied Ecology, **34**(1), 131–142.

655 Woods, N., Gebril, A., Mitchell, A., Iannetta, P. P. M., Kenicer, G., Tate, R. J., Pickard, A.I.,
656 Gray, A.I., Ferro, V. A. (2012) Brain changing tubers: gene expression changes
657 following mediaeval tuber consumption. Neuroscience, **15**, 1343-1349.
658 Young, J.P.W., Crossman, L.C., Johnston, A.W., Thomson, N.R., Ghazoui, Z.F., Hull, K.H.,
659 Wexler, M., Curson, A.R.J., Todd, J.D., Poole, P.S., Mauchline, T.H., East, A.K.,
660 Quail, M.A., Churcher, C., Arrowsmith, C., Cherevach, I., Chillingworth, T., Clarke,
661 K., Cronin, A., Davis, P., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S.,
662 Mungall, K., Norbertczak, H., Rabbinowitsch, E., Sanders, M., Simmonds, M.,
663 Whitehead, S., Parkhill, J. (2006) The genome of *Rhizobium leguminosarum* has
664 recognizable core and accessory components. Genome biology, **7**(4), R34.Zézé, A.,
665 Mutch, L.A., Young, J.P.W. (2001). Direct amplification of nodD from community
666 DNA reveals the genetic diversity of *Rhizobium leguminosarum* in soil.
667 Environmental Microbiology, **3**(6), 363-370.
668

669 **Table 1.** Characterisation of the heteromorphic seeds of *Lathyrus linifolius* L.. Results present
670 average data (± SE) on the basis of their relative: proportion of total seed complement (%),
671 fresh and dry weight (mg seed^{-1}) and moisture content (%) of fresh weight after equilibration
672 at 5 ^{o}C and 15 % relative humidity. Physical qualities of each seed type are presented as
673 length and width across seed major and minor axis, respectively (mm), circularity coefficient
674 (where, 1 = perfect circle, 0 elongated shape), surface area (SA; mm^2), volume (V; mm^3) and
675 SA : V ratio. Germination for each morph type is presented for scarified and non-scarified
676 seeds (%). Germination and germination rate (t_{50}) are presented for the optimal germination
677 temperature for each morph type. Seed carbon (C) and nitrogen (N) content are expressed in
678 proportional and absolute terms. *P value*, denotes values for statistical significance test.

Parameter			**Seed heteromorph (colour type)**		***P value***
			Green	**Brown**	
Proportion of batch (%)			31 ± 2.89	69 ± 2.89	<0.001
Fresh weight (mg seed^{-1})			16.10 ± 0.21	19.62 ± 0.77	0.012
Dry weight (mg seed^{-1})			14.87 ± 0.49	18.28 ± 1.12	0.050
Moisture content (%)			8.45 ± 2.35	7.59 ± 2.51	0.815
Physical qualities (fresh seeds)	**Length (mm)**		2.66 ± 0.03	2.72 ± 0.02	0.017
	Width (mm)		2.44 ± 0.04	2.49 ± 0.04	0.042
	Area (mm^2)		5.14 ± 0.13	5.33 ± 0.12	0.013
	Cirularity		0.896 ± 0.002	0.891 ± 0.001	0.003
	SA (mm^2)		13.13 ± 0.03	13.17 ± 0.03	0.018
	V (mm^3)		8.46 ± 0.34	8.93 ± 0.31	0.017
	SA : V ratio		1.62 ± 0.07	1.53 ± 0.06	0.040
Colour discrimination	**Red**	**Integrated density**	583 ± 14	523 ± 12	<0.001
	Green		453 ± 17	352 ± 15	
	Blue		207 ± 20	178 ± 20	
	Red	**Avg mm^2**	113 ± 2	97 ± 2	
	Green		88 ± 3	66 ± 3	
	Blue		40 ± 4	33 ± 4	
Germination (%)	**Not scarified seeds**		81.6 ± 7.4	70.0 ± 5.8	<0.001
	Scarified seeds		91.6 ± 1.67	86.7 ± 4.7	
Germination rate (t_{50}, d)	**Not scarified seeds**		22.8 ± 1.8	14.6 ± 0.69	
	Scarified seeds		15.8 ± 1.8	9.37 ± 0.94	
Carbon and nitrogen	**% Carbon**		45.09 ± 0.61	45.04 ± 0.43	0.948
	Carbon (mg seed^{-1})		6.70 ± 0.26	8.24 ± 0.57	0.0717
	% Nitrogen		4.16 ± 0.50	4.26 ± 1.10	0.936
	Nitrogen (mg seed^{-1})		0.61 ± 0.06	0.77 ± 0.16	0.463
	C:N Ratio		11.24 ± 1.64	12.85 ± 4.46	0.752

