

## Phylogenetic diversity of *Mesorhizobium* in chickpea

DONG HYUN KIM<sup>1</sup>, MAYANK KAASHYAP<sup>1</sup>, ABHISHEK RATHORE<sup>1</sup>, ROMA R DAS<sup>1</sup>, SWATHI PARUPALLI<sup>1</sup>, HARI D UPADHYAYA<sup>1</sup>, S GOPALAKRISHNAN<sup>1</sup>, POORAN M GAUR<sup>1</sup>, SARVJEET SINGH<sup>2</sup>, JAGMEET KAUR<sup>2</sup>, MOHAMMAD YASIN<sup>3</sup> and RAJEEV K VARSHNEY<sup>1,4,\*</sup>

<sup>1</sup>International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, India

<sup>2</sup>Punjab Agricultural University, Ludhiana 141 027, India

<sup>3</sup>RAK College of Agriculture, Sehore 466 001, India

<sup>4</sup>CGIAR Generation Challenge Program, C/o CIMMYT, Mexico, DF 06600, Mexico

\*Corresponding author (Fax, 91-40-30713074; Email, [r.k.varshney@cgiar.org](mailto:r.k.varshney@cgiar.org))

Crop domestication, in general, has reduced genetic diversity in cultivated gene pool of chickpea (*Cicer arietinum*) as compared with wild species (*C. reticulatum*, *C. bijugum*). To explore impact of domestication on symbiosis, 10 accessions of chickpeas, including 4 accessions of *C. arietinum*, and 3 accessions of each of *C. reticulatum* and *C. bijugum* species, were selected and DNAs were extracted from their nodules. To distinguish chickpea symbiont, preliminary sequences analysis was attempted with 9 genes (16S rRNA, *atpD*, *dnaJ*, *glnA*, *gyrB*, *nifH*, *nifK*, *nodD* and *recA*) of which 3 genes (*gyrB*, *nifK* and *nodD*) were selected based on sufficient sequence diversity for further phylogenetic analysis. Phylogenetic analysis and sequence diversity for 3 genes demonstrated that sequences from *C. reticulatum* were more diverse. Nodule occupancy by dominant symbiont also indicated that *C. reticulatum* (60%) could have more various symbionts than cultivated chickpea (80%). The study demonstrated that wild chickpeas (*C. reticulatum*) could be used for selecting more diverse symbionts in the field conditions and it implies that chickpea domestication affected symbiosis negatively in addition to reducing genetic diversity.

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### 1. Introduction

Crop domestication, a process of selection of the sustainable plants with desirable features such as high productivity, short duration, reduced seed dispersal/dormancy and branching, uniform flowering/fruitletting and seed maturation, etc., happened several thousand years ago (Doebley *et al.* 2006; Lenser and Theissen 2013; Olsen and Wendel 2013). During this process, many genes responsible for the desirable traits and agriculturally favourable alleles were eliminated. So the resultant major upshot of domestication is the reduction of crop genetic diversity relative to their wild progenitors (Doebley *et al.* 2006; Olsen and Wendel 2013). Chickpea (*Cicer arietinum* L.), like other domesticated crops, has less genetic diversity in cultivars in comparison

to their wild relatives (Agarwal *et al.* 2012; Varshney *et al.* 2013). In recent years, efforts have been initiated towards regaining the lost genes/alleles by introgressing them from crop wild relatives (CWRs) in elite varieties of plant species (McCouch *et al.* 2013). Some plant species where some success have been achieved include tomato (Fridman *et al.* 2004), barley (Schmalenbach *et al.* 2009), chickpea and other crops (Hajjar and Hodgkin 2007; Thompson *et al.* 2011).

