Phylogenetic diversity of *Mesorhizobium* in chickpea

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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/fruiting 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 phylogenic 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: TGCGAGGAGAATTTCGGRTC), *nodD* (Forward: CTCGTCGCGCTCGACGCATTGA; Reverse: TGCCCCATGGACATGTA), *nifH* (Forward: GTCTCCTATGACGTGCTCGG; Reverse: GCTTCCATGGTGATCGGGGT), *nifK* (Forward: TTCGAGCGAACCATGTCCTT; 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	*C. arietinum*	ICCW 6	*C. reticulatum*	ICC 17290	*C. bijugum*
ICC 1710	*C. arietinum*	ICCW 8	*C. reticulatum*	ICC 17296	*C. bijugum*
ICC 9755	*C. arietinum*	ICCW 9	*C. reticulatum*	ICC 17304	*C. bijugum*
ICC 8350	*C. arietinum*				

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

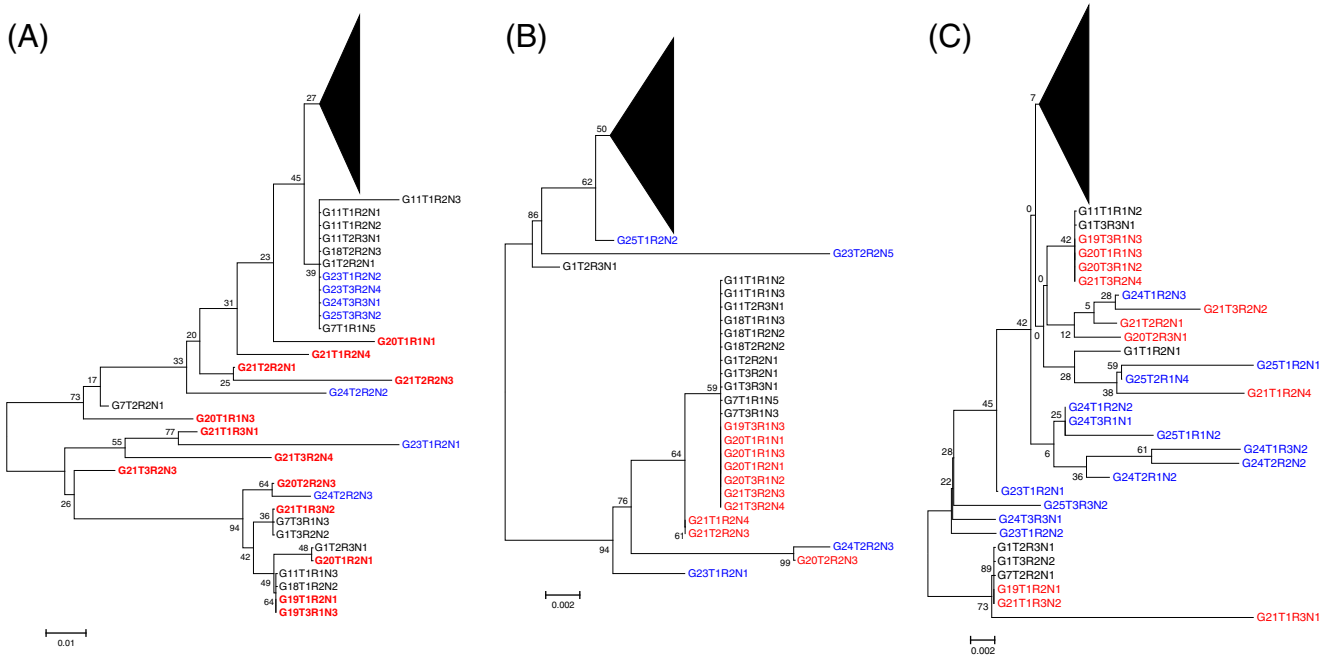

Figure 1. Phylogenetic tree based on three gene sequences of *gyrB*(A), *nifK*(B) and *nodD*(C) from nodules sampled from three *Cicer* species using neighbour-joining method. Black triangle represent major group of sequences. Sequences from nodules of cultivated chickpea (*C. arietinum*), *C. reticulatum* 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 (%)		
	gyrB	*nifK*	*nodD*	*gyrB*	*nifK*	*nodD*
C. arietinum	0.024	0.004	0.005	80.61 (50/62*)	80.61 (50/62)	90.32 (56/62)
C. reticulatum	0.066	0.009	0.009	59.37 (19/32)	68.75 (22/32)	65.62 (21/32)
C. bijugum	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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