679 **Figure Legends**

680

681 **Figure 1.** The heteromorphic seeds of *Lathyrus linifolius* L. (bitter vetch) can be
682 discriminated on the basis of their different colours, as either brown or green. They are shown
683 here partitioned on this basis to either the right-, or left-hand sides of the image, respectively.
684 Scale bar small divisions = mm.

685

686 **Figure 2.** The response to seed incubation temperature of non-scarified, green (○) and brown
687 (■) seed morphotypes showing: **A**) the percentage of total non-scarified seeds germinated and
688 **B)** seed germination rate ($1/t_{50}$). Vertical bars show SE of the means of 3 replicates. Solid and
689 dashed lines show curves of models fitted to describe data for the green- and brown coloured
690 seed morphs, respectively, where: A) shows lines fitted using generalised linear modelling;
691 and B), shows the sigmoidal models.

692

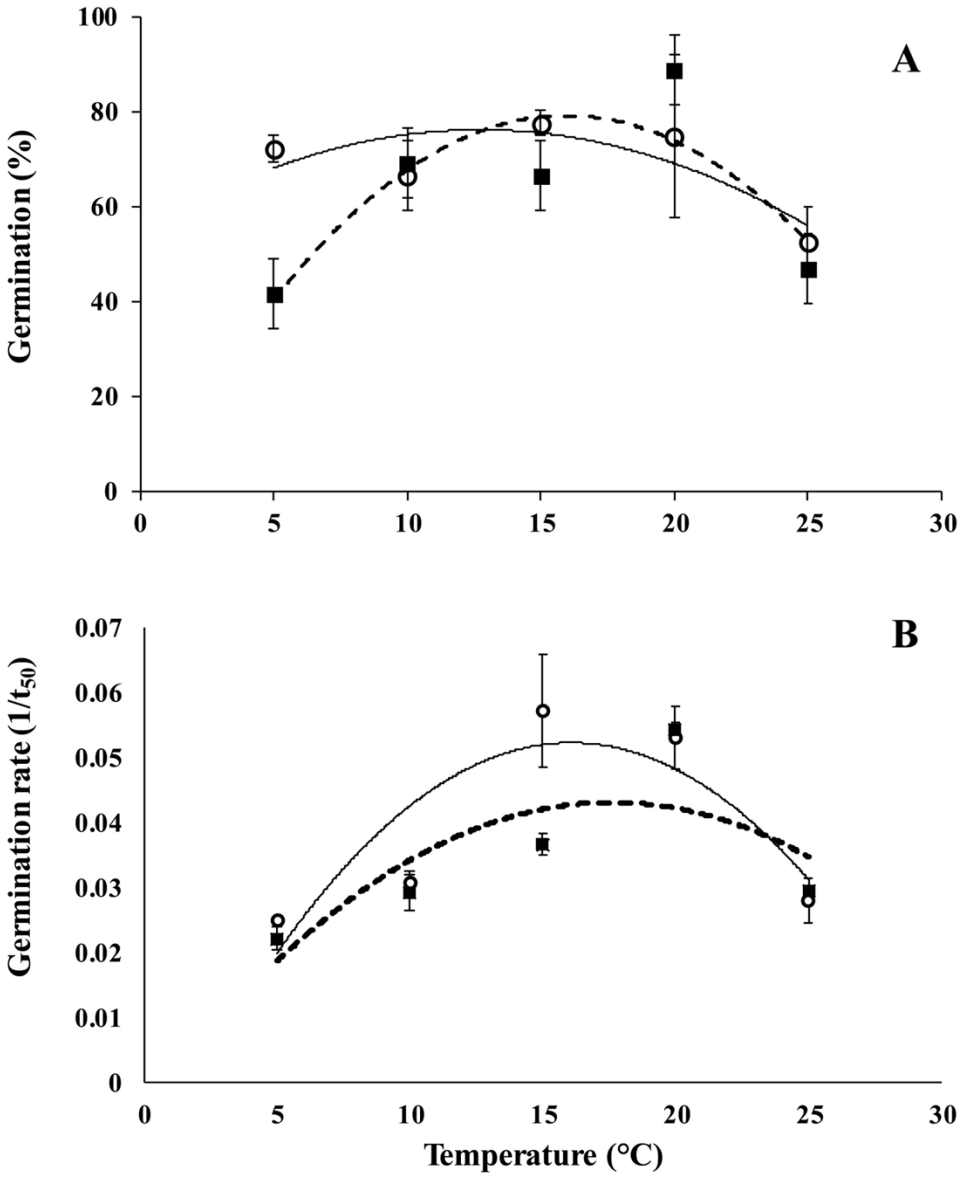
693 **Figure3.** Micrographs of *Lathyrus linifolius L.* nodule sections, showing: **A**) light-
694 micrograph of a mature nodule in longitudinal profile showing all stages of development from
695 the meristem (m) and the invasion zone (iz) through to the N-fixing (*) and the senescent
696 zones (s). Bar = 200 μm; **B**) higher magnification light micrograph showing a view of the
697 meristem (m), invasion (iz) and, N-fixing zones (*). Bar = 100 μm; **C**) rhizobial infected cells
698 (*) in the N-fixing zone; these are packed with bacteroids labelled b in panel D). The infected
699 cells are interspersed with uninfected interstitial cells which are full of amyloplasts/starch
700 grains (s). Bar = 20 μm; **D**) transmission electron micrograph of bacteroids (b) in an infected
701 cell; the section has been immunogold labelled with an antibody against the Fe-(nifH) protein
702 of nitrogenase (arrows). Bar = 1 μm.

