Characterization of Rhizobia for the Improvement of Soybean Cultivation at Cold Conditions in Central Europe

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In central Europe, soybean cultivation is gaining increasing importance to reduce protein imports from overseas and make cropping systems more sustainable. In the field, despite the inoculation of soybean with commercial rhizobia, its nodulation is low. In many parts of Europe, limited information is currently available on the genetic diversity of rhizobia and, thus, biological resources for selecting high nitrogen-fixing rhizobia are inadequate. These resources are urgently needed to improve soybean production in central Europe. The objective of the present study was to identify strains that have the potential to increase nitrogen fixation by and the yield of soybean in German soils. We isolated and characterized 77 soybean rhizobia from 18 different sampling sites. Based on a multilocus sequence analysis (MLSA), 71% of isolates were identified as Bradyrhizobium and 29% as Rhizobium. A comparative analysis of the nodD and nifH genes showed no significant differences, which indicated that the soybean rhizobia symbiotic genes in the present study belong to only one type. One isolate, GMF14 which was tolerant of a low temperature (4°C), exhibited higher nitrogen fixation in root nodules and a greater plant biomass than USDA 110 under cold conditions. These results strongly suggest that some indigenous rhizobia enhance biological nitrogen fixation and soybean yield due to their adaption to local conditions.

Key words: Germany, Glycine max, nitrogen fixation, rhizobia, MLSA

Rhizobia form a symbiotic association with leguminous plants, which results in the formation of an organ called a nodule on the root of the host plant. Inside the root nodule, rhizobia convert atmospheric nitrogen to ammonia, which may then be utilized for plant growth. Biological nitrogen fixation (BNF) may reduce chemical fertilization and its detrimental effects on soil properties and the environment (Zahran, 1999). Approximately 120 rhizobial species in 15 genera of α-proteobacteria and β-proteobacteria have been documented for diverse legumes (Andrews and Andrews, 2017). According to the literature, slow growers include Bradyrhizobium japonicum, B. elkanii, B. yuanmingense, and B. liaoningense, while fast growers comprise Mesorhizobium tianhanense and Ensifer fredii, which are symbionts of soybean (Glycine max [L.]) grown in different regions of the world (Jordan, 1982; Kuykendall et al., 1992; Xu et al., 1995; Chen et al., 2000; Camacho et al., 2002; Yang et al., 2006; Man et al., 2008; Yang and Zhou, 2008; Han et al., 2009; Wang et al., 2009; Andrews and Andrews, 2017).

Soybean is the most important legume in the world and its seeds contain 40% protein and 20% oil (Arslanoglu et al., 2011). The northeastern region of China, the Korean peninsula, and Japan are the origin of soybean (Li et al., 2010). China, USA, Brazil, and Argentina are the largest producers in the world. In recent years, the demand for soybean has increased in Europe due to increasing consumer demand for soy as a healthy alternative to meat as well as to reduce the import of genetically modified soybean products from USA and South America. However, soybean cultivation is still relatively new in central Europe (Zimmer et al., 2016) even though areas of soybean cultivation increased two-fold between 2013 and 2018 (Krautgartner et al., 2018). There is also large potential to grow soybean under the cooler and less favorable environments in northern parts of Europe (Singleton et al., 1985). The rhizobia inoculation of soybean is a sustainable practice to induce atmospheric nitrogen fixation and subsequently improve crop productivity and soil fertility (Keyser and Li, 1992). Commercialized rhizobia have been utilized to inoculate soybeans in several countries across Europe (Zimmer et al., 2016). Nevertheless, when grown for the first time under these conditions, soybean showed low nodule formation and a poor yield (Giller, 2001). Commercial inoculants containing B. japonicum are used in Germany; however, even after inoculation, soybean often forms a smaller number of nodules (Zimmer et al., 2016) than that in Japan. These findings indicate that the rhizobia used as inoculants are not suitable for the local environmental conditions of Germany, and, thus, the development of biofertilizers using native rhizobia that are more appropriate for local conditions is needed. Dif-
Different environmental factors affect legume and rhizobia symbioses, such as temperature (Zhang and Smith, 1996; Zimmer et al., 2016), pH (Lin et al., 2012), salinity (Song et al., 2012), and the amount of nitrogen in soil (Ray et al., 2006). A low soil temperature also affects nodulation (Zhang and Smith, 1996; Zhang et al., 1996). The optimal temperature for BNF ranges between 20 and 30°C (Bordeleau and Prévost, 1994). Therefore, soybean does not show good nodulation or nitrogen fixation when soil temperature is between 8 and 15°C (Zimmer et al., 2016). Soybean cultivars that adapt to low temperatures have been successfully generated through breeding in central Europe; however, efficient symbionts have not yet been established (Zimmer et al., 2016). Therefore, it is important to isolate German soybean-rhizobia that are highly efficient at fixing nitrogen and increasing biomass at low temperatures, which may reduce the use of chemical fertilizers as well as negative environmental impacts (Watson et al., 2017).

