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Genetic and phenotypic diversity of rhizobia nodulating Chickpea (*Cicer arietinum* L.) in soils from southern and central Ethiopia

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Abstract: Forty-two chickpea-nodulating rhizobia were isolated from soil samples collected from diverse agro-ecological locations of Ethiopia, and characterized based on 76 phenotypic traits. Furthermore, 18 representative strains were selected and characterized using multilocus sequence analyses (MLSA) of core and symbiotic gene loci. Numerical analysis of the phenotypic characteristics grouped the 42 strains into four distinct clusters. The analysis of the 16S rRNA gene of the 18 strains showed that they belong to the *Mesorhizobium* genus. Based on phylogenetic tree constructed from the combined genes sequences (*recA*, *atpD*, *glnII*, and *gyrB*), the test strains were distributed into four genospecies (designated as genospecies I – IV). Genospecies I, II, and III could be classified with *M. ciceri*, *M. abyssinicae*, and *M. shonense*, respectively while genospecies IV might represent an unnamed *Mesorhizobium* genospecies. Phylogenetic reconstruction based on the symbiosis-related (*nifH* and *nodA*) genes supported a single cluster together with previously described symbiont of chickpea (*M. ciceri* and *M. mediterraneum*). Overall, our results corroborate earlier findings that Ethiopian soils harbor phylogenetically diverse *Mesorhizobium* species, justifying further explorative studies. The observed differences in symbiotic effectiveness indicated the potential to select effective strains for use as inoculants and to improve the productivity of chickpea in the country.

Keywords: *Mesorhizobium*, MLSA, Core genes, Symbiotic genes, Symbiotic effectiveness

Introduction

Chickpea (*Cicer arietinum* L.) is the world's third most important food legume crop. Ethiopia ranks first in chickpea production and hectareage in Africa (FAO 2015), where it plays an important role in the local economy and contributes to sustainable agriculture. Because of higher protein contents as compared to cereals, legumes such as chickpea have attained significance for having excellent food value for the human diet, particularly for developing countries such as Ethiopia where animal protein sources are unaffordable for most people. However, the national average chickpea yield in Ethiopia is 1.7 t ha⁻¹ (CSA 2013), which is far below the potential yield of 5.0 t ha⁻¹, suggested by others (Keneni et al. 2011). Low soil fertility is one of the most important yield limiting factors in sub-Saharan Africa (SSA). Among essential plant nutrients, nitrogen (N) is the most important limiting nutrient for crop production in Ethiopia. Use of nitrogenous fertilizers is limited because it is poorly accessible and costly to smallholder farmers (Yanggen et al. 1998). Thus, increased use of legume crops is a viable alternative due to their ability to fix atmospheric N in symbiotic associations with rhizobia, forgoing the need for inorganic N inputs. The successful production of legumes including chickpea thus depends on the availability of compatible rhizobia in the soil with superior symbiotic performance.

Rhizobia are soil-inhabiting Gram-negative bacteria that form root nodules on legume plants and exist as intracellular symbionts within the developed nodules, converting atmospheric N into ammonia for assimilation by the plant in exchange for plant-derived organic acids (Abaidoo et al. 1990, Sprent 2007). Chickpea has been considered specific for its rhizobial requirement (Gaur and Sen 1979) nodulating with only two species, *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* (Nour et al. 1994a, 1994b, 1995). However, recently use of molecular methods and characterization of samples from more geographical locations revealed that chickpea nodulating rhizobia belong to several species within the genus *Mesorhizobium* (Alexandre et al. 2009; Laranjo et al. 2012; Rai et al. 2012). Importantly, many current chickpea producing areas may be devoid of rhizobia capable of nodulating chickpea. For example, until chickpea was introduced in Western Canada in the early 1990's, soils there contained no indigenous rhizobia capable of nodulating chickpea (Kyei-Boahen et al. 2002). While chickpea production has increased in this area, only introduced rhizobia are able to nodulate chickpea, making the choice of rhizobial strain particularly important as both the crop and the soil microbial community are effectively inoculated by the introduced strains. In contrast, it is presumed that soils in Ethiopia support a vast array of rhizobial

strains capable of nodulating chickpea, reflecting the long history of cultivation of chickpea landraces and likely co-evolution of the symbionts.

Modern bacterial taxonomy is based on the integration of phenotypic and genotypic data for a more stable classification (Vandamme et al. 1996; Zakhia and De Lajudie 2001). Phenotypic studies are necessary for characterization and selection of rhizobial strains adapted to marginal edapho-climatic conditions, and to provide preliminary information about their genetic diversity (Howieson and Dilworth 2016; Zahran et al. 2003). Sequence analysis of the 16S rRNA gene has been used as a regular protocol for bacterial taxonomic study. Rhizobial genomes often carry several copies of the 16S rRNA gene; however, they usually are 100% similar. The largest limitation of using the 16S rRNA gene in rhizobia taxonomy is its low discrimination level for closely related species (Acinas et al. 2004; van Berkum et al. 2006). To overcome these drawbacks, protein-coding genes have been proposed as alternative phylogenetic markers to discriminate between closely related species (Stackebrandt et al. 2002). These genes have a faster rate of evolution than the 16S rRNA gene, but are conserved enough to retain genetic information useful for taxonomy purposes. Consequently, new techniques such as Multilocus Sequence Analysis (MLSA) based on the analysis of several housekeeping genes have been applied in phylogenetic analyses and identification of concrete groups of rhizobia (Menna et al. 2009; Rivas et al. 2009; Wang et al. 1998). Besides these housekeeping genes, other genes involved in the legume symbiosis called “accessory” genes are commonly included in rhizobial species description and in some MLSA analyses (Rivas et al. 2009).

Ethiopia has a large biodiversity of rhizobial resources (Wolde-meskel 2007); however, only a few of them have been extensively explored using a molecular approach (Wolde-meskel et al. 2005; Degefu et al. 2011; Asrese et al. 2012; Asrese et al. 2013; Degefu et al. 2013), and the genetic diversity of chickpea nodulating rhizobia remains largely unexplored. In order to improve the beneficial effect of chickpea inoculation, it is important to determine the characteristics of the indigenous rhizobial population. Therefore, the aims of the experiments reported in this paper were: (i) to study the symbiotic and phenotypic characteristics of chickpea nodulating rhizobia isolated from soil samples collected from chickpea growing locations in southern and central Ethiopia; and ii) to evaluate genetic diversity among the chickpea rhizobial strains. For this purpose we determined the phylogeny of housekeeping genes coding for recombination protein (*recA*), 16S rRNA, glutamine synthase II (*glnII*), ATP synthase beta subunit (*atpD*) and DNA gyrase subunit B (*gyrB*). The phylogeny of symbiotic genes for nodulation (*nodA*) and nitrogen fixation (*nifH*) also were studied for the test strains.

Materials and methods

Bacterial strains and culture conditions

The 42 rhizobial isolates (Table 1) used in this study were isolated from root nodules of chickpea, *Cicer arietinum* (cultivar Nattoli) grown in soil samples collected from chickpea growing farmers' fields at diverse agro-ecological locations in southern and central Ethiopia (Fig. 1). These areas have a history of chickpea cultivation that extends for centuries, and to our knowledge they have never been inoculated with rhizobial strains. Rhizobia were isolated, purified and cultivated on Yeast extract Mannitol Agar (YMA) following Vincent (1970). Primary isolates from YMA containing Congo Red (CR) plates were re-streaked on YMA containing $25 \mu\text{g mL}^{-1}$ Bromothymol Blue (BTB), $25 \mu\text{g mL}^{-1}$ CR and Pentose Glucose Agar containing $25 \mu\text{g mL}^{-1}$ Bromocresol Purple (PGA-BCP) and incubated at 28°C for differentiation purposes (Howieson and Dilworth 2016). All strains were stored in 15% (v/v) glycerol at 80°C .