703

Figure 4. Molecular phylogenetic analysis using the maximum likelihood (ML) method of *Lathyrus linifolius* root nodule isolates for the *nodA* gene (442 positions in the final dataset). The sequences shown are for representative isolates only (not duplicates), believed to be potentially nodulating on the basis of their probable species identity discerned by 16S rRNA BLAST results. Only bootstrap values >50 % (1000 bootstrap replicates) are shown in the tree. The type strains are shown by a "T" at the end of each strain code. The tree is rooted with *Azorhizobium caulinodans* ORS 571^{T}. The tree is drawn to scale, with branch lengths measured in the number of substitutions *per* site. All positions containing gaps and missing data were eliminated.

Figure 5. Molecular phylogenetic analysis using the Maximum Likelihood (ML) method of *Lathyrus linifolius* root nodule isolates for**: A**) 16S rRNA (255 positions in the final dataset); and, **B)** *nodD* genes (512 positions in the final dataset). Only bootstrap values >50 % (1000 bootstrap replicates) are shown in the tree. The type strains are shown by a "T" at the end of each strain code. The tree was rooted with *Azorhizobium caulinodans* ORS 571^{T}. The trees are drawn to scale, with branch lengths measured in the number of substitutions *per* site. All positions containing gaps and missing data were eliminated.