An analysis of the genetic diversity of rhizobia is important for increasing rhizobial inoculation efficiency under various environmental conditions. There is currently no information on the genetic resources of native rhizobia in Germany.

The objectives of the present study were to investigate the genetic background of soybean-associated root nodule bacteria in some German soils and identify efficient isolates that adapt and increase soybean production under local conditions.

We isolated soybean rhizobia from soil samples derived from soybean-cultivated and non-cultivated locations in the northern part of Germany. The isolates obtained were subjected to a phenotypic characterization of their stress tolerance (temperature, salt, and pH) and symbiotic performance. To understand the genetic background of these isolates, we analyzed three housekeeping genes (16S rRNA, recA, and atpD) and two symbiotic genes (nodD and nifH). The symbiotic properties of some isolates were assessed under optimal temperature conditions and cold conditions.

Materials and Methods

Soil samples and collection sites

Eighteen soil samples were collected in November 2017 from 9 locations in the northern part of Germany (Fig. 1, Table 1). Samples were taken from different fields belonging to two different categories based on the presence (soil samples No. 10–18 in Table 1) or absence (No. 1–9) of a soybean cultivation history. At these locations, the pH of the corresponding soil samples ranged between 6.0 and 7.7, namely, slightly acidic to neutral. Prior to the isolation of rhizobia, all soil samples were transferred to the laboratory and kept at 4°C.

Isolation of rhizobia from German soils

Two soybean cultivars “Merlin” and “Enrei” were used as trap hosts to isolate rhizobial strains. “Enrei” is a Japanese cultivar that is used worldwide under optimal cultivation. “Merlin” is a domesticated cultivar under German conditions and the most common cultivar for relatively cool growing conditions in Germany (Zimmer et al., 2016). Soybean seeds were surface sterilized by immersion in 70% ethanol for 1 min, followed by immersion in 3% sodium hypochlorite for 2 min and rinsing five times with sterile water. Sterilized nodules were crushed in 0.5 mL of 15% glycerol and aliquots of the resultant suspensions were streaked on 1.5% YEM medium containing 0, 1, 2, 3, and 4% (w/v) NaCl. The growth of rhizobia was examined after 7–14 d of incubation at 28°C. The temperature tolerance with distilled water. Thereafter, surface-sterilized seeds were incubated at 28°C for 3 d under dark conditions for germination. Germinated seeds were then transferred to 300-mL glass jars containing sterile vermiculite (Hirukon S, Hiruishi Kagaku Kogyo) watered with a sterilized nitrogen-free solution and irrigated to 60% of field capacity (Broughton and Dilworth, 1970). Inoculation was performed by the addition of 5 g of the soil sample diluted in 10 mL of sterilized water to each glass jar. Inoculated jars were transferred to a growth chamber and soybean plants were grown at 25°C for 4 weeks under a 16-h light/8-h dark photoperiod. After 28 d, prior to root nodule collection, entire soybean plants were removed from the glass jars and washed with tap water to remove vermiculite. Root nodules were detached and surface sterilized by immersion in 70% ethanol for 1 min, followed by immersion in 3% sodium hypochlorite for 2 min and rinsing five times with sterile water. Sterilized nodules were crushed in 0.5 mL of 15% glycerol and aliquots of the resultant suspensions were streaked on 1.5% yeast extract mannitol agar (YEM) plates (Somasegaran and Hoben, 1994). Plates were incubated at 28°C for 3–7 d. Single colonies were picked up, checked for purity by repeated streaking onto fresh YEM plates, and then used for further analyses.

Abiotic stress tolerance test

The different phenotypic characteristics of the isolated bacteria, such as tolerance to low and high temperatures, NaCl levels, and low and high pH, were assessed as described previously (Djididi et al., 2011). The NaCl stress tolerance of isolates was tested on YEM medium containing 0, 1, 2, 3, and 4% (w/v) NaCl. Acid and alkaline stress tolerance was tested on YEM plates in which pH was adjusted to 4.5, 5, 6.8, 8, and 10 by the addition of HCl (1 M) or NaOH (1 M), respectively. The growth of rhizobia was examined after 7–14 d of incubation at 28°C. The temperature tolerance
Table 1. Soil information from sampling sites