Rhizobia are known to fix atmospheric nitrogen through their symbiotic association with legumes and each species has different host range for plants with various metabolic capabilities (Margaret *et al.* 2011; Wielbo *et al.* 2010). Although there are many parameters postulated for affecting the symbiotic efficiency of rhizobia under field environments, two key factors

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were found to be important in chickpea symbiosis: (i) nodule occupancy, which illustrates the survival competitiveness among the bacterial community (Ben Romdhane *et al.* 2008; Ben Romdhane *et al.* 2007) and (ii) effectiveness of nitrogen fixation, which intensifies the plant growth and yield (Ben Romdhane *et al.* 2008). However, there are many rhizobia partnered with legume plants that were found to be ineffective nitrogen fixers, and one such partnership was revealed by Sachs *et al.* (2010) on *Lotus strigosus* with *Bradyrhizobium* sp. Crop domestication also contributes to this phenomenon and this was supported by Kiers *et al.* (2007), who found effective symbiosis with mixture of rhizobial strains by older cultivars of soybean than their modern elite cultivars obtained by 60 years of breeding. This suggests that the elite cultivars bred intensively for high fertility conditions have lost their ability to select efficient rhizobial strains under low fertile field conditions.

Sequence analysis of 16S ribosomal RNA (rRNA) has emerged as one of the most important methods in taxonomy and phylogenetic analysis of bacteria (Weisburg *et al.* 1991). However, due to the high sequence conservation, 16S rRNA sequence is insufficient to distinguish the strains of one species or even closely related species (Laranjo *et al.* 2012). So, the use of additional diverse genes apart from 16S rRNA for narrowing the phylogenetic analysis is a prerequisite. Recently, housekeeping genes, located on the bacterial chromosome, have been used in combination with 16S rRNA for elucidation of taxonomic relationships (Martens *et al.* 2007; Laranjo *et al.* 2012). In *Mesorhizobium* spp., symbiotic genes, present in their chromosomes, have also been characterized alongside housekeeping genes to understand phylogenetic relationships (Laranjo *et al.* 2008; Chen *et al.* 2010).

In view of above, the present study was undertaken to examine the effects of chickpea domestication on symbiosis by comparison of phylogenetic differences among the rhizobial strains of cultivated and wild chickpea.

## 2. Materials and methods

### 2.1 Plant materials and sample preparation

A total of 10 accessions including 4 from cultivated species (*C. arietinum*) and 6 from wild species (*C. reticulatum* and

*C. bijugum*) were included in the study (table 1). The experiment was conducted under field conditions (17°30' N; 78°16' E; altitude 549 m) in vertisols during the post-rainy season of 2010–2011 (sowing date: 15-11-2010) at ICRISAT, Patancheru, India. The field location of chickpea changes every season in ICRISAT and experimental field was solarized for 6 months before starting the experiment. The field was designed as a randomized split plot with three replications for each accession. Nine plants of each accession and five nodules from each plant were randomly selected for DNA extraction with MACHEREY-NAGEL Plant II kits (MACHEREY-NAGEL, Germany) as per the manufacturer's protocol. A total of 143 DNA samples were selected based on their quality by 1% agarose gel for PCR and sequencing.

### 2.2 PCR amplification and sequencing

For sequence analysis, following primer pairs were designed for selected genes of *Mesorhizobium ciceri* for PCR amplification: *gyrB* (Forward: GAGCTTGCCTTCCTSAATTC; Reverse: TGCGAGGAGAATTTTCGGRTC), *nodD* (Forward: CTCGTCGCGCTCGACGCATTGA; Reverse: TGCCCCATGGACATGTA), *nifH* (Forward: GTCTCCTATGACGTGCTCGG; Reverse: GCTTCCATGGTGATCGGGGT), *nifK* (Forward: TTCGAGCGAACCATGTCCCTT; Reverse: CAGTGTCTTTCGGGTGTTGT).

PCR was performed at the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s and final extension at 72°C for 10 min. The amplicons were sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems Inc., USA) at MacroGen Inc., Seoul, Korea. Although we sampled similar number of nodules from each cultivated and wild species, we dropped few samples from the analysis because of elution and poor sequence quality. The sequences in this study have been deposited in the GenBank database as *gyrB* (KF710045–KF710174), *nifK* (KF710175–KF710317) and *nodD* (KF710318–710460).