Figure 2



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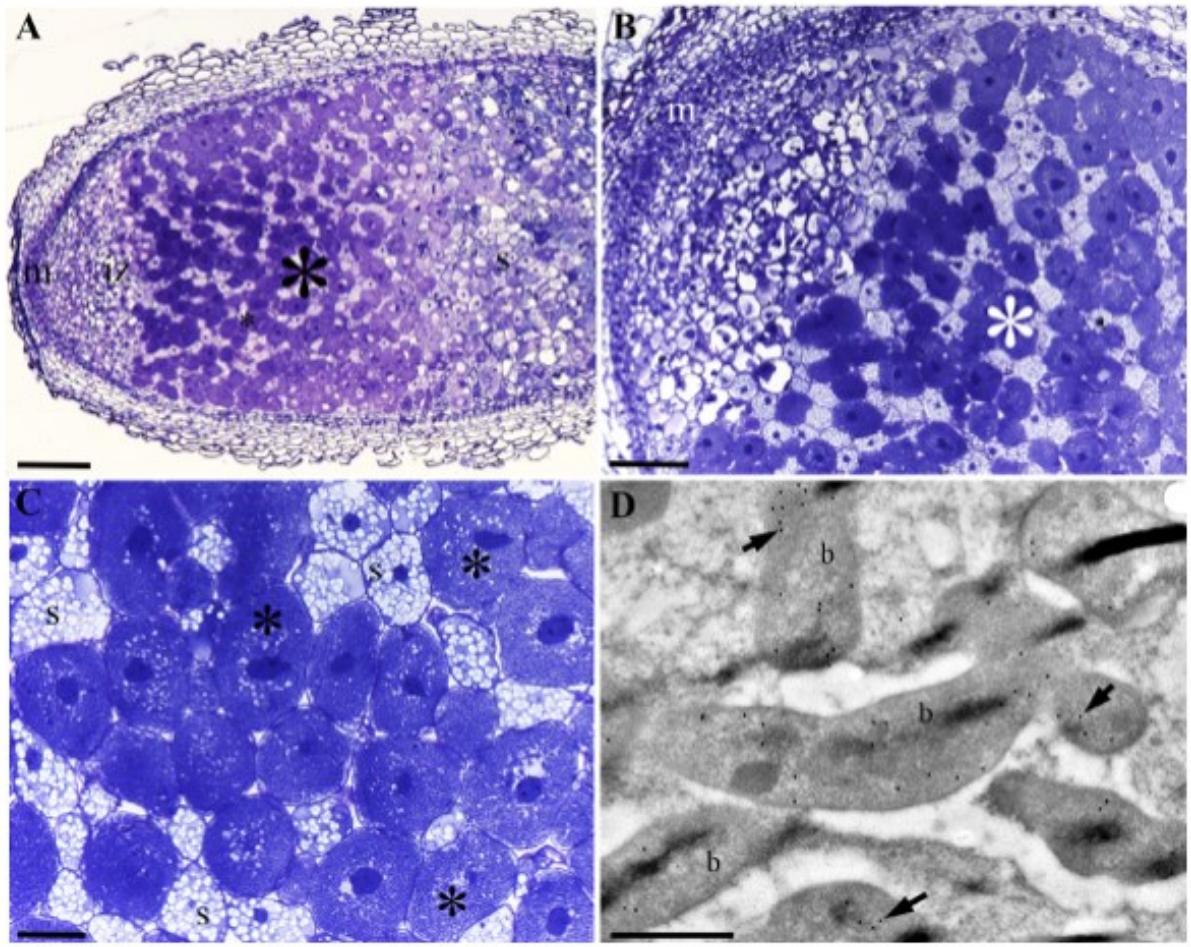