| Soil sample no. | Site Name       | Collection Date | Longitude X | Latitude Y | Soil rating index | Clay (%) | Silt (%) | Sand (%) | Crop sequence history | Soybean cultivation | pH      |
|-----------------|-----------------|-----------------|-------------|------------|-------------------|----------|----------|----------|-----------------------|--------------------|---------|
| 1               | Sachsendorf     | 2017/11/30      | 14.4690     | 52.5045    | 54                | 27       | 49       | 25       | Oiled rape - winter wheat | No                 | 7.0±0.15 |
| 2               | Dedelow         | 2017/11/9       | 13.8016     | 53.3696    | 45                | 10       | 32       | 58       | Winter wheat - maize - winter wheat - oiled rape - winter barley - winter wheat | No                 | 6.9±0.05 |
| 3               | Paulineau       | 2017/11/22      | 12.6689     | 52.6474    | 45                | 9        | 20       | 72       | Oiled rape - winter barley - winter wheat | No                 | 6.8±0.02 |
| 4               | Nossen          | 2017/11/17      | 13.2693     | 51.0567    | 63                | 16       | 81       | 4        | Pea - oat - clover - winter wheat - potato | No                 | 6.5±0.06 |
| 5               | Köllitsch       | 2017/11/17      | 13.0942     | 51.5136    | 64                | 14       | 34       | 52       | Pea - winter wheat - alfalfa - alfalfa - alfalfa - maize - winter wheat | No                 | 6.6±0.02 |
| 6               | Gollmbach       | 2017/11/28      | 9.5831      | 51.9029    | 75                | 15       | 77       | 9        | Maize - winter wheat - winter barley - maize | No                 | 6.8±0.02 |
| 7               | Schneega        | 2017/11/28      | 10.8330     | 52.9016    | 25                | 3        | 5        | 93       | Flower mixtures - crops | No                 | 6.0±0.18 |
| 8               | Müncheberg      | 2017/11/30      | 14.1274     | 52.1516    | 29                | 4        | 10       | 86       | Grass - grass - grass | No                 | 6.4±0.07 |
| 9               | Müncheberg      | 2017/11/30      | 14.1287     | 52.5205    | 30                | 4        | 10       | 86       | Maize - alfalfa - winter rye - alfalfa - alfalfa - alfalfa | No                 | 6.7±0.07 |
| 10              | Müncheberg      | 2017/11/30      | 14.1227     | 52.5185    | 30                | 4        | 10       | 86       | Oat - grass/clover - oat - soybean - maize - alfalfa | 2014               | 6.8±0.06 |
| 11              | Müncheberg      | 2017/11/30      | 14.1281     | 52.5214    | 30                | 4        | 10       | 86       | Alfalfa - oat - soybean - maize - alfalfa - winter rye | 2015               | 6.5±0.06 |
| 12              | Müncheberg      | 2017/11/30      | 14.1326     | 52.5227    | 30                | 4        | 10       | 86       | Oat - soybean - winter barley - winter wheat | 2016               | 6.1±0.03 |
| 13              | Müncheberg      | 2017/11/30      | 14.1340     | 52.5223    | 30                | 4        | 10       | 86       | Soybean - winter wheat - oiled rape - winter rye | 2017               | 6.3±0.04 |
| 14              | Felrow          | 2017/11/29      | 14.2504     | 51.8359    | 27                | 5        | 15       | 80       | Winter rye - soybean | 2013               | 6.6±0.02 |
| 15              | Felrow          | 2017/11/29      | 14.2759     | 51.8526    | 27                | 5        | 15       | 80       | Maize - grass/clover - winter wheat - soybean - maize - grass/clover | 2014               | 6.3±0.02 |
| 16              | Felrow          | 2017/11/29      | 14.2720     | 51.8293    | 43                | 5        | 15       | 80       | Grass/clover - winter wheat - soybean - maize - grass/clover - grass/clover | 2015               | 6.3±0.02 |
| 17              | Felrow          | 2017/11/29      | 14.2782     | 51.8543    | 31                | 5        | 15       | 80       | Winter wheat - soybean - winter wheat - grass/ clover - winter wheat - lupini | 2016               | 6.4±0.02 |
| 18              | Felrow          | 2017/11/29      | 14.2412     | 51.8362    | 36                | 5        | 15       | 80       | Winter wheat - grass/clover - winter wheat - soybean | 2017               | 6.3±0.01 |

* The German soil rating index describes the yield potential of soil ranging from very poor soil with a rating of 10 to very good soil with a rating of 100.

Data on the soil rating index, soil texture, and crop sequence history were measured and monitored by research stations at each location.

of isolates was tested at 4, 15, 28, 37, and 45°C on YEM medium and bacterial growth was scored 7–14 d after the inoculation (Djedidi et al., 2011). These experiments were performed in three replications.

Genomic DNA preparation

Isolates were grown in YEM broth at 28°C for 2–7 d. Rhizobial cells were collected using a centrifuge and then washed twice with sterile distilled water. Genomic DNA was extracted using a Wizard Genomic DNA purification kit (Promega).