**Table 1.** List of 10 accessions of cultivated and wild chickpeas analysed

Cultivated chickpeas		Wild chickpeas			
Accession name	Species	Accession name	Species	Accession name	Species
ICC 4958	<i>C. arietinum</i>	ICCW 6	<i>C. reticulatum</i>	ICC 17290	<i>C. bijugum</i>
ICC 1710	<i>C. arietinum</i>	ICCW 8	<i>C. reticulatum</i>	ICC 17296	<i>C. bijugum</i>
ICC 9755	<i>C. arietinum</i>	ICCW 9	<i>C. reticulatum</i>	ICC 17304	<i>C. bijugum</i>
ICC 8350	<i>C. arietinum</i>				

### 2.3 Sequence and statistical analysis

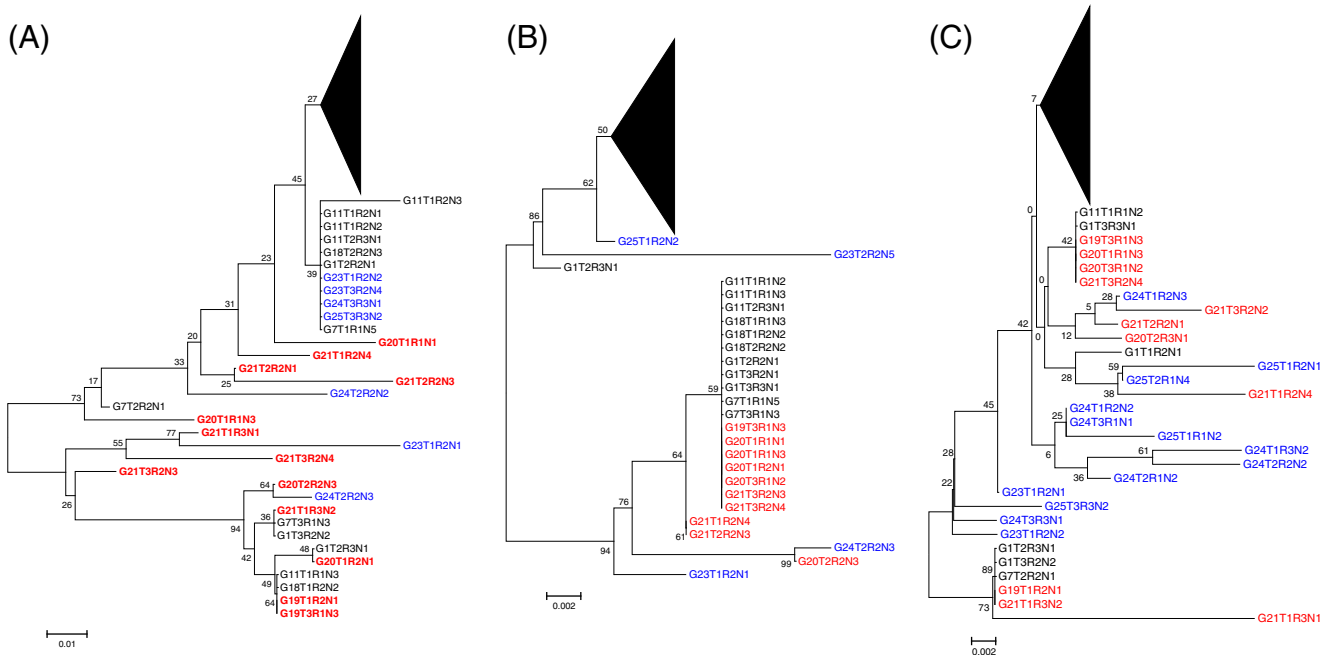
Sequences of 16S rRNA, five housekeeping genes (*atpD*, *dnaJ*, *glnA*, *gyrB* and *recA*) and three symbiosis genes (*nifH*, *nifK* and *nodD*) of *Mesorhizobium* species were downloaded from NCBI Genbank for preliminary sequence analysis. BLASTN was used for comparison of sequences among *Mesorhizobium* species. Three sequences (*gyrB*, *nifK* and *nodD*) were selected for further analysis based on sequence similarity and aligned with Clustal 2 (<http://www.clustal.org/clustal2>) using default parameters. Phylogenetic trees were constructed by MEGA5 program using neighbour-joining and maximum-likelihood methods with 1000 replicates in the bootstrap test (Tamura *et al.* 2011). Sequence diversity was measured according to plant species from which DNAs were extracted. The numbers of base differences per sequence from mean diversity calculations for each genotype are referred as sequence diversity. Standard error was obtained by a bootstrap procedure (1000 replicates).