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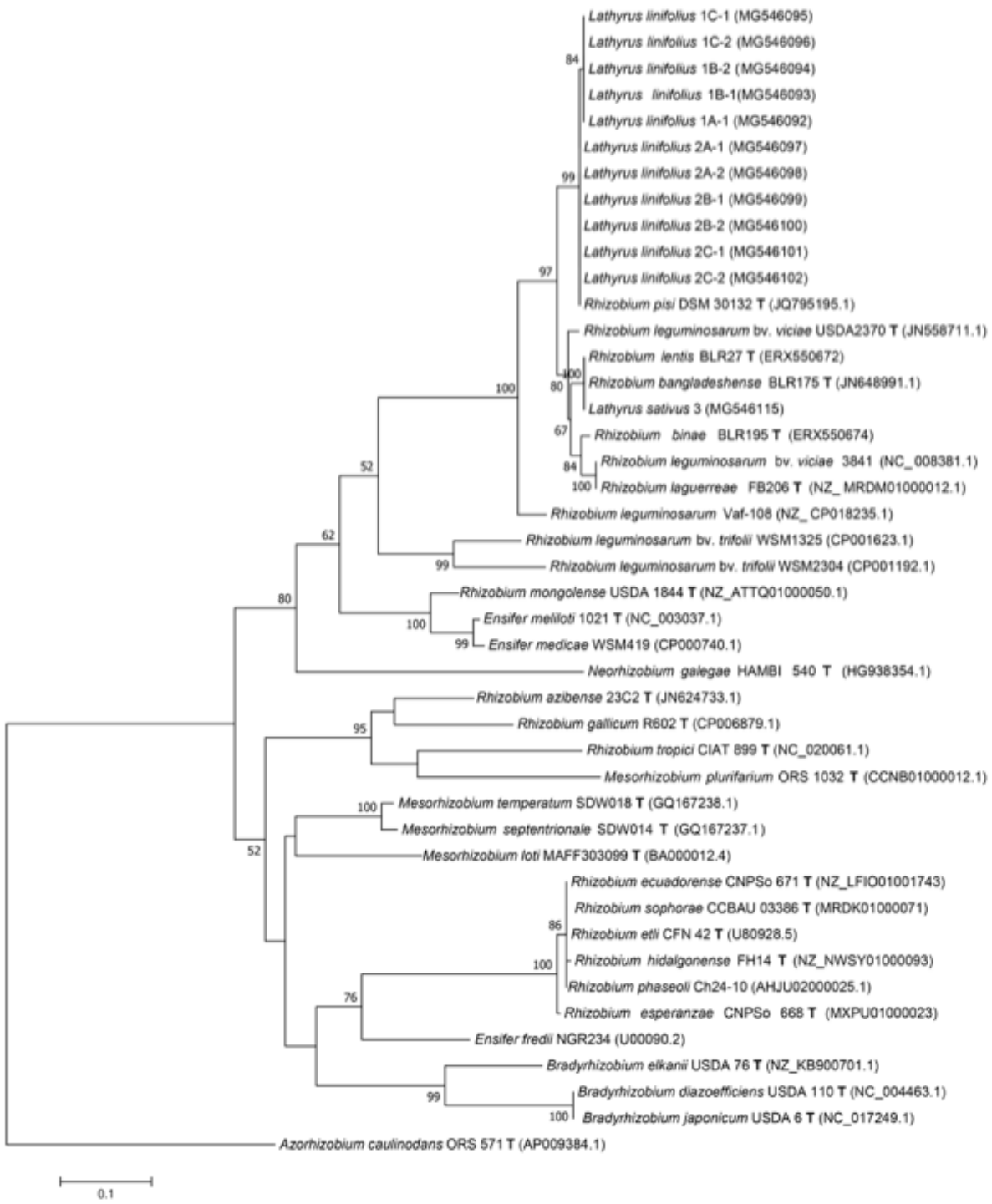
Lathyrus linifolius 1C-1 (MG546095)
Lathyrus linifolius 1C-2 (MG546096)
Lathyrus linifolius 1B-2 (MG546094)
Lathyrus linifolius 1B-1(MG546093)
Lathyrus linifolius 1A-1 (MG546092)
Lathyrus linifolius 2A-1 (MG546097)
Lathyrus linifolius 2A-2 (MG546098)
Lathyrus linifolius 2B-1 (MG546099)
Lathyrus linifolius 2B-2 (MG546100)
Lathyrus linifolius 2C-1 (MG546101)
Lathyrus linifolius 2C-2 (MG546102)
Rhizobium pisi DSM 30132 T (JQ795195.1)
Rhizobium leguminosarum bv. viciae USDA2370 T (JN558711.1)
Rhizobium lentis BLR27 T (ERX550672)
Rhizobium bangladeshense BLR175 T (JN648991.1)
Lathyrus sativus 3 (MG546115)
Rhizobium binae BLR195 T (ERX550674)
Rhizobium leguminosarum bv. viciae 3841 (NC_008381.1)
Rhizobium laguerreae FB206 T (NZ_ MRDM01000012.1)
Rhizobium leguminosarum Vaf-108 (NZ_ CP018235.1)
Rhizobium leguminosarum bv. trifolii WSM1325 (CP001623.1)
Rhizobium leguminosarum bv. trifolii WSM2304 (CP001192.1)
Rhizobium mongolense USDA 1844 T (NZ_ATTQ01000050.1)
Ensifer meliloti 1021 T (NC_003037.1)