DNA amplification and sequencing

Polymerase chain reaction (PCR) amplification and sequencing of the DNA fragments of the 16S rRNA, recA, atpD, nodD, and nifH genes were performed. All genes were amplified by PCR using 10 ng purified DNA, EX taq™ (TaKaRa Bio). The pairs of primers utilized and amplification conditions are shown in Table S1. PCR products were purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics) and directly sequenced using the same primers used for PCR (Table S1). PCR products were sequenced using an ABI Prism 3500 Genetic Analyzer (Applied Biosystems), according to the manufacturer’s protocols.

Phylogenetic analyses

Nucleotide sequences from the 16S rRNA, recA, atpD, nodD, and nifH genes of the isolates were used to evaluate their phylogenetic relationships. To identify the most closely related DNA sequences, they were compared to the GenBank nucleotide sequence database using BLAST. Multiple sequence alignment of the nucleotide sequences and phylogenetic trees were performed using the following software: Genetyx version 11, Clustal W, and MEGA version 7 (Tamura et al., 2013). Phylogenetic trees were generated using the neighbor-joining method, the Kimura 2-parameter model. In the multilocus sequence analysis (MLSA) (Nzoué et al., 2009), the phylogenetic tree was constructed using the 16S rRNA, recA, and atpD gene sequences obtained.

Plant test

Rhizobial strains were tested for their nodulation ability and symbiotic performance on the soybean cultivars “Merlin” and “Enrei”. Seeds were surface-sterilized, as indicated in the isolation section. Seeds were allowed to germinate at 25°C for 3 d in the dark. Seedlings were then transferred to glass jars containing sterile vermiculite and a nitrogen-free solution. A bacterial suspension containing approximately 107 cells (2 mL plant–1) was used to inoculate each soybean plant. Plants were grown in a plant growth chamber at 25°C under a 16-h light/8-h dark photoperiod for 4 weeks.

To imitate soybean growth conditions in Germany, another plant test was performed under low temperature conditions. Since the soybean growing season (between May and July, during which the average temperature is 17–24°C in daytime and 7–12°C in nighttime) in Germany has a 10°C difference between daytime and nighttime, plants were grown at 20°C under 16 h of light and 10°C under 8 h of dark for 40 d. Two soybean cultivars, “Merlin” and “Sultana”, were cultivated under low temperature conditions. These two cultivars are common for relatively cool growing conditions in Germany.

After harvesting the plants, shoot dry weight, root dry weight, root nodule dry weight, and nodule number were assessed. Plant shoots, roots, and nodules were dried at 80°C for 48 h to obtain dry weights. In the acetylene reduction assay (ARA), after harvesting the plants, roots that contained root nodules were placed in 300-mL glass jars, which had 10% of the air removed and replaced with 10% acetylene (v/v). Root nodules were then incubated at 25°C for 30 min. The amount of ethylene was measured using a Shimadzu GC-2014 gas chromatograph (Shimadzu), equipped with a Porapak N column (Agilent Technologies).

Nucleotide sequence accession numbers

DNA sequences were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers LC434646 to LC434728 for
Results

Isolation of rhizobia from German soils

A total of 454 indigenous isolates were obtained from the root nodules of the two soybean cultivars, with the following repartition: 264 isolates from “Merlin” and 190 strains from “Enrei”. Plants inoculated with soil samples with no soybean cultivation history, such as soil sample no. 1 (Sachsendorf), 2 (Dedelow), 3 (Paulinenaue), 4 (Nossen), 6 (Golmbach), and 7 (Schnega), did not show root nodule formation. Additionally, inoculations with soil samples 5 (Köllitsch), 8 (Müncheberg), and 9 (Müncheberg) resulted in only a few nodules (Table S2). On the other hand, we observed nodules and isolated rhizobia from soils with a soybean cultivation history, such as soils from the Müncheberg (Broughton and Dilworth, 1970; Chen et al., 1991; Chen et al., 2000; Camacho et al., 2002) and Fehrow areas (Eckhardt et al., 1931; Chen et al., 1988; Frank et al., 2008; Djeididi et al., 2011; Chibeba et al., 2017), using the two soybean cultivars “Merlin” and “Enrei” (Table S2). All the isolates produced similar creamy colonies, with a transparent light-yellow color when grown on YEM medium at 28°C. Regarding their growth properties, 85.2% of isolates were slow glowing (5–7 d) and the remaining 14.8% had fast growing (1–3 d) characteristics (Table S2).