### 3. Results

Preliminary *in silico* sequence analysis based on 9 genes, including 16S rRNA, five housekeeping genes (*atpD*, *dnaJ*, *glnA*, *gyrB* and *recA*) and three symbiosis genes (*nifH*, *nifK*

and *nodD*), was performed to find genes with sufficient variability to distinguish the strains of *Mesorhizobium ciceri*. Four housekeeping genes (*atpD*, *dnaJ*, *glnA* and *recA*) which had sequence similarities ranging 92% to 100% among *Mesorhizobium* species and 16S rRNA sequences which showed 98–100% similarity were not included for further analysis. Similarly, sequences of *nifH*, a gene involved in nitrogen fixation, were also not included because of high sequence similarity (96–100%). Three remaining genes displayed sufficient sequence divergence to distinguish strains of *M. ciceri*. As a result, one housekeeping gene (*gyrB*) and two symbiosis-related genes (*nifK* and *nodD*) were selected for further analysis. Each sequence of three genes were deposited in the Genbank of NCBI as following accession, *gyrB* (KF710045–KF710174), *nifK* (KF710175–KF710317) and *nodD* (KF710318–710460). These genes had 86–100% similarities in 143 sequences while individual sequence similarities were varied: *gyrB* (86–100%), *nifK* (87–100%) and *nodD* (90–100%).

Phylogenetic tree was individually constructed based on 143 sequences for 3 genes (figure 1). Phylogenetic tree depicted that more than half sequences belonged to one big clade (dominant cluster) which contained about 80% of sequences from *C. arietinum*, about 60% of sequences from *C. reticulatum* and about 80% of sequences from *C. bijugum* (Table 2). Nodule occupancy by dominant symbiont



**Figure 1.** Phylogenetic tree based on three gene sequences of *gyrB*(A), *nifK*(B) and *nodD*(C) from nodules sampled from three *Cicer* species using neighbour-joining method. Black triangle represent major group of sequences. Sequences from nodules of cultivated chickpea (*C. arietinum*), *C. reticulatum* and *C. bijugum* have been shown in black, red and blue colours, respectively. The names of sequences included chickpea accessions: G1 (ICC 4958), G7 (ICC 1710), G11 (ICC 9755), G18 (ICC 8350), G19 (ICCW 6), G20 (ICCW 8), G21 (ICCW 9), G23 (ICC 17290), G24 (ICC 17296) and G25 (ICC 17304).

**Table 2.** Sequence diversity and nodule occupancy by dominant symbiont for three genes in three *Cicer* species

Species	Sequence diversity			Nodule occupancy by dominant symbiont (%)		
	<i>gyrB</i>	<i>nifK</i>	<i>nodD</i>	<i>gyrB</i>	<i>nifK</i>	<i>nodD</i>
<i>C. arietinum</i>	0.024	0.004	0.005	80.61 (50/62*)	80.61 (50/62)	90.32 (56/62)
<i>C. reticulatum</i>	0.066	0.009	0.009	59.37 (19/32)	68.75 (22/32)	65.62 (21/32)
<i>C. bijugum</i>	0.014	0.006	0.007	85.71 (42/49)	91.83 (45/49)	73.49 (36/49)

\* Number of sequences in dominant cluster by total number of sequences in each species for each gene accordingly.

indicated how many nodules were occupied by dominant strain of rhizobia. Most of *C. arietinum* and *C. bijugum*'s sequences belonged to dominant cluster, which corresponds with their lower sequence diversity than *C. reticulatum* (Table 2). Rhizobia associated with *C. reticulatum* displayed the highest sequence diversity where as rhizobia from cultivars and *C. bijugum* showed similar sequence diversity. Phylogenetic trees of neighbour-joining and maximum-likelihood methods (data not shown) were very similar with a little variation in minor branching pattern.