Ensifer medicae WSM419 (CP000740.1)
Neorhizobium galegae HAMBI 540 T (HG938354.1)
Rhizobium azibense 23C2 T (JN624733.1)
Rhizobium gallicum R602 T (CP006879.1)
Rhizobium tropici CIAT 899 T (NC_020061.1)
Mesorhizobium plurifarium ORS 1032 T (CCNB01000012.1)
Mesorhizobium temperatum SDW018 T (GQ167238.1)
Mesorhizobium septentrionale SDW014 T (GQ167237.1)
Mesorhizobium loti MAFF303099 T (BA000012.4)
Rhizobium ecuadorense CNPSo 671 T (NZ_LFIO01001743)
Rhizobium sophorae CCBAU 03386 T (MRDK01000071)
Rhizobium etli CFN 42 T (U80928.5)
Rhizobium hidalgonense FH14 T (NZ_NWSY01000093)
Rhizobium phaseoli Ch24-10 (AHJU02000025.1)
Rhizobium esperanzae CNPSo 668 T (MXPU01000023)
Ensifer fredii NGR234 (U00090.2)
Bradyrhizobium elkanii USDA 76 T (NZ_KB900701.1)
Bradyrhizobium diazoefficiens USDA 110 T (NC_004463.1)
Bradyrhizobium japonicum USDA 6 T (NC_017249.1)
Azorhizobium caulinodans ORS 571 T (AP009384.1)
84
99
97
100
80
100
67
84
100
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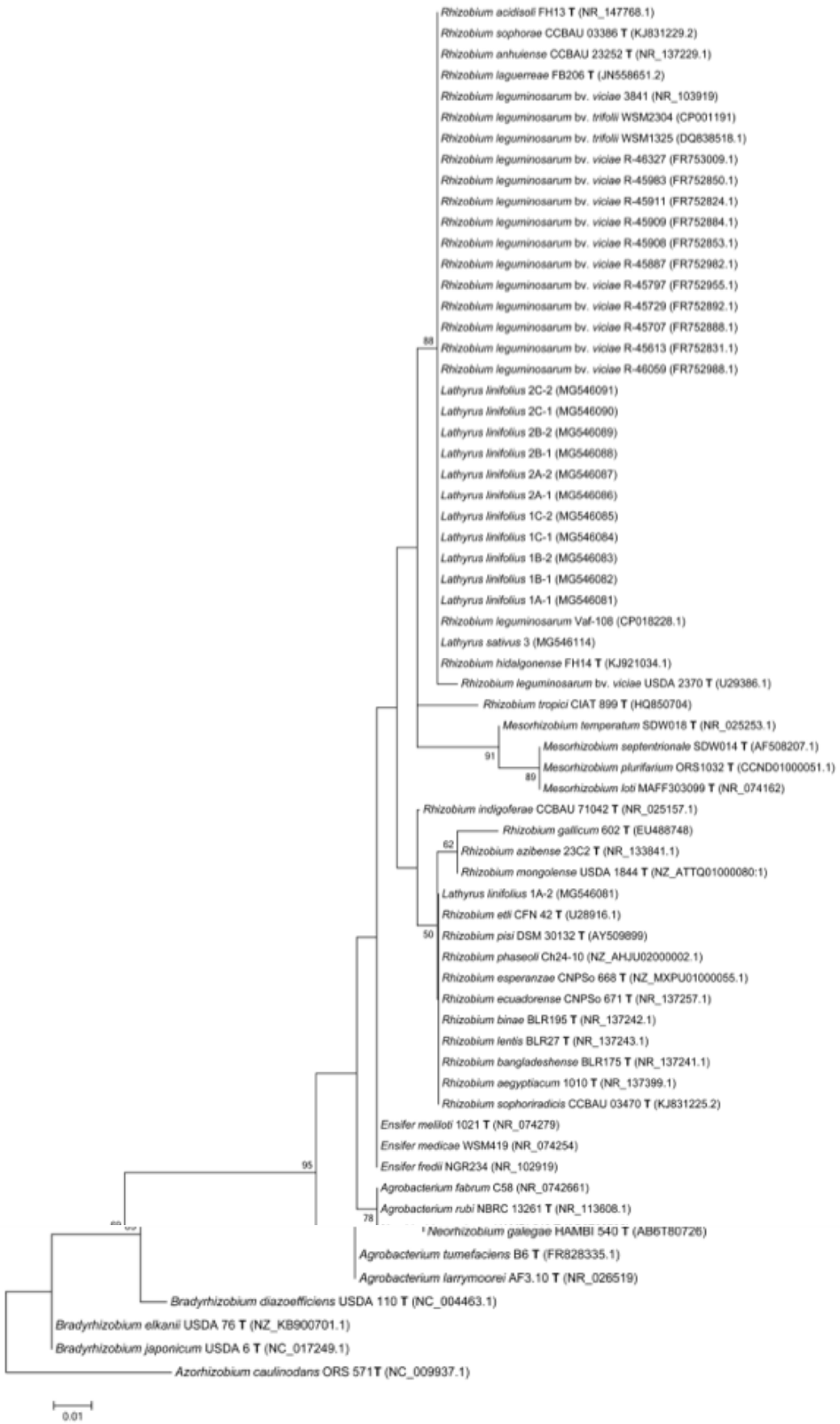
99
80
100
99
95
100
52
86
100
76
99
100
0.1