<<Insert Figure 1 here>>

<<Insert Table 1 here>>

Symbiotic traits

To assess infectivity and effectiveness in fixing atmospheric N, each strain was grown in Yeast extract Manitol Broth (YMB) to the logarithmic phase ($\pm 10^9 \text{ cell mL}^{-1}$) and aseptically inoculated on germinated seeds of chickpea planted in modified Leonard jars containing sterilized and N-free growth media (Howieson and Dilworth 2016). The experiment was carried out with three replicates using a randomized complete design. As controls, each block contained two jars (N free and with N) with non-inoculated seedlings. The seedlings were watered weekly with sterile, one-quarter strength Jensen's modified N-free nutrient solution and with sterilized distilled water as necessary, according to Howieson and Dilworth (2016). Furthermore, the positive control (with N) received weekly additions of 0.5% (w/v) KNO_3 as a N source. Plants were maintained in a greenhouse and harvested 45 days after planting and nodule number, nodule dry weight, and shoot dry biomass were recorded. The plant shoot samples were oven dried at 70°C to a constant weight and ground to pass through a 2 mm sieve. Nitrogen accumulation in the plant shoot (percent N) was determined using a LECO CNS-2000 carbon, nitrogen, and sulfur analyzer. The data were analyzed by one-way analysis of variance

using the general linear model procedure of the SAS software package (SAS/STAT; version 9.3) and mean values were separated according to Duncan's multiple range test at $p = 0.05$ (SAS Institute 2012). The percent symbiotic effectiveness (SE) of the isolates was expressed as a percentage of the shoot dry biomass of each treatment compared with the shoot dry biomass of the positive control (with N). Finally, the SE values of the isolates were rated as highly effective, >80%; effective, 50–80%; poorly effective, 35–50%; or ineffective, <35% (Purcino et al. 2000).

Physiological and biochemical tests

Except for carbohydrate and N assimilation, all the following tests were carried out in triplicate on YMA plates. The plates were divided into 64 equal parts. Each square was spot-inoculated with 10 μL of the strain culture previously grown on YMB to the exponential phase ($\pm 10^9$ cells·mL⁻¹). Inoculated plates were incubated at 28°C and, after 7 days, the growth of colonies was monitored visually (Howieson and Dilworth 2016). The following reference strains were subjected to phenotypic characterization together with the test strains to elucidate the relatedness with the reference strains: *M. loti* HAMBI (1129^T), *Ensifer meliloti* (HAMBI 2148^T), *Rhizobium leguminosarum* (HAMBI12^T), *Bradyrhizobium japonicum* (HAMBI 2314^T), *M. mediterraneum* (HAMBI 2096^T), and *Rhizobium huautlense* (HAMBI 2409^T).

Physiological Characteristics

Bacterial growth at different temperatures was determined on YMA plates inoculated as described above and incubated at the following temperatures: 5°C, 15°C, 20 to 30°C, 35°C, 40°C, and 45°C (Maatallah et al. 2002). The isolates were tested for their tolerance to salinity on YMA media supplemented with NaCl at concentrations of 1%, 2%, 3%, 4%, and 5% (w/v) (Amarger et al. 1997). The ability of the isolates to grow on acidic and alkaline media were tested by inoculating them onto YMA with the pH adjusted to 4.0, 4.5, 5.0, 5.5, 8.0, 8.5, 9.0, 9.5 or 10.0 with a 10% sterile solution of HCl or NaOH (Amarger et al. 1997).

Intrinsic antibiotic and heavy metal resistance

The intrinsic antibiotic and heavy metal resistances of the isolates were tested on YMA with various concentrations of antibiotics or metal compounds incorporated. The stock solutions of the antibiotics or heavy metals were sterilized using 0.2 μm membrane filters and added to YMA at 55°C via filter paper with a Luer-Lock system using hypodermal syringes in a laminar hood flow. The antibiotics used were as follows: chloramphenicol (5 and 15 $\mu\text{g}\cdot\text{mL}^{-1}$), erythromycin (10 and 20 $\mu\text{g}\cdot\text{mL}^{-1}$), streptomycin (40 and 80 $\mu\text{g}\cdot\text{mL}^{-1}$), spectinomycin (250 and 500 $\mu\text{g}\cdot\text{mL}^{-1}$), kanamycin (15 $\mu\text{g}\cdot\text{mL}^{-1}$), lincomycin (100 $\mu\text{g}\cdot\text{mL}^{-1}$), trimethoprim (200 $\mu\text{g}\cdot\text{mL}^{-1}$), neomycin (5 and 20 $\mu\text{g}\cdot\text{mL}^{-1}$), and novobiocin (5 $\mu\text{g}\cdot\text{mL}^{-1}$). Similarly, the heavy metals used were $\text{MnSO}_4\cdot\text{H}_2\text{O}$ (500 $\mu\text{g}\cdot\text{mL}^{-1}$), $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$ (500 $\mu\text{g}\cdot\text{mL}^{-1}$), $\text{Pb}(\text{CH}_3\text{COOH})\cdot 3\text{H}_2\text{O}$ (500 $\mu\text{g}\cdot\text{mL}^{-1}$), $\text{CoSO}_4\cdot 7\text{H}_2\text{O}$ (100 $\mu\text{g}\cdot\text{mL}^{-1}$), CuCl_2 (100 $\mu\text{g}\cdot\text{mL}^{-1}$), and ZnCl_2 (500 $\mu\text{g}\cdot\text{mL}^{-1}$) (Howieson and Dilworth 2016). Strains were considered resistant when growth occurred, and sensitive when no growth was detected.

Carbon source utilization

The following carbon sources were used to determine whether they could be used as the sole carbon source for the isolates following Howieson and Dilworth (2016): D-trehalose, dextrin, D-maltose, sucrose, D-xylose, D-rhamnose, D-raffinose, sorbitol, citric acid, dulcitol, L-arabinose, malonic acid, D-fructose, xylitol, D-mannitol, myo-inositol, D-galactose, D-mannose, and glycine. Carbohydrate solution [10 μL of a 10% (m/v) solution in distilled water] was prepared in sterilized plastic vials. Heat stable carbon sources (fructose, glucose, sucrose, lactose and mannitol) were added to the carbohydrate-free basal medium (essentially similar to YM with a concentration of yeast-extract reduced to 0.05 $\text{g}\cdot\text{L}^{-1}$) at a final concentrations of 1 $\text{g}\cdot\text{L}^{-1}$ and autoclaved together with the media. The other heat labile carbon sources were sterilized using 0.2 μm mesh sterile filter papers with a Luer-Lock system and added to the basal medium at 55°C aseptically. The solutions were then aseptically dispensed into sterile petri dishes and allowed to solidify in the hood.

Nitrogen source utilization

The following N sources were tested as sole N sources for the isolates according to Amarger et al. (1997): D-serine, L-histidine, L-pyroglutamic acid, L-phenylalanine, L-proline, uridine, thymidine, inosine, L-arginine, L-leucine, L-aspartic acid, L-glutamic acid, L-threonine, L-alanine, L-asparagine,

and L-isoleucine. These N sources were added to a basal medium at a final concentration of $0.5 \text{ g} \cdot \text{L}^{-1}$. The basal medium was adjusted to be 1g of K_2HPO_4 , 1g of KH_2PO_4 , 0.01g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.2g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of CaCl_2 , 1g of $(\text{NH}_4)_2\text{SO}_4$ and 15g of agar per liter of distilled water.

Phosphate solubilization

The basal medium ($10 \text{ g} \cdot \text{L}^{-1}$ glucose, $0.5 \text{ g} \cdot \text{L}^{-1}$ yeast-extract, $0.1 \text{ g} \cdot \text{L}^{-1}$ CaCl_2 , $0.25 \text{ g} \cdot \text{L}^{-1}$ $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and $15 \text{ g} \cdot \text{L}^{-1}$ agar dissolved in distilled water) was used to test the phosphate solubilizing ability of the isolates. The medium was supplemented with $2.5 \text{ g} \cdot \text{L}^{-1}$ of $\text{Ca}_3(\text{PO}_4)_2$ as a source of phosphate. The pH of the medium was adjusted to 7.2 before autoclaving (Alikahani et al. 2006). The appearance and formation of the clear zone around the colony was checked within 7 to 10 days of inoculation (Xie et al. 2009). Isolates that formed clear zones around the colony were considered phosphate solubilizers.

Numerical analysis

Traits were coded 1 for positive and 0 for negative. The final matrix contained 42 isolates, 6 reference strains (altogether 48), and 76 traits. A computer cluster analysis of 76 phenotypic variables was carried out using a similarity coefficient and a dendrogram was constructed by the unweighted pair group method with average (UPGMA) clustering with the NTSYSpc21 program.

DNA preparation

Total genomic DNA was isolated from $3 \times 1.5 \text{ mL}$ cultures using the procedure described previously by Boom et al. (1990) with a slight modification using diatomaceous earth or a Celite analytical filter aid as a DNA binding solid support (Terefework et al. 2001).