Abiotic stress tolerance of soybean rhizobia

The responses of the 454 isolates to the different levels of temperature, salinity, and pH varied. Extreme (low/high) temperature, high salinity, and acidic/alkaline pH tolerant strains were identified among the screened soybean rhizobia (Fig. 2). Their phenotypic characteristics were investigated in more detail. The majority of the strains were able to grow at a temperature range of 15–37°C (Fig. 2). However, only 33 strains grew at 4°C and 13 strains were able to grow at a...
high temperature of 44°C. These low and high temperature tolerant strains were isolated from the “Merlin” cultivar. None of the strains isolated from “Enrei” soybeans grew at the low (4°C) or high temperature (44°C). A large number, corresponding to 98.5%, of strains were able to grow in a wide pH range, varying between 4.5 and 10. Additionally, only 31 strains were able to grow on medium amended with 1% NaCl; 28 were isolated from “Merlin” and 3 from “Enrei”. Twenty-one strains grew at salinity levels ranging between 1 and 4% NaCl; 20 were isolated from the “Merlin” cultivar and only 1 from “Enrei” (Fig. 2).

Phylogenetic analysis of MLSA

Based on MLSA, 77 isolates were divided into two groups. The majority of the isolates were assigned to Bradyrhizobium (71.4%), and the remaining to Rhizobium (28.6%) (Fig. 3). In the Bradyrhizobium group, two subgroups were observed: subgroup A, containing 6 isolates from this study with reference stains B. japonicum USDA 6 and B. diazoefficiens USDA 110; subgroup B, containing 49 isolates with reference stains B. lupini, B. yuanningsense, B. pachyrhizzi, B. elkanii, and Bradyrhizobium sp. VAF1269. Twenty-two isolates belonged to the Rhizobium cluster, which was also separated into three subgroups: subgroup C, containing 12 strains with the reference strains R. tropici USDA 9030 and R. lusitanum P1-7; subgroup D, containing 6 strains with the reference strain R. pusense NRCPB 10; and subgroup E, containing 4 strains with the reference strain R. alamii.

Phylogenetic analysis of symbiotic genes for nodD and nifH

We were unable to amplify the nodD and nifH genes in 21 out of 22 Rhizobium isolates (based on MLSA). Therefore, these isolates were not included in the phylogenetic trees in Fig. 4 for nodD and Fig. 5 for nifH. The phylogenetic tree, built with the partial sequences of the nodD genes for 56 isolates, showed two groups (Fig. 4). The first group of Bradyrhizobium was composed of 55 isolates and the reference strains of Bradyrhizobium sp. VAF1269, USDA 6, and USDA 4. The second group consisted of only one Rhizobium isolate, GMM49, which had a nodD gene that was very similar to that of B. elkanii. The phylogenetic analysis of the nifH genes showed similar results (Fig. 5). All 56 isolates, except for GMM49, had identical nifH genes, forming a large cluster together with B. diazoefficiens strains. A nifH gene of GMM49 showed lower (98%) similarity to that of B. diazoefficiens.

Symbiotic performance under optimal temperature conditions

To elucidate the symbiotic properties of isolates, 44 representative strains; 22 belonging to Bradyrhizobium and 22 to Rhizobium, were selected based on MLSA phylogeny and inoculated into “Merlin” and “Enrei” (Fig. 3, Table 2). All plants inoculated with Bradyrhizobium isolates formed nodules (Table 2). Strains GMM71 and GMF14 induced a high biomass in both soybean cultivars. Regarding the “Merlin” cultivar inoculated with Bradyrhizobium isolates, the root nodule number ranged between 18.2±9.7 and 61.3±26.0 and ARA between 38.3±12.6 and 140.6±11.6 μmol C₂H₄ h⁻¹ g⁻¹ dry weight of nodules (Table 2). Concerning “Enrei”, the root nodule number ranged between 24.3±10.7 and 48.7±16.2 and ARA between 11.1±7.2 and 94.8±7.3 μmol C₂H₄ h⁻¹ g⁻¹ dry weight of nodules (Table 2). However, no significant differences were observed in root nodule numbers or ARA between the two soybean cultivars.

Strain GMF14, isolated from soil sample 18, showed the highest root nodule number (61.3±26.0) when inoculated into “Merlin”. The plant biomass induced by this isolate was significantly higher than that of control plants for both soybean cultivars. The highest ARA for “Merlin” was obtained with isolate GMF24 (subgroup B) with 140.6±11.6 μmol C₂H₄ h⁻¹ g⁻¹ per dry weight of nodules. Isolates GEM96, GMM71, GMM36, GMM 34, GEM108, GMF 42, and GMF14 were the most effective in terms of plant growth promotion because they increased the soybean biomass by almost 2-fold from that of the non-inoculated control.

In the 22 isolates belonging to the Rhizobium group, only one isolate, GMM49, which was closely related to R. tropici and R. lusitanum, induced nodule formation on “Merlin” soybeans (Table 2). Regarding the “Merlin” cultivar inoculated with strain GMM49, the root nodule number was 35.7±13.2 and ARA was 119±28.6 μmol C₂H₄ h⁻¹ g⁻¹ dry weight of nodules.