728
729


Rhizobium acidisoli FH13 T (NR_147768.1)
Rhizobium sophorae CCBAU 03386 T (KJ831229.2)
Rhizobium anhuiense CCBAU 23252 T (NR_137229.1)
Rhizobium laguerreae FB206 T (JN558651.2)
Rhizobium leguminosarum bv. viciae 3841 (NR_103919)
Rhizobium leguminosarum bv. trifolii WSM2304 (CP001191)
Rhizobium leguminosarum bv. trifolii WSM1325 (DQ838518.1)
Rhizobium leguminosarum bv. viciae R-46327 (FR753009.1)
Rhizobium leguminosarum bv. viciae R-45983 (FR752850.1)
Rhizobium leguminosarum bv. viciae R-45911 (FR752824.1)
Rhizobium leguminosarum bv. viciae R-45909 (FR752884.1)
Rhizobium leguminosarum bv. viciae R-45908 (FR752853.1)
Rhizobium leguminosarum bv. viciae R-45887 (FR752982.1)
Rhizobium leguminosarum bv. viciae R-45797 (FR752955.1)
Rhizobium leguminosarum bv. viciae R-45729 (FR752892.1)
Rhizobium leguminosarum bv. viciae R-45707 (FR752888.1)
Rhizobium leguminosarum bv. viciae R-45613 (FR752831.1)
Rhizobium leguminosarum bv. viciae R-46059 (FR752988.1)
Lathyrus linifolius 2C-2 (MG546091)
Lathyrus linifolius 2C-1 (MG546090)
Lathyrus linifolius 2B-2 (MG546089)
Lathyrus linifolius 2B-1 (MG546088)
Lathyrus linifolius 2A-2 (MG546087)
Lathyrus linifolius 2A-1 (MG546086)
Lathyrus linifolius 1C-2 (MG546085)
Lathyrus linifolius 1C-1 (MG546084)
Lathyrus linifolius 1B-2 (MG546083)
Lathyrus linifolius 1B-1 (MG546082)
Lathyrus linifolius 1A-1 (MG546081)
Rhizobium leguminosarum Vaf-108 (CP018228.1)
Lathyrus sativus 3 (MG546114)
Rhizobium hidalgonense FH14 T (KJ921034.1)
Rhizobium leguminosarum bv. viciae USDA 2370 T (U29386.1)
Rhizobium tropici CIAT 899 T (HQ850704)
Mesorhizobium temperatum SDW018 T (NR_025253.1)
Mesorhizobium septentrionale SDW014 T (AF508207.1)
Mesorhizobium plurifarium ORS1032 T (CCND01000051.1)
Mesorhizobium loti MAFF303099 T (NR_074162)
Rhizobium indigoferae CCBAU 71042 T (NR_025157.1)
Rhizobium gallicum 602 T (EU488748)
Rhizobium azibense 23C2 T (NR_133841.1)
Rhizobium mongolense USDA 1844 T (NZ_ATTQ01000080:1)
Lathyrus linifolius 1A-2 (MG546081)
Rhizobium etli CFN 42 T (U28916.1)
Rhizobium pisi DSM 30132 T (AY509899)
Rhizobium phaseoli Ch24-10 (NZ_AHJU02000002.1)
Rhizobium esperanzae CNPSo 668 T (NZ_MXPU01000055.1)
Rhizobium ecuadorense CNPSo 671 T (NR_137257.1)
Rhizobium binae BLR195 T (NR_137242.1)
Rhizobium lentis BLR27 T (NR_137243.1)
Rhizobium bangladeshense BLR175 T (NR_137241.1)
Rhizobium aegyptiacum 1010 T (NR_137399.1)
Rhizobium sophoriradicis CCBAU 03470 T (KJ831225.2)
Ensifer meliloti 1021 T (NR_074279)
Ensifer medicae WSM419 (NR_074254)
Ensifer fredii NGR234 (NR_102919)
Agrobacterium fabrum C58 (NR_0742661)
Agrobacterium rubi NBRC 13261 T (NR_113608.1)
Neorhizobium galegae HAMBI 540 T (AB6T80726)
Agrobacterium tumefaciens B6 T (FR828335.1)
Agrobacterium larrymoorei AF3.10 T (NR_026519)
Bradyrhizobium diazoefficiens USDA 110 T (NC_004463.1)
Bradyrhizobium elkanii USDA 76 T (NZ_KB900701.1)
Bradyrhizobium japonicum USDA 6 T (NC_017249.1)
Azorhizobium caulinodans ORS 571T (NC_009937.1)
88
91
89
62
50
95
78
0.01

730

731
732

733