PCR amplification and gene sequencing

The following genes were studied: 16S rRNA, partial internal sequences of protein coding housekeeping genes (*recA*, *gyrB*, *atpD*, and *glnII*), and the symbiosis related genes (*nodA* and *nifH*). The partial 16S rRNA gene was amplified and sequenced using the primers fD1 and rD1 as described previously Weisburg et al. (1991). PCR amplification and sequencing of partial *recA* and *atpD* genes were undertaken according to Gaunt et al. (2001), and *glnII* and *gyrB* gene amplification and

sequencing were as carried out following the PCR conditions previously specified (Martens et al. 2008). Partial amplification and sequencing of the symbiotic gene, *nodA*, was carried out as previously described (Laguerre et al. 2001). For amplification and sequencing of the partial *nifH* gene sequence, primers and PCR condition specified by Rivas et al. (2002) were used.

Phylogenetic data analysis

The quality of the sequences was checked and edited using BioEdit 7.2.5 (Hall 2004). For preliminary identification, the 16S rRNA gene sequences of the test strains were compared to the Genbank database using nucleotide BLAST. The 16S rRNA sequences obtained from the 18 rhizobia strains used in the present study were compared with those of *Mesorhizobium* reference strains available in the GenBank database. Multiple nucleotide alignments for the test isolates and reference species in the genus *Mesorhizobium* were carried out using the CLUSTAL W program (Thompson et al. 1994) from MEGA version 6.0 (Tamura et al. 2013). Phylogenetic trees were constructed using the neighbor-joining method with Kimura's two-parameter model, nucleotide distances and discarding positions with gaps in any sequence. The robustness of the tree topology was calculated from bootstrap analysis with 1000 replications of the sequences for neighbor-joining. The percentage similarity of the genes was estimated using the Kimura-2 distance matrix correction model as implemented in MEGA version 6 (Tamura et al. 2013).

Results

Isolation and symbiotic effectiveness test on sand culture

Forty-two chickpea isolates formed nodules when re-inoculated on host plants using sterile sand culture under controlled environmental conditions. All 42 isolates were Gram-negative, rod-shaped bacteria that did not grow on Peptone Glucose Agar containing Bromo Cresol Purple (PGA-BCP) and absorbed Congo Red (CR) on YEM-CR media (data not shown). Furthermore, all isolates turned the Bromo Thymol Blue (BTB)-YEM media from moderately yellow to deep yellow.

Symbiotic effectiveness of the test strains showed considerable variations when inoculated to the host plant (Chickpea, var. Nattoli). The uninoculated control was not nodulated. The test strains of chickpea rhizobia showed great variation in their ability to fix atmospheric N and consequent ability to enhance shoot dry weight, shoot N uptake, nodule number, and nodule dry weight per plant

($p < 0.001$; Table 2). The highest shoot dry matter accumulation was recorded from plants inoculated with the isolate CP41 followed by plants treated with isolates CP97, CP105, and CP08, with shoot dry matter accumulation of 0.98, 0.83, 0.81 and 0.81 g·plant⁻¹, respectively. The lowest dry mass was recorded from plants inoculated with isolates CP65, CP84, and CP109, which all yielded 0.33 g·plant⁻¹. Similarly, the effect of indigenous rhizobial strains on shoot N uptake varied in the range of 7.8 (CP65) to 41.8 (CP41) mg·plant⁻¹. The relative differences of N accumulation increase in shoot by CP41, CP97, CP105 and CP08 strains were 98, 78, 76 and 75%, respectively over the uninoculated N fertilized control.

<<Insert Table 2 here>>

The mean nodule number per plant varied from 17 to 60, which were induced by isolates CP74 and CP151, respectively. Despite the large number of nodules per plant recorded by isolate CP151, this isolate had low symbiotic effectiveness (67%) as shown in Table 2. The highest nodule dry weight of 208 mg·plant⁻¹ was recorded from plants inoculated with isolate CP41, whereas the lowest nodule dry weight of 41 mg·plant⁻¹ was shown by the host inoculated with isolate CP140. The relative effectiveness, expressed in percent of the N-fed control, showed that isolates CP08, CP41, CP97, CP105 and CP133 were the most efficient, with more than 80% dry matter yield relative to the uninoculated N-fed control while CP65, CP84 and CP109 were the least efficient with only 33% effectiveness.

Physiological traits and numerical analysis

The phenotypic traits of the 42 chickpea rhizobia are summarized in Table S1 and S2. The optimum temperature for growth of all isolates was in the range 20 to 35°C. Below and beyond this range, the isolates showed variations in their growth. The salt inhibitory concentrations varied among strains. All bacterial isolates survived at 1% NaCl, and 85.7% of the isolates survived at 2% NaCl. However, at higher concentrations, the percentage of tolerant strains decreased rapidly and only two strains showed a moderate growth at 4% and 5% NaCl. All isolates grew at pH values ranging between 8 and 8.5. The reference strains were very sensitive to extreme temperatures, high NaCl concentrations, and extremes of pH as compared to the indigenous strains (data not shown). All tested strains grew on trehalose, sucrose, galactose, glucose, mannitol, inosine, histidine, asparagine, glutamic acid, threonine, alanine, uridine, and leucine. None of the strains utilized citric acid. The

evaluation of intrinsic resistance to antibiotics (according to their concentrations) of chickpea nodulating rhizobia showed that all of the tested isolates exhibited high resistance to erythromycin and neomycin. All tested strains were found to be sensitive to streptomycin, spectinomycin, and lincomycin, and thus were not able to grow. All test strains were found to be sensitive to zinc.

The dendrogram obtained from the computer numerical analysis of 76 phenotypic traits of the 42 chickpea nodulating rhizobia including the six reference strains showed that they could be grouped into at least four clusters at 30% similarity level (supplemental Fig. 1). Cluster I was the largest with 30 test strains originating from various locations (Table 1) and clustered independently by excluding the reference species. Cluster I is again grouped into 8 sub clusters at 75% similarity level. The majority of strains in Cluster I was able to grow at pH ranging between 5 and 9.5, at temperatures between 10°C and 35°C, and tolerated NaCl concentrations ranging between 1% and 2%. The growth of these isolates was fully inhibited by kanamycin, streptomycin, ampicillin, chloramphenicol, spectinomycin, nickel, zinc, aluminum, cobalt, and lead, but they were insensitive to nalidixic acid, mercury, and manganese. Most of inorganic phosphate-solubilizer (67%) strains were observed in this cluster. Regarding carbohydrate assimilation, these bacteria were able to metabolize a wide range of carbon substrates.

Cluster II has only three isolates (CP98, CP125, and CP136) and exhibited similar characteristics to the reference strains *M. loti* and *M. mediterraneum*. Unlike strains of other clusters, they could not tolerate a NaCl concentration greater than 2% nor extreme temperature. The majority of Cluster II strains were able to grow within a pH range between 4 and 8.5. It seems that this group resists acidic conditions. Inorganic phosphate-solubilizing activity was not observed in this cluster. The growth of these isolates was fully inhibited by raffinose and malonic acid but they grew well in most carbohydrate, N and antibiotic sources.

Cluster III consists of six isolates. They have distinct characteristics in that they tolerate a 5% NaCl concentration and most of them are capable of solubilizing inorganic phosphate. Cluster III isolates exhibited similar characteristics to the reference strains *Rhizobium leguminosarum* and *Rhizobium huautlense*. Most of the strains of Cluster III grew well in most carbohydrate, N and antibiotic sources. They were resistant to Al and Co but sensitive to Zn and Cu. Cluster IV has only three isolates (CP74, CP99, and CP100). These were sensitive to low and high pH and temperature extremes, as well as high salinity. They also were sensitive to kanamycin and copper. Cluster IV was also characterized by the ability to solubilize inorganic phosphate. Similar to the other clusters most of the strains of Cluster IV grew well in most carbohydrate and N sources and tolerated most

antibiotic sources. The two reference strains (*Ensifer meliloti* and *Bradyrhizobium japonicum*) were clustered alone on the dendrogram.

Genetic diversity and identification

Of the 42 test strains, 18 representative strains based on phenotypic characteristic and geographical location of soil sampling sites were selected (Table 1) and characterized using multilocus sequence analyses (MLSA) of 16S rRNA, core (*recA*, *atpD*, *glnII*, and *gyrB*) and symbiotic (*nodA* and *nifH*) genes loci. The identification process started with the sequencing of the partial 16S rRNA gene (1322 - 1362bp). For each gene, sequence data were aligned and used to infer phylogenetic trees and to compare the placement of the test strains with respect to currently (at the time of writing this manuscript) designated *Mesorhizobium* reference species.