#### 4. Discussion

Sequences of 16S rRNA and housekeeping genes have been used for bacterial taxonomic or phylogenetic analysis (Chen *et al.* 2010; Martens *et al.* 2007; Laranjo *et al.* 2012;). Preliminary *in silico* searches demonstrated that these genes showed very high sequence similarities with sequences of *Mesorhizobium* species in NCBI Genbank. Three genes (*gyrB*, *nifK* and *nodD*) were selected out of 9 genes (16S rRNA, *atpD*, *dnaJ*, *glnA*, *gyrB*, *nifH*, *nifK*, *nodD* and *recA*) based on sufficient sequence diversity, which suggested that conserved genes could not be used for taxonomic and phylogenetic analysis within bacterial species. Six accessions of wild chickpeas (*C. reticulatum* and *C. bijugum*) and 4 accessions of cultivated chickpeas (*C. arietinum*) were selected to assess the differences in symbiosis with rhizobial bacteria between cultivated and wild chickpeas. Clustering based on the phylogenetic tree contained one big clade (dominant symbiont) and several small minor clades (figure 1). Rhizobia belonging to dominant cluster could be of same operational taxonomic unit because they have 99% similar sequence (White *et al.* 2010). Nodule occupancy by dominant symbiont indicated that nodules of *C. reticulatum* could be occupied by diverse rhizobia (Table 2). Based on sequence diversity and distribution of sequences in phylogenetic tree, *C. reticulatum* harboured the most diverse *Mesorhizobium*, which is consistent with the previous studies (Zahran 2001). These observations imply that *C. reticulatum*, the ancestor of cultivated chickpea (Ohri and Pal 1991), could possess symbiotic relationship with more diverse *Mesorhizobium*, and that during domestication the capacity to associate with a

diverse range of rhizobia had been lost, probably through the loss of alleles of nod factor signalling genes (Ormeno-Orrillo *et al.* 2012). This observation could be connected to previous work that showed nodulation genes were involved in determination of host range because sequence diversity of *nodD* was the highest and nodule occupancy by dominant symbiont in *nodD* was the lowest in *C. reticulatum* (Laguerre *et al.* 2001; Ormeno-Orrillo *et al.* 2012).

Many studies of domestication have documented a genetic bottleneck in crops compared to their CWRs (Doebley *et al.* 2006; Olsen and Wendel 2013). Chickpea domestication effects were explored by the analysis of rhizobial sequences. In summary, this study demonstrated that rhizobia from *C. reticulatum* showed more sequence diversity and chickpea domestication might have negative influence on symbiosis in addition to reducing genetic diversity. Therefore, it will be important to identify and transfer those useful alleles from wild species to leading varieties of chickpea, which will enhance both symbiotic efficiency as well as genetic diversity for crop improvement.

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#### References

- Agarwal G, Jhanwar S, Priya P, Singh VK, Saxena MS, Parida SK, Garg R, Tyagi AK and Jain M 2012 Comparative analysis of kabuli chickpea transcriptome with desi and wild chickpea provides a rich resource for development of functional markers. *PLoS ONE* 7 e52443