Symbiotic performance under low temperature conditions

To assess the abilities of some isolates for nodulation and nitrogen fixation on soybean plants under cold conditions, a second plant test was performed in which inoculated plants were maintained at low temperatures. Temperatures were set at 20°C in the daytime and 10°C at night, which corresponded to the average temperatures in Germany during the soybean sowing season (May to June). In this test, two German soybean cultivars “Merlin” and “Sultana”, known to be adapted to cold conditions, were inoculated with seven strains; GMF14, GMM71, GEM96, GMF42, GMM34, GMM36, and GMF57. These strains were selected because in the plant test under optimal temperature conditions, they significantly increased plant biomass from that with the control non-inoculated treatment, with the exception of strain GMF57 (Table 2). Among the selected strains, strains GMF14 and GMF57 grew at 4°C (Table S3). Additionally, when inoculated into “Merlin”, these strains showed no significant difference in terms of dry weight from the positive control USDA 110.

Under cold conditions, the inoculation of “Merlin” with strain GMF14 significantly increased the shoot dry weight of the “Merlin” cultivar from that with USDA 110 (Fig. 6A). However, the root dry weight induced by strain GMF14 was not significantly different from that induced by USDA 110 (Fig. 6A). The nodule dry weights induced by GMF14, GMM71, and GMM96, GMF42, and GMF57 were significantly higher than those induced by USDA 110 (Fig. 6B). Regarding total nodule numbers, no significant difference was observed between any strains, and GEM96, GMF57, and GMM36 formed more medium-sized nodules than USDA 110 (Fig. 6B); most of the nodules formed with USDA 110 were small. Regarding ARA g⁻¹ of nodule dry weight, no significant difference was observed between any strains, whereas
ARA (g⁻¹ of plant dry weight) of plants inoculated with GMF14, GMF57, and GMM34 were significantly higher than those of USDA 110-inoculated plants (Fig. 6C). Plants inoculated with GMF14 and GMF57 appeared to have darker green leaves than those inoculated with USDA110 (Fig. 6D) as well as a significantly higher nodule dry weight.

Fig. 3. Phylogenetic tree of MLSA based on concatenated sequences of 16S rRNA (1,299 bp), recA (541 bp), and atpD (453 bp) genes. The nucleotide sequences from 77 representative isolates and reference strains of each species belonging to different genera. The tree was constructed by the neighbor-joining method. Bootstrap values are shown as percentages from 1,000 replicates. Scale bars represent 1% of the nucleotide substitutions.

Strains isolated from “Merlin” were marked with a bold letter. Strains that survived at 4°C were marked with stars and strains that grew at 4% NaCl were marked with black dots.
and ARA g⁻¹ of plant dry weight than USDA 110-inoculated plants (Fig. 6B and C).

In contrast, in the Sultana cultivar (Fig. S1), shoot dry weight, root dry weight, nodule dry weight, nodule number, ARA g⁻¹ of nodule dry weight, and ARA g⁻¹ of plant dry weight were not significantly higher than those of USDA 110-inoculated plants.

**Discussion**

*Distribution of soybean rhizobia from the northern part of Germany*

In the present study, rhizobia were isolated from eighteen soil samples collected from different areas in the northern part of Germany using two soybean cultivars, “Merlin” and “Enrei”, as trap hosts (Fig. 1). Soybean rhizobial strains
Fig. 5. Phylogenetic tree of the nifH gene constructed using a 666-bp partial nucleotide sequence from 56 isolates and reference strains of each species belonging to different genera. Bootstrap values are shown as percentages from 1,000 replicates. Strains that survived at 4°C were marked with stars and strains that grew at 4% NaCl were marked with black dots.