16S rRNA gene sequence analysis

The partial 16S rRNA gene sequences of the 18 chickpea rhizobia test strains were obtained and compared with *Mesorhizobium* strains available in the GenBank database. In the 16S rRNA gene phylogenetic tree, 18 test strains belonged to the branch defining the genus *Mesorhizobium* (Fig. 2a). The phylogenetic tree built with the 16S rRNA sequences split the *Mesorhizobium* test strains in three groups. The first group comprised three test strains grouped with *M. ciceri* USDA3383 and *M. cantuariense* ICMP 19515 with sequence similarity of 100%. The second group included eight strains showing closer relation with *M. shonense* HAMBI 3295 with sequence similarity of 99.8% and the third group included seven test strains showing closer relation with *M. plurifarum* LMG 11892 with sequence similarity of 99.6%. The taxonomic positions of the strains were further investigated by *recA*, *atpD*, *glnII*, and *gyrB* gene sequence analysis.

<<Insert Figure 2 here>>

Analysis of concatenated gene sequences

Phylogenetic trees were constructed based on the combined *recA*, *atpD*, *glnII* and *gyrB* gene sequences (in total 1635bp in length). The phylogenetic tree of concatenated genes (Fig. 2b) generated from sequence alignments of the test strains and the described reference species, revealed

four clusters with high bootstrap support (99% to 100 %) at each branch. The different genospecies were placed into separate four clades within *Mesorhizobium* genus. Genospecies I comprising CP123, CP125 and CP148 were found to be closely associated with previously described microsymbiont of chickpea, *M. ciceri* USDA3383 with Average Nucleotide Identity (ANI value) of 98.2%. Genospecies II comprising two strains (CP41 and CP49) were closely related to the *M. abyssinicae* type strain HAMBI 3306 (one of the three described new species from Ethiopia) with ANI value of 98.7%. Genospecies III, containing six strains (CP08, CP10, CP12, CP30, CP51, and CP69), were closely related to type strains *M. shonense* HAMBI 3295 (96.3% ANI) and *M. hawassense* HAMBI 3301 (94.1% ANI). Genospecies IV contains seven strains (CP84, CP98, CP105, CP130, CP134, CP136 and CP151). The phylogenetic analyses based on concatenated genes identified *M. plurifarum* LMG 11892 to be the closest related type strain to genospecies IV, with ANI value that did not exceed 94%.

Analysis of individual house-keeping genes

The phylogenetic trees of the individual house-keeping genes (Fig. 3 a-d) generated from sequence alignments of the test strains and the described reference species revealed that the 18 isolates from Ethiopian soils group in four different clusters. The lengths of the alignments were 435bp, 490bp, 616bp and 750bp for *recA*, *atpD*, *glnII*, and *gyrB*, respectively. In the individual housekeeping phylogenetic trees, in agreement with the concatenated gene phylogeny, the test strains were consistently grouped into four separate clades (I–IV) within the *Mesorhizobium* genus. In general, the tree topologies for all house-keeping genes, and thus the placement of the test strains within the trees, were consistent for the four house-keeping genes (*recA*, *atpD*, *glnII* and *gyrB*). With respect to the *recA*, *atpD*, *glnII* and *gyrB* genes sequences, the test strains within genospecies I were found to be closely associated with type strain *M. ciceri* (either USDA3383 or HAMBI 1750) with sequence similarity of 97.7%, 98.31%, 96.6%, and 100%, respectively. Genospecies I were closely related to *M. cantuariense* ICMP 19515 for *recA* and *glnII* with sequences similarity of 95.3% and 96.7%, respectively.

<<Insert Figure 3 here>>

Genospecies II were closely related to the *M. abyssinicae* type strain HAMBI 3306 based on *recA*, *atpD*, *glnII*, and *gyrB* gene with sequence similarity of 99.7%, 99.5%, 99.3%, and 97.1%,

respectively. Genospecies III were closely related to *M. hawassense* (HAMBI 3301) for *recA* and *atpD* with sequences similarity of 94.0% and 95.5%, respectively. Meanwhile it showed a close phylogenetic relatedness to *M. shonense* for *glnII* and *gyrB* with sequences similarity of 95.2%, and 99.1%, respectively. Similarly, genospecies III was clustered with *M. hawassense* for *glnII* with sequences similarity of 94.0%. The phylogenetic analyses based on *recA* (96% similarity), *atpD* (96.6% similarity), *glnII* (92.5% similarity) and *gyrB* (91% similarity) genes identified *M. plurifarum* LMG 11892 to be the most closely related reference species to genospecies IV.

Phylogeny of symbiotic genes

The nucleotide sequences of about 600bp for *nodA* and 400bp for *nifH* genes were obtained for 18 test strains that belonged to the genus *Mesorhizobium*. The *nodA* and *nifH* phylogenetic trees are presented in Fig. 4a and b. The grouping of the strains on the trees generated from these genes was different from those of the core genes. In the *nodA* tree, the entire group of test strains were placed into one well-supported (100% bootstrap value) closely related monophyletic clade along with previously described symbionts of chickpea, *M. ciceri* (UPM-Ca7^T) (100% sequence similarity) and *M. mediterraneum* (UPM-Ca36^T) (99.8% similarity). Similarly, in the *nifH* phylogenetic tree all test strains were tightly clustered into a single clade with the *M. ciceri* (UPM-Ca7^T) and *M. mediterraneum* (UPM-Ca36^T) type strains identified to be the closest neighbors (100% sequence similarity to both).

<<Insert Figure 4 here>>

Discussion

The results obtained from Gram staining, growth on YMA-CR medium and PGA-BCP medium confirmed the standard cultural and morphological characteristics of rhizobia. Furthermore, all isolates turned the BTB-YEM media from moderately yellow to deep yellow, indicating the acid-producing ability of the isolates, which is a common characteristic of fast growing *Mesorhizobium* species. In view of this characteristics feature, we further characterized the test strains using physiological and biochemical tests.

Physiological characteristics and numerical analysis

Physiological and biochemical studies are the basis for detailed polyphasic taxonomy and have been used to make taxonomic analysis of strains belonging to the family rhizobiaceae (Zhang et al. 1991). Phenotypic characteristics including temperature and pH profiles are useful parameters to characterize new test strains within the family rhizobiaceae, as these parameters were shown to have a great effect on rhizobial growth and symbiotic performance (Maâtallah et al. 2002; Zahran 1999). The test strains showed differences in terms of temperature and pH (Table S1). A similar wide variation in phenotypic characteristic has been reported for chickpea rhizobial isolates from different countries including Ethiopia (Alexandre et al. 2009; Jida and Assefa 2012; Maâtallah et al. 2002; Rai et al. 2012; Romdhane et al. 2009). The Ethiopian chickpea nodulating strains were able to use several compounds as sole C and N sources and were variable in their response to different antibiotic and heavy metal resistance. The new strains, which were shown to utilize several C and N sources, with their abilities to resist different levels of antibiotics and heavy metals, can be considered promising candidates for inoculant development in areas where C, N, antibiotics and heavy metals are limiting factors. However, phenotypic characterization provided only an initial insight into the diversity of the new strains and molecular techniques should be employed to elucidate their exact phylogenetic positions with respect to the reference species within the genus *Mesorhizobium*.

The chickpea microsymbionts represent four *Mesorhizobium* genospecies

Phylogenetic analyses of multi-genes found on chromosomes can clarify bacterial relationships better than the analysis of a single locus (Farida et al. 2009; Rivas et al. 2009). The phylogenetic analyses based on 16S rRNA gene sequences indicated that the rhizobial strains nodulating chickpea in Ethiopia were distributed under the genus *Mesorhizobium*. These results were consistent with previous reports that showed chickpea rhizobia were more closely related to *Mesorhizobium* species (Jarvis et al. 1997; Kim et al. 2014; Laranjo et al. 2012; Rai et al. 2012). Previously, bacteria that could establish an effective symbiosis with chickpeas were considered a single group because of the specificity for the plant host (Gaur and Sen 1979), growth characteristics, and polymorphism of *nifH* genes (Cadahia et al. 1986). Later, based on a genotypic and phenotypic diversity studies, Nour et al. (1994a, 1994b, 1995) identified and proposed two new genomic species: *Mesorhizobim ciceri* and *Mesorhizobium mediterraneum*. Traditionally, chickpea has been considered as restrictive host for nodulation (Broughton and Perret 1999) nodulating with only these two species. However, recent

phylogenetic studies on isolates from different geographical locations has shown that other species of *Mesorhizobium* may effectively nodulate the legume including *M. tianshanense*, *M. amorphae*, *M. loti*, *M. huakuii*, *M. opportunistum* and *M. muleiense* (Laranjo et al. 2004, 2008; Rivas et al. 2007; Suneja et al. 2016). In our study, we demonstrated that even within the relatively small sampling of the collection that were analyzed, the chickpea nodulating strains in Ethiopia represented four unique taxonomic positions in this genus, where only three strains were closely related to *M. ciceri* and no isolates grouped with *M. mediterraneum*. This observation justifies further exploration of the region for new chickpea nodulating strains.