- Ben Romdhane S, Aouani ME, Trabelsi M, De Lajudie P and Mhamdi R 2008 Selection of high nitrogen-fixing Rhizobia nodulating chickpea (*Cicer arietinum*) for semi-arid Tunisia. *J. Agron. Crop Sci.* **194** 413–420
- Ben Romdhane S, Tajini F, Trabelsi M, Aouani M and Mhamdi R 2007 Competition for nodule formation between introduced strains of *Mesorhizobium ciceri* and the native populations of rhizobia nodulating chickpea (*Cicer arietinum*) in Tunisia. *World J. Microbiol. Biotechnol.* **23** 1195–1201
- Chen W-M, Zhu W-F, Bontemps C, Young JPW and Wei GH 2010 *Mesorhizobium alhagi* sp. nov., isolated from wild *Alhagi sparsifolia* in north-western China. *Int. J. Syst. Evol. Microbiol.* **60** 958–962
- Doebley JF, Gaut BS and Smith BD 2006 The molecular genetics of crop domestication. *Cell* **127** 1309–1321
- Fridman E, Carrari F, Liu Y-S, Fernie AR and Zamir D 2004 Zooming in on a quantitative trait for tomato yield using interspecific introgressions. *Science* **305** 1786–1789
- Hajjar R and Hodgkin T 2007 The use of wild relatives in crop improvement: a survey of developments over the last 20 years. *Euphytica* **156** 1–13
- Kiers ET, Hutton MG and Denison RF 2007 Human selection and the relaxation of legume defences against ineffective rhizobia. *Proc. R. Soc. B.* **274** 3119–3126
- Laguette G, Nour SM, Macheret V, Sanjuan J, Drouin P and Amarger N 2001 Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology* **147** 981–993
- Laranjo M, Alexandre A, Rivas R, Velázquez E, Young JPW and Oliveira S 2008 Chickpea rhizobia symbiosis genes are highly conserved across multiple *Mesorhizobium* species. *FEMS Microbiol. Ecol.* **66** 391–400
- Laranjo M, Young JPW and Oliveira S 2012 Multilocus sequence analysis reveals multiple symbiovars within *Mesorhizobium* species. *Syst. Appl. Microbiol.* **35** 359–367
- Lenser T and Theissen G 2013 Molecular mechanisms involved in convergent crop domestication. *Trends Plant Sci.* doi: [10.1016/j.tplants.2013.08.007](https://doi.org/10.1016/j.tplants.2013.08.007)
- Margaret I, Becker A, Blom J, Bonilla I, Goesmann A, Göttfert M, Lloret J, Mittard-Runte V, et al. 2011 Symbiotic properties and first analyses of the genomic sequence of the fast growing model strain *Sinorhizobium fredii* HH103 nodulating soybean. *J. Biotechnol.* **155** 11–19
- Martens M, Delaere M, Coopman R, De Vos P, Gillis M and Willems A 2007 Multilocus sequence analysis of *Ensifer* and related taxa. *Int. J. Syst. Evol. Microbiol.* **57** 489–503
- McCouch S, Baute GJ, Bradeen J, Bramel P, Bretting PK, Buckler E, Burke JM, Charest D, et al. 2013 Agriculture: Feeding the future. *Nature* **499** 23–24
- Ohri D and Pal M 1991 The origin of chickpea (*Cicer arietinum* L.): karyotype and nuclear DNA amount. *Heredity* **66** 367–372
- Olsen KM and Wendel JF 2013 A bountiful harvest: genomic insights into crop domestication phenotypes. *Annu. Rev. Plant Biol.* **64** 47–70
- Ormeno-Orrillo E, Menna P, Almeida LG, Ollero FJ, Nicolas MF, Pains Rodrigues E, Shigueyoshi Nakatani A, Silva Batista JS, et al. 2012 Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris* L.). *BMC Genomics* **13** 735
- Sachs JL, Ehinger MO and Simms EL 2010 Origins of cheating and loss of symbiosis in wild *Bradyrhizobium*. *J. Evol. Biol.* **23** 1075–1089
- Schmalenbach I, Léon J and Pillen K 2009 Identification and verification of QTLs for agronomic traits using wild barley introgression lines. *Theor. Appl. Genet.* **118** 483–497
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28** 2731–2739
- Thompson J, Reen R, Clewett T, Sheedy J, Kelly A, Gogel B and Knights E 2011 Hybridisation of Australian chickpea cultivars with wild *Cicer* spp. increases resistance to root-lesion nematodes (*Pratylenchus thornei* and *P. neglectus*). *Australas. Plant Pathol.* **40** 601–611
- Varshney RK, Song C, Saxena RK, Azam S, Yu S, Sharpe AG, Cannon S, Baek J, et al. 2013 Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. *Nat. Biotech.* **31** 240–246
- Weisburg WG, Barns SM, Pelletier DA and Lane DJ 1991 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173** 697–703
- Wielbo J, Marek-Kozaczuk M, Mazur A, Kubik-Komar A and Skorupska A 2010 Genetic and metabolic divergence within a *Rhizobium leguminosarum* bv. *trifolii* population recovered from cover nodules. *Appl. Environ. Microbiol.* **76** 4593–4600
- White J, Navlakha S, Nagarajan N, Ghodsi M-R, Kingsford C and Pop M 2010 Alignment and clustering of phylogenetic markers - implications for microbial diversity studies. *BMC Bioinformatics* **11** 152
- Zahran HH 2001 Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *J. Biotechnol.* **91** 143–153

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