were mostly isolated from fields in the Müncheberg and Fehrow areas that belong to Brandenburg State, which have soybean cultivation histories. In these areas, farmers use commercial rhizobial fertilizers from the BASF company (https://www.basf.com/global/en.html), such as HiStick®-soy containing B. japonicum. This fertilizer needs to be inoculated into soybean seeds at a rate of $4 \times 10^9$ viable cells g$^{-1}$ before sowing to fix nitrogen and meet yield potentials (Zimmer et al., 2016). A commercial rhizobial strain was introduced into these two areas as inoculants between 2013 and 2017, after which soybean was not cultivated. Soil from the remaining 7 locations with no soybean cultivation his-
The stress tolerance characteristics of rhizobial strains are important for adaptation to the local environmental conditions of a given soil. In the present study, we attempted to identify strains with multiple stress tolerance and efficient nitrogen fixation abilities, with the major aim of selecting a rhizobial inoculant for soybean cultivation under field conditions in Germany. One of the key features that needed to be fulfilled by the screened isolates was cold tolerance because rhizobial inoculants need to adapt to the low temperatures that range between 8 and 15°C in German fields (Zimmer et al., 2016). At the same time, high temperatures affect rhizobia infection and nitrogen fixation in several legume species, including soybean, peanut, and cowpea (Munevar and Wollum, 1982; Rainbird et al., 1983; Kishinevsky et al., 1992). Soil degradation, due to salinization, alkalization, and acidification, is one of the most serious issues affecting the fertility of soils, particularly in arid and semi-arid areas. Therefore, given the importance of the stress tolerance abilities of rhizobial strains, the isolates of the present study were screened for their growth under different stress conditions of temperature, salinity, and pH. Thirty-three isolates (15.1% of the total) trapped on the “Merlin” cultivar had the ability to grow at a low temperature, in contrast to none from “Enrei”. The cold tolerant rhizobium in German soils may exhibit stronger symbiotic ability to the German cultivar “Merlin” than to the Japanese cultivar “Enrei”; however, the mechanisms by which the survival ability at a low temperature of isolates correlates with symbiotic ability with soybean have not yet been elucidated in detail. However, to develop agricultural practices for soybean cultivation in Europe, a clearer understanding of this interaction is very important.

Furthermore, the majority of the low temperature and high NaCl tolerant strains were isolated from “Merlin” (Table S2, Fig. 2). This indicates that “Merlin” is a better trap host for isolating candidates of soybean inoculants being adapted to European soybean cultivation. This is consistent with previous findings by Artigas et al. (2019), which showed that domesticated soybean is a better trap host and its rhizobial strains are adapted to native abiotic stress conditions (Artigas et al., 2019).
Fig. 6. Shoot dry weight, root dry weight, root nodule dry weight, root nodule number, and acetylene reduction activity per nodule and plant of “Merlin” soybean inoculated with 7 isolates under cold conditions (the control is a non-inoculated plant). Large nodules >5 mm, medium-sized nodules 2–5 mm, and small nodules <2 mm. a) Shoot dry weight and root dry weight, b) root nodule dry weight and root nodule number, c) acetylene reduction activity nodule⁻¹ dry weight and plant⁻¹ dry weight, d) images of plants, and e) images of nodules. Results for each strain were compared to those for strain USDA 110 using the Student’s t-test; *P<0.05, **P<0.01.
**Phylogenetic properties of isolates**

To elucidate the genetic background of soybean rhizobia showing tolerance to various abiotic stresses, 77 isolates were selected and assessed for their phylogenetic properties based on a sequence analysis of their 16S rRNA, recA, and atpD genes. Therefore, we performed MLSA of the 16S rRNA, recA, and atpD genes, as shown in Fig. 3. In subgroup A, 6 isolates from the present study belonged to type strains of *B. diazoefficiens* USDA 110 and *B. japonicum* USDA 6. These type strains are currently agriculturally important rhizobia worldwide because they have superior N2 fixation abilities (Singleton et al., 1985; Pazdernik et al., 1997; Klogo et al., 2015). *B. japonicum* strains are being used in the global production of commercial inoculants for soybeans, including Germany (Zimmer et al., 2016). This result indicates that these *Bradyrhizobium* strains originated from commercial inoculants. In group B, the majority of strains belong to *B. lupini, B. yuanmingense*, and *B. elkanii*. In Germany, lupini is a common plant. Wiehe and Höflich (Wiehe and Höflich, 1995) previously reported that lupini plants were cultivated in the Müncheberg area. Lupini is nodulated by rhizobia belonging to the genus *Bradyrhizobium* (Eckhardt et al., 1931; Andam and Parker, 2007). A total of 28.6% isolates were assigned to the *Rhizobium* species in the present study. Previous studies also reported the isolation of rhizobia belonging to *Rhizobium/Agrobacterium* from soybean plants (Abaidoo et al., 2000; Chen et al., 2000; Hong et al., 2010; Youseif et al., 2014; Alam et al., 2015). The present results revealed that *Bradyrhizobium* is the predominant symbiont of soybean in the northeastern part of German soils. Furthermore, these German indigenous rhizobia are distributed in fields with soybean cultivation histories, which indicates that the natural population of rhizobia have been affected by both the cultivation of different legume crops and the application of inoculants. These results are supported by other investigations in Europe, such as Poland and Spain, and also in Africa (Madrzak et al., 1995; Bourebaba et al., 2016; Chibeba et al., 2017).