Based on the concatenated genes phylogenetic tree (including *recA*, *atpD*, *glnII*, and *gyrB*), strains in genospecies I, II, and III could be classified with *M. ciceri* (De Lajudie et al. 1998), *M. abyssinicae*, and *M. shonense*, (Degefu et al. 2013). In all cases, the ANI values between the three groups and the reference species was higher than the cut off value (96%) proposed by Konstantinidis et al. (2006). However, we propose genospecies IV to be unnamed *Mesorhizobium* genospecies because in all cases, the ANI value between genospecies IV and the defined species of *Mesorhizobium* was found to be lower than 96% (Konstantinidis et al., 2006) for species identity. In our analysis, strains belonging to a single species (i.e., genospecies IV), in addition to having high intra-species sequence identity (ANI values), formed separate and highly supported monophyletic groups, distinct from the reference *Mesorhizobium* species. However, the placements of genospecies IV on the phylogenetic tree varied depending on the different housekeeping genes considered. Horizontal gene transfer and subsequent recombination may be a possible explanation for the inconsistent placement of this genospecies (Acinas et al. 2004; van Berkum et al. 2006). This indicated that analysis of more variable housekeeping genes could give a better correlation with the 96% ANI value as suggested by Martens et al. (2008). In our analysis, a clear relationship was detected between the grouping of the strains and their geographic origins. Thus, genospecies I and IV are mainly from central Ethiopia, whereas genospecies II and III are from southern Ethiopia, indicating the possibility that soils at different geographic locations may harbor different population of chickpea nodulating rhizobia.

Symbiosis-related gene: implication on host range

In this study, *nodA* and *nifH* genes were analyzed in order to determine the identity of the symbiosis-related genes and the diversity of chickpea nodulating strains in terms of these genes.

Irrespective of their chromosomal backgrounds, the designated genospecies in this study shared high sequence similarities for their *nodA* and *nifH* genes among themselves and the two defined *Mesorhizobium* species as references. For example, phylogenetic analyses based on *nodA* genes revealed that the new test strains showed close resemblance to the previously described symbionts of chickpea, *M. ciceri* and *M. mediterraneum*, and thus tightly clustered together (100% BT value, Fig 4a). Similarly, the *nifH* gene sequence-based phylogenetic tree (Fig. 4b) also demonstrated the tight clustering (99% BT value) of the new test strains with the *M. ciceri* and *M. mediterraneum*.

Taken together, we clearly demonstrated that the phylogeny derived from the symbiosis-related genes were not consistent with the phylogenetic tree constructed from the house-keeping genes, suggesting the different evolutionary history between chromosomal and symbiosis-related genes. Chickpea is confirmed as a non-promiscuous host, and thus can be nodulated by strains of several species of *Mesorhizobium* harboring nearly identical nodulation genes (Rivas et al. 2007; Laranjo et al. 2008). In agreement with this, our results indicated that the four genospecies that nodulate chickpea shared common *NodA* genes, suggesting recognition of only a few *Nod* genes by chickpea. This study has contributed to the knowledge that rhizobia with different chromosomal backgrounds may carry similar symbiosis genes, explaining how a restrictive host for nodulation, such as chickpea, is nodulated by several different *Mesorhizobium* species.

On analysis of results on symbiotic effectiveness, all tested strains were able to infect their host plant and fix atmospheric N, leading to more plant shoot production and N uptake than in the uninoculated and N-free controls. The correlation analyses revealed positive and highly significant ($p < 0.001$) associations of nodule number and nodule dry weight with shoot dry weight ($r = 0.49$ and 0.52 , respectively). In earlier investigations in Ethiopia, Mnalku et al. (2009) and Argaw (2012) reported significant correlations of nodule number and dry weights with shoot dry biomass on faba bean inoculated with compatible rhizobia.

Howieson and Dilworth (2016) and Peoples et al. (2002) suggested that shoot dry matter is a good indicator of relative effectiveness of a rhizobial isolate. The relative dry matter accumulation of chickpea in relation to the N-fertilized positive control showed that five (12%) of our isolates were highly effective, while twenty-four (57%), nine (21.5%) and four (9.5%) of them were considered effective, poorly effective or ineffective, respectively. This is in agreement with the findings of Jida and Assefa (2012) who reported that Ethiopian soils harbor symbiotically diverse chickpea-nodulating rhizobia, hence indicating possibilities to select effective strains to improve N fixation and grain yield of the crop. Higher N accumulation in shoots of inoculated plants, as compared to

uninoculated controls, suggests greater N fixation and is a useful parameter to select strains for commercial inoculant production (Wani et al. 2007; Rokhzadi and Toashih 2011). The highly effective strains identified in this study (CP08, CP41, CP97, CP105, and CP133) can be considered potentially elite strains for chickpea inoculant production. Earlier, four out of the five strains that the present manuscript highlights as highly effective were already tested on pot and field trials and the result indicated that the chickpea indigenous rhizobial strain Cp41 was superior to all the others for almost all parameters (Tena et al. 2016), thus can be promoted for large scale inoculant production.

Conclusion

The symbiotic, phenotypic and genotypic characterization of chickpea nodulating rhizobia isolated from different chickpea growing area of Ethiopia revealed a wide diversity. Indigenous rhizobial isolates were phenotypically characterized in order to assess their ability to tolerate abiotic stress conditions including salinity, pH and high temperature. A dendrogram constructed from physiological and biochemical data placed the test strains into four main clusters. The phylogenetic analyses based on 16S rRNA gene sequences indicated that the rhizobial strains nodulating chickpea in Ethiopia were distributed under the genus *Mesorhizobium*. Furthermore, the phylogenetic trees of concatenated genes (*recA*, *atpD*, *glnII* and *gyrB*) generated from sequence alignments of the test strains and the described reference species, revealed four groups (designated as genospecies I – IV) supported with high bootstrap values. Strains in genospecies I, II, and III could be classified with *M. ciceri*, *M. abyssinicae*, and *M. shonense*, respectively, whereas genospecies IV might be an unnamed *Mesorhizobium* genospecies. Our result indicated that the four genospecies that nodulate chickpea share common symbiosis genes. Overall, our results corroborated earlier findings that Ethiopian soils harbor phylogenetically diverse *Mesorhizobium* species, justifying further explorative studies. Some indigenous rhizobial isolates showed significant performance on nodulation status and plant growth parameters on inoculated chickpea plants compared to the uninoculated control treatments. The observed difference in symbiotic effectiveness underscores the potential to select effective strains for use as inoculants and to improve the productivity of chickpea within the country.

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Appendix A. Supplementary data

Supplementary data associated with this manuscript are Table S1, S2 and Figure S1.

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Table and Figure Legends

Table 1. List of rhizobial strains isolated from root nodules of chickpea in Ethiopia and their phenotypic groupings and phylogenetic positions.

Table 2. The effect of inoculation of rhizobia nodulating chickpea on shoot dry weight per plant (SDWP), shoot nitrogen content per plant (SNCP), nodule number per plant (NNP), nodule dry weight per plant (NDWP) and the percent symbiotic effectiveness (SE%) under control conditions using sand culture

Figure 1. Map of Ethiopia showing soil sampling sites

Figure 2. Neighbor-joining phylogenetic tree based on *16S rRNA* (a) and concatenated (*atpD*, *glnII*, *recA* and *gyrB*) (b) gene sequences showing the relationships among chickpea microsymbionts tested strains, and recognized species of the genus *Mesorhizobium*. The group (I – IV) generated on the concatenated gene-based phylogeny. Bootstrap values 70% (based on 1000 replications) are shown at each node. Bars indicate the % of estimated nucleotide substitutions per site. *M*: *Mesorhizobium*. *R*: *Rhizobium*

Figure 3. Neighbor-joining phylogenetic trees based on *recA* (a), *atpD* (b), *glnII* (c) and *gyrB*(d) gene sequences showing the relationships among the different chickpea microsymbionts and recognized species of the genus *Mesorhizobium*. Bootstrap values over 70% (based on 1000 replications) are shown at each node. Bars indicate the % of estimated nucleotide substitutions per site. *M*: *Mesorhizobium*.

Figure 4. Neighbor-joining phylogenetic trees based on sequences of symbiosis-related genes *nodA* (a) and *nifH*(b) genes, showing the relationships among the different chickpea isolates and recognized species of the genus *Mesorhizobium*. Bootstrap values over 70% (based on 1000 replications) are shown at each node. Bars indicate the % of estimated substitutions expected number of changes per site. *M*: *Mesorhizobium*.

234x171mm (96 x 96 DPI)

573x602mm (78 x 78 DPI)

254x190mm (96 x 96 DPI)

Strain	Geographic origin	Dendrogram cluster ^a	Phylogenetic assignments	
			Genospecies ^b	Assignments
CP1	Ele (SE)	I	–	–
CP6	Ele (SE)	I	–	–
CP8	Ele (SE)	I	III	<i>M. shonense</i>
CP10	Ele (SE)	I	III	<i>M. shonense</i>
CP12	Gogeti (SE)	I	III	<i>M. shonense</i>
CP19	Gogeti (SE)	I	–	–
CP20	Gogeti (SE)	I	–	–
CP27	Gogeti (SE)	I	–	–
CP30	Taba (SE)	I	III	<i>M. shonense</i>
CP41	Taba (SE)	I	II	<i>M. abyssinica</i>
CP44	Taba (SE)	I	–	–
CP49	Taba (SE)	I	II	<i>M. abyssinica</i>
CP51	Bodity (SE)	I	III	<i>M. shonense</i>
CP61	Bodity (SE)	III	–	–
CP65	Bodity (SE)	I	–	–
CP69	Jole (SE)	I	III	<i>M. shonense</i>
CP74	Jole (SE)	IV	–	–
CP79	Jole (SE)	I	–	–
CP82	Jole (SE)	I	–	–
CP84	Akaki (CE)	I	IV	<i>Mesorhizobium</i> sp.
CP97	Akaki (CE)	I	–	–
CP98	Akaki (CE)	II	IV	<i>Mesorhizobium</i> sp.
CP99	Akaki (CE)	IV	–	–
CP100	Dukem (CE)	IV	–	–
CP105	Dukem (CE)	I	IV	<i>Mesorhizobium</i> sp.
CP109	Dukem (CE)	I	–	–
CP122	Seladingay (CE)	I	–	–
CP123	Seladingay (CE)	I	I	<i>M. ciceri</i>
CP125	Seladingay (CE)	II	I	<i>M. ciceri</i>
CP126	Ataye (CE)	III	–	–
CP130	Ataye (CE)	III	IV	<i>Mesorhizobium</i> sp.
CP132	Ataye (CE)	I	–	–
CP133	Asfachew (CE)	I	–	–
CP134	Asfachew (CE)	I	IV	<i>Mesorhizobium</i> sp.
CP135	Asfachew (CE)	I	–	–
CP136	Armania (CE)	II	IV	<i>Mesorhizobium</i> sp.
CP139	Armania (CE)	I	–	–
CP140	Armania (CE)	I	–	–
CP143	Legeberi (CE)	III	–	–
CP148	Legeberi (CE)	I	I	<i>M. ciceri</i>
CP150	Aleltu (CE)	III	–	–
CP151	Aleltu (CE)	III	IV	<i>Mesorhizobium</i> sp.

SE, South Ethiopia; and CE, Central Ethiopia; *M.*, *Mesorhizobium*; ^a Dendrogram highlighting the phenotypic similarities among rhizobial strains; ^b Genospecies defined based on phylogenetic tree constructed from the concatenated genes sequences (*recA*, *atpD*, *glnII*, and *gyrB*) tree.

Isolate Code	SDWP ^a (g)	SE	SNCP ^a (mg)	NNP ^a	NDWP ^a (mg)
CP1	0.42 ± 0.08 ^{H-L}	43	11.4 ± 2.9 ^{P-U}	18.3 ± 2.0 ^N	65.0 ± 9.5 ^{J-O}
CP6	0.54 ± 0.09 ^{D-L}	54	16.2 ± 3.3 ^{L-T}	46.0 ± 8.2 ^{A-G}	84.3 ± 20.2 ^{F-O}
CP8	0.81 ± 0.07 ^{A-C}	82	32.2 ± 2.0 ^{C-F}	26.0 ± 3.0 ^{L-M}	187.0 ± 58.0 ^{AB}
CP10	0.43 ± 0.04 ^{H-L}	43	11.8 ± 1.5 ^{P-U}	17.3 ± 4.3 ^N	68.3 ± 7.9 ^{I-O}
CP12	0.46 ± 0.08 ^{G-L}	46	13.0 ± 3.1 ^{P-U}	27.7 ± 6.7 ^{E-M}	80.7 ± 5.3 ^{H-O}
CP19	0.56 ± 0.16 ^{C-K}	56	16.9 ± 5.1 ^{L-S}	28.3 ± 2.5 ^{I-N}	110.0 ± 8.9 ^{D-K}
CP20	0.34 ± 0.05 ^{K-L}	34	9.7 ± 1.4 ^{R-U}	20.0 ± 3.3 ^{MN}	57.7 ± 18.7 ^{K-O}
CP27	0.51 ± 0.08 ^{E-J}	51	14.9 ± 1.5 ^{P-U}	28.0 ± 9.8 ^{I-M}	97.0 ± 12.1 ^{F-N}
CP30	0.44 ± 0.02 ^{H-L}	44	12.2 ± 1.7 ^{P-U}	30.0 ± 3.8 ^{E-L}	101.3 ± 1.5 ^{E-N}
CP41	0.98 ± 0.28 ^A	99	41.8 ± 10.2 ^{AB}	59.7 ± 15.2 ^A	207.7 ± 36.2 ^A
CP44	0.57 ± 0.17 ^{B-K}	58	18.4 ± 6.7 ^{K-R}	30.7 ± 7.1 ^{F-N}	111.3 ± 8.6 ^{D-K}
CP49	0.72 ± 0.15 ^{B-F}	72	26.4 ± 5.5 ^{D-J}	26.7 ± 8.6 ^{I-N}	153.3 ± 51.2 ^{B-E}
CP51	0.46 ± 0.03 ^{F-L}	46	13.4 ± 1.8 ^{O-U}	40.0 ± 5.8 ^{C-L}	106.7 ± 10.6 ^{F-L}
CP61	0.63 ± 0.04 ^{B-I}	64	22.5 ± 1.8 ^{H-N}	29.3 ± 4.8 ^{G-L}	143.3 ± 47.0 ^{B-F}
CP65	0.33 ± 0.02 ^{KL}	33	7.8 ± 0.9 ^{TU}	42.7 ± 3.9 ^{B-J}	45.7 ± 9.1 ^{NO}
CP69	0.36 ± 0.09 ^{J-K}	36	9.1 ± 1.8 ^{S-U}	44.7 ± 3.5 ^{A-H}	49.0 ± 7.6 ^{MO}
CP74	0.35 ± 0.04 ^{J-K}	36	8.7 ± 1.4 ^{S-U}	17.0 ± 3.9 ^N	52.3 ± 5.5 ^{L-O}
CP79	0.62 ± 0.14 ^{B-I}	63	22.0 ± 6.1 ^{H-O}	29.7 ± 8.6 ^{H-N}	135.7 ± 34.2 ^{B-H}
CP82	0.52 ± 0.04 ^{E-L}	53	15.6 ± 2.2 ^{L-U}	47.3 ± 8.2 ^{A-D}	116.3 ± 10.3 ^{C-J}
CP84	0.33 ± 0.06 ^{K-L}	33	10.8 ± 3.5 ^{Q-U}	18.7 ± 1.1 ^N	70.0 ± 6.9 ^{I-O}
CP97	0.83 ± 0.08 ^{AB}	84	12.2 ± 3.7 ^{P-U}	34.7 ± 5.4 ^{D-L}	103.7 ± 30.1 ^{E-M}
CP98	0.56 ± 0.10 ^{C-K}	57	17.3 ± 3.6 ^{K-T}	42.3 ± 9.0 ^{B-K}	142.3 ± 39.8 ^{B-F}
CP99	0.44 ± 0.10 ^{H-L}	44	33.2 ± 2.4 ^{DC}	31.3 ± 14.6 ^{D-N}	63.0 ± 23.9 ^{J-O}
CP100	0.56 ± 0.17 ^{C-K}	57	17.2 ± 5.8 ^{K-T}	43.3 ± 9.2 ^{B-I}	168.3 ± 47.0 ^{A-C}
CP105	0.81 ± 0.09 ^{A-C}	82	32.3 ± 5.2 ^{C-E}	47.0 ± 4.6 ^{A-F}	163.3 ± 16.2 ^{A-D}
CP109	0.33 ± 0.02 ^{KL}	33	18.0 ± 1.6 ^{J-R}	34.6 ± 4.7 ^{D-L}	48.9 ± 8.1 ^{MO}
CP122	0.54 ± 0.08 ^{D-L}	54	19.5 ± 1.5 ^{I-Q}	43.3 ± 7.2 ^{B-I}	67.3 ± 26.9 ^{I-O}
CP123	0.57 ± 0.03 ^{B-K}	58	27.0 ± 3.7 ^{C-I}	43.0 ± 5.2 ^{B-I}	94.4 ± 26.3 ^{F-O}
CP125	0.59 ± 0.07 ^{B-J}	60	29.7 ± 1.2 ^{C-H}	42.7 ± 8.4 ^{B-J}	86.7 ± 6.2 ^{G-O}
CP126	0.73 ± 0.10 ^{B-E}	74	23.7 ± 9.9 ^{H-M}	51.3 ± 9.0 ^{A-C}	61.9 ± 17.1 ^{JK}
CP130	0.79 ± 0.03 ^{A-D}	79	31.2 ± 3.8 ^{C-G}	58.3 ± 15.4 ^{AB}	95.5 ± 41.5 ^{F-O}
CP132	0.66 ± 0.30 ^{B-H}	66	25.7 ± 6.7 ^{D-K}	46.3 ± 7.5 ^{A-F}	121.0 ± 21.9 ^{C-I}
CP133	0.80 ± 0.11 ^{A-C}	81	29.5 ± 4.3 ^{C-H}	57.7 ± 8.6 ^{AB}	179.8 ± 42.0 ^{AB}
CP134	0.70 ± 0.17 ^{B-G}	71	22.4 ± 8.5 ^{H-N}	51.7 ± 9.5 ^{A-C}	117.2 ± 16.2 ^{C-J}
CP135	0.79 ± 0.08 ^{A-D}	79	22.8 ± 4.0 ^{H-N}	37.7 ± 5.5 ^{C-L}	56.6 ± 22.9 ^{K-O}
CP136	0.63 ± 0.24 ^{B-I}	64	20.2 ± 5.7 ^{I-P}	47.0 ± 12.5 ^{A-E}	67.3 ± 26.9 ^{I-O}
CP139	0.64 ± 0.11 ^{B-H}	65	27.2 ± 7.8 ^{C-H}	47.3 ± 11.1 ^{A-D}	138.6 ± 44.2 ^{B-G}
CP140	0.60 ± 0.19 ^{B-J}	60	18.7 ± 1.5 ^{I-Q}	26.3 ± 1.6 ^{K-N}	40.5 ± 18.4 ^O
CP143	0.73 ± 0.18 ^{B-E}	74	15.2 ± 2.7 ^{M-U}	42.3 ± 6.6 ^{B-K}	69.7 ± 19.9 ^{I-O}
CP148	0.58 ± 0.01 ^{B-K}	59	24.2 ± 2.7 ^{P-U}	32.7 ± 4.7 ^{E-N}	52.7 ± 15.8 ^{L-O}
CP150	0.48 ± 0.09 ^{E-L}	49	35.2 ± 5.2 ^{BC}	35.0 ± 0.5 ^{D-M}	52.8 ± 11.7 ^{L-O}
CP151	0.67 ± 0.06 ^{B-H}	67	11.8 ± 2.2 ^{P-U}	60.3 ± 7.6 ^A	93.0 ± 11.5 ^{F-O}