**Genetic characterization of symbiotic genes**

The phylogenetic tree, built with the partial sequences of the nodD and nifH genes, showed that all isolates, except for GMM49, possessed identical nodD and nifH genes, suggesting a common origin for these symbiotic genes. However, these symbiotic genes may be derived from a single strain that may be the commercial inoculants previously introduced in these fields. These results are consistent with previous findings between non-*Bradyrhizobium* strains and introduced *B. japonicum* inoculants in Brazil, Poland, and Africa (Martinez-Hidalgo and Hirsch, 2017). Regarding the MLSA of the 16S rRNA, atpD, and recA genes shown in Fig. 3, a large number of isolates were classified into subgroup B, which is genetically remote from subgroup A including *B. japonicum* and *B. diazoefficiens*. These results imply that the horizontal gene transfer (HGT) of symbiotic genes occurred from the commercial inoculant to indigenous soil bacteria with different genetic backgrounds of *Bradyrhizobium*. HGT may occur within several years of the introduction of commercial inoculants, suggesting rapid and frequent genome rearrangement through HGT among inoculants and soil bacteria (Barcellos et al., 2007). GMM49 has the unique characteristic of possessing *R. latusatum*-type housekeeping genes, *B. elkanii*-type nodD genes, and *B. diazoefficiens*-type nifH genes. This supports HGT potentially occurring among inoculants and soil bacteria in the fields tested, and imply the complex rearrangement of symbiotic genes in isolates recovered from the field. The HGT of nodulation and nitrogen fixation genes in a symbiosis island is well known between the same or different rhizobial genera, which supports the present results (Parker et al., 2002; Barcellos et al., 2007; Martinez-Hidalgo and Hirsch, 2017; Artigas et al., 2019). Similar findings were reported by Artigas et al. (2019), who isolated *R. alamii* species with nod and nifH genes derived from *Bradyrhizobium* sp. (Artigas et al., 2019). Furthermore, the complex HGT of symbiotic genes occurred in rhizobia in Brazilian Savannah soils, showing the genetic variability due to adaptation to stressful environmental conditions (Barcellos et al., 2007). These findings highlight the strategies that bacteria may often use as ecological advantages, such as the acquisition of genes for symbiotic ability with an exotic host legume.

**Symbiotic performance of isolates**

Soybean requires warm growing conditions with temperatures ranging between 25 to 30°C for optimal symbiotic activity. Under cold conditions, soybean shows a decrease in both nodulation and nodule function, which will directly affect soybean yield. Cold conditions negatively affect nitrogen-fixing symbiosis, mainly due to the sensitivity of the host plant and this effect may be alleviated by using cold tolerant legume and rhizobial partners. Prevost et al. (1987) reported that the nitrogenase activities of rhizobia isolated from cold areas were higher at low temperatures than those from warm areas. They isolated a strain from a cold environment that formed nodules under low temperatures, whereas a strain isolated from a warmer environment did not (Prevost et al., 1987). Zhang et al. (1996) reported that two *B. japonicum* rhizobia, isolated from cool soil conditions in Canada, increased the nodule number, nodule weight, shoot nitrogen, and yield of soybean in field experiments, and also found that the implementation of rhizobia under cold conditions was affected by their geographical origin (Zhang et al., 1996). Collectively, these findings imply that the optimal strategy to promote soybean nodulation and, thus, its yield in German soils is to isolate local rhizobial strains that are tolerant to German cold conditions. Under optimal temperature conditions, plant biomass was not significantly different between the seven selected isolates and USDA 110 (Table 2). On the other hand, when strain GMF14 was inoculated into “Merlin” soybean under low temperature conditions, strain GMF14 stimulated the plant biomass, particularly shoot dry weight, and its effects were different from USDA 110 (Fig. 6). This result indicates that these bacteria isolated from cold environments stimulate soybean biomass. The cultivar “Merlin” inoculated with the *Bradyrhizobium* strain GMF14 (belonging to *Bradyrhizobium* sp. based on MLSA) increased plant growth and was tolerant of low temperate conditions. Nev-
ertheless, when GMF 14 was inoculated into the other cultivar “Sultana”, it did not show the same results (Fig. S1). This indicates that GMF 14, which was isolated from “Merlin”, had a better symbiotic relationship with this host plant than “Sultana” under cold conditions. Based on the present study, which focused on abiotic stress, such as low temperatures, GMF14 is the most promising candidate for soybean inoculation at Brandenburg State. In the next step, we need to investigate inoculation effects using these candidate strains in field experiments under German conditions.

In conclusion, the present study is the first to attempt to identify the genetic background of indigenous soybean rhizobia isolated from northern Germany. Stress tolerance and plant test results demonstrated that there are several promising isolates for soybean inoculation in European soybean cultivation systems. Furthermore, these candidates may adapt to local environments. GMF14 induced the highest plant growth, root nodule number, and nitrogen fixation among all the isolates obtained. Further field studies are needed for the development of suitable inoculation technology for soybean cultivation in European soils, including Germany.

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