Control ^b	0.30± 0.02 ^L	30	6.9 ±0.5 ^U		
N ^c +	0.99± 0.12 ^A	100	42.8 ± 3.9 ^A		
CV (%) ^d	21.6		21.6	21.4	27.7
Significant ^e	***		***	***	***

^a Values are mean ± SE of three replicates, mean values followed by the same letters in each column and treatment showed no significant difference by DMRT (p = 0.05). ^b Control: without chemical and biological fertilizers. ^c N: with optimum amount of nitrogen fertilizer. ^d CV (%): percent coefficient of variability. ^e Significant: probability level. *** showed significant difference at 0.001 probability levels.

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Genetic and phenotypic diversity of rhizobia nodulating Chickpea (*Cicer arietinum* L.) in soils from southern and central Ethiopia

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Table S1. Differentiating phenotypic traits of 42 chickpea isolates

Cluster ^a					Cluster				
Characteristic	I (n ^b = 30)	II (n= 3)	III (n= 6)	IV (n= 3)	Characteristic	I (n= 30)	II (n= 3)	III (n= 6)	IV (n= 3)
Temperature tolerance					Carbohydrate Utilization ^c				
5°C	0	0	0	0	Dextrin	26	2	5	2
15°C	12	0	4	0	Maltose	19	1	4	3
20-30°C	30	3	6	3	Xylose	19	3	6	3
35°C	25	2	6	3	Rhamnose	21	3	6	3
40°C	5	0	1	0	Raffinose	10	0	5	2
NaCl tolerance					Soribitol	27	3	6	3
1%	30	3	6	3	Dulcitol	27	3	6	3
2%	25	3	6	2	Arabinose	15	2	4	3
3%	0	0	3	1	Malonic Acid	6	0	1	1
4%	0	0	2	0	Xylitol	9	1	5	2
5%	0	0	2	0	Myo-inositol	28	2	6	3
pH tolerance					Glycine	8	1	2	2
4	1	2	1	0	Phosphate solubilization				
4.5	7	2	4	0	Phosphate	12	0	4	2
5	13	3	6	0	Antibiotics Resistance in µg ml^{-1c}				
5.5	16	3	6	3	Chloramphenicol (5)	29	3	6	3
8	30	3	6	3	Chloramphenicol (15)	14	3	6	3
8.5	30	3	6	3	Kanamycin (15)	19	2	4	0
9	26	1	3	1	Trimethoprim (200)	24	3	2	3
9.5	19	1	3	0	Novobiocin (5)	9	1	6	3
10	17	0	1	0	Heavy Metal Resistance in µg ml^{-1c}				
Nitrogen utilization ^c					Mn (500)	4	1	3	3
Serine	6	2	3	3	Al (500)	7	3	6	1
Thymidine	27	3	6	3	Pb (500)	7	1	1	3
Phenylalanine	29	3	6	3	Co (100)	10	1	5	3
Isoleucine	27	2	6	3	Cu (100)	2	1	1	0
Aspartic Acid	28	2	6	3					
Pyroglutamic Acid	29	3	6	3					
Proline	27	2	6	3					
Arginine	20	2	6	3					

^a Clusters I, II, III and IV are as defined in Fig. 3. ^b total number of isolates per cluster and the column numbers are those of the isolates giving a positive reaction. ^c substrates utilized or not utilized by all strains were omitted from the table.

Table S2. Physiological and biochemical individual strain data.

Table S2.1. Physiological characteristics of the chickpea isolates

Isolate code	Temperature						Salt					pH									
	5OC	15OC	20-30OC	35OC	40OC	45OC	1NaCl	2 NaCl	3 NaCl	4 NaCl	5 NaCl	4.0pH	4.5pH	5.0pH	5.5pH	8.0pH	8.5pH	9.0pH	9.5pH	10.0pH	
CP01	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	
CP06	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	
CP08	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	
CP10	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	
CP12	-	-	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	
CP19	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	
CP20	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	
CP27	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	
CP30	-	-	+	-	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	
CP41	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	
CP44	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	
CP49	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	
CP51	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	
CP61	-	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	
CP65	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	
CP69	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	
CP74	-	+	+	+	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	-	
CP79	-	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	-	
CP82	-	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	
CP84	-	+	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	
CP97	-	+	+	+	+	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	
CP98	-	+	+	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	
CP99	-	-	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	
CP100	-	-	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	
CP105	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	
CP109	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	
CP113	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	
Cp123	-	-	+	+	-	-	+	+	-	-	-	+	-	+	-	+	+	+	+	+	
Cp125	-	-	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	-	
Cp126	-	-	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	-	-	
Cp130	-	-	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	
Cp132	-	-	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	
Cp133	-	-	+	+	-	-	+	+	-	-	-	-	-	+	+	+	+	+	-	-	

Cp134	-	-	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-
Cp135	-	-	+	+	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-
Cp136	-	+	+	+	+	-	+	+	-	-	-	+	-	+	+	+	+	+	-	-
Cp139	-	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-
Cp140	-	+	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+
Cp143	-	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Cp148	-	+	+	+	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+
Cp150	-	+	+	+	+	-	+	+	+	-	+	-	-	+	+	+	+	+	+	+
Cp151	-	-	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+
HAMBI 1129	-	-	-	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+
HAMBI 2148	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
HAMBI 12	-	-	-	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+
HAMBI 2314	-	-	-	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+
HAMBI 2096	-	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	-	-	-
HAMBI 2409	-	+	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-

Table S2.2 Carbohydrate Utilization Test of the chickpea isolates

Isolate code	Threhalose	Dextrin	Maltose	Sucrose	Xylose	Rhamnose	Raffinose	Sorbitol	Citric Acid	Dulcitol	Arabinose	Malonic Acid	Fructose	Xylitol	Mannitol	Myo-isitol	Galactose	Glycin	Glucose
CP01	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+
CP06	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	+	-	+
CP08	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+
CP10	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+
CP12	+	+	+	+	-	+	-	+	-	+	+	-	+	-	+	+	+	+	+
CP19	+	+	+	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+
CP20	+	+	+	+	-	+	-	-	-	+	-	-	+	-	+	+	+	-	+
CP27	+	+	-	+	-	+	-	+	-	+	-	-	+	-	+	+	+	-	+
CP30	+	+	+	+	-	+	-	+	-	+	-	+	+	-	+	+	+	-	+
CP41	+	+	-	+	-	+	-	+	-	+	-	+	+	-	+	+	+	-	+
CP44	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	+
CP49	+	+	+	+	-	-	+	+	-	+	-	-	+	-	+	+	+	+	+
CP51	+	+	+	+	-	-	-	+	-	+	-	+	+	-	+	+	+	-	+
CP61	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	+
CP65	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
CP69	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
CP74	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	+
CP79	+	+	-	+	+	-	-	+	-	-	-	-	+	-	+	+	+	-	+
CP82	+	+	-	+	+	-	-	+	-	+	-	-	+	-	+	+	+	-	+
CP84	+	+	+	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+
CP97	+	+	+	+	+	+	-	+	-	+	-	-	+	+	+	-	+	+	+
CP98	+	+	+	+	+	+	-	+	-	+	-	-	+	+	+	-	+	+	+
CP99	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+
CP100	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
CP105	+	+	+	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+
CP109	+	+	+	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+
CP113	+	-	+	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+
Cp123	+	-	-	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+
Cp125	+	-	-	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+
Cp126	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
Cp130	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+
Cp132	+	+	-	+	+	-	-	+	-	-	-	-	+	+	+	+	+	-	+
Cp133	+	+	-	+	+	+	-	+	-	-	-	-	-	-	+	+	+	-	+
Cp134	+	+	-	+	+	-	+	-	-	+	-	-	+	-	+	+	+	-	+
Cp135	+	-	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-	+
Cp136	+	+	-	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	+

Cp139	+	-	-	+	+	-	-	-	-	+	-	-	+	-	+	-	+	-	+
Cp140	+	+	-	+	+	-	-	+	-	+	-	-	+	+	+	+	+	-	+
Cp143	+	+	-	+	+	+	-	+	-	+	-	-	+	+	+	+	+	-	+
Cp148	+	+	-	+	+	+	-	+	-	+	+	-	+	+	+	+	+	-	+
Cp150	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+
Cp151	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+
HAMBI1 129	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	-	+
HAMBI2 148	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	+
HAMBI1 2	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+
HAMBI2 314	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	-	+
HAMBI2 096	+	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+	-	+	+
HAMBI2 409	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	-

Table S2.3. Nitrogen Source Utilization and phosphate solubilization test of the chickpea isolates

Isolate code	Clusters	Serine	Inosine	Tristamine	Asparagine	Glutamic Acid	Threonine	Thymidine	Alanine	Phenylalanine	Isoleucine	Aspartic Acid	Pyroglutamic Acid	Proline	Uredine	Arginine	Leucine	Phosphate solu
CP01	I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
CP06	I	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-
CP08	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP10	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP12	I	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
CP19	I	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
CP20	I	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
CP27	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP30	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP41	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP44	I	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
CP49	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP51	I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
CP61	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP65	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP69	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP74	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP79	I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
CP82	I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
CP84	I	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
CP97	I	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	-
CP98	II	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	-
CP99	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP100	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CP105	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CP109	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CP113	I	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	-
Cp123	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cp125	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Cp126	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Cp130	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Cp132	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Cp133	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cp134	I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Cp135	I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+

Cp136	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cp139	I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Cp140	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cp143	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cp148	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cp150	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cp151	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HAMBI 1129	II	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
HAMBI 2148	U C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
HAMBI 12	III	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
HAMBI 2314	U C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
HAMBI 2096	II	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
HAMBI 2409	III	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-

Table S2.4. Intrinsic Antibiotic and Heavy Metal Resistance ($\mu\text{g/ml}$) of the chickpea Isolates

Isolate code	Antibiotics Resistance														Heavy Metal Resistance					
	Chl		Ery		Str		Spec		Kan	Linc	Trim	Neo		Nov	Mn	Al	Pb	Co	CuCl ₂	ZnCl ₂
	5	15	10	20	40	80	250	500	15	15	200	5	20	5						
CP01	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP06	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP08	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP10	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP12	+	-	+	+	-	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-
CP19	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	+	-	-	-
CP20	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	+	-	-	-	-
CP27	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP30	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
CP41	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	+	+	-	-
CP44	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	+	-	-
CP49	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	+	-	-
CP51	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	+	-	+	-	-
CP61	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-
CP65	+	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-
CP69	+	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-
CP74	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-
CP79	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-
CP82	+	+	+	+	-	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-
CP84	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP97	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-
CP98	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-
CP99	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	-
CP100	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	-
CP105	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP109	+	-	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-
CP113	+	+	+	+	-	-	-	-	+	-	+	+	+	-	+	-	+	+	-	-
Cp123	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	+	-	-	-	-
Cp125	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	+	-	-	-	-
Cp126	+	+	+	+	-	-	-	-	+	-	-	+	+	+	-	+	-	-	-	-
Cp130	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	-
Cp132	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-
Cp133	+	-	+	+	-	-	-	-	+	-	+	+	+	-	+	-	-	-	-	-
Cp134	-	-	+	+	-	-	-	-	+	-	-	+	+	+	-	-	-	+	+	-
Cp135	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-
Cp136	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	+	-	-	-	-

Cp139	+	-	+	+	-	-	-	-	+	-	-	+	+	+	+	-	-	-	-	-
Cp140	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	+	-	+	-	-
Cp143	+	+	+	+	-	-	-	-	+	-	-	+	+	+	-	+	-	+	+	-
Cp148	+	+	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-
Cp150	+	+	+	+	-	-	-	-	+	-	-	+	+	+	+	+	-	+	-	-
Cp151	+	+	+	+	-	-	-	-	+	-	-	+	+	+	+	+	-	+	-	-
HAMBI 1129	+	-	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	+	-	-
HAMBI 2148	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
HAMBI 12	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
HAMBI 2314	+	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	-
HAMBI 2096	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+
HAMBI 2409	-	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+	+

Figure S1. Dendrogram highlighting the phenotypic similarities among rhizobial strains isolated from chickpeas from different areas of Ethiopia. Numbers I, II, III and IV denote the phenotypic clusters. The reference strains are: ML: *Mesorhizobium loti* HAMBI (1129T), EM: *Ensifer meliloti* (HAMBI 2148T), RL: *Rhizobium leguminosarum* (HAMBI 12T), BJ: *Bradyrhizobium japonicum* (HAMBI 2314T), MM: *M. mediterraneum* (HAMBI 2096T) and RH: *Rhizobium huautlense* (HAMBI 2409T).