Bradyrhizobium vignae sp. nov., a nitrogen-fixing symbiont isolated from effective nodules of Vigna and Arachis

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Twenty one strains of symbiotic bacteria from root nodules of local races of cowpea (Vigna unguiculata), Bambara groundnut (Vigna subterranea) and peanuts (Arachis hypogaea) grown on subsistence farmers' fields in the Kavango region of Namibia, were previously characterized as a novel group within the genus Bradyrhizobium. To verify their taxonomic position, the strains were further analysed using a polyphasic approach. 16S rRNA gene sequences were most similar to Bradyrhizobium manausense BR 3351T, with Bradyrhizobium ganzhouense RTF806T being the most closely related type strain in the phylogenetic analysis, and Bradyrhizobium yuanmingense CCBAU 10071T in the ITS sequence analysis. Phylogenetic analysis of concatenated glnII-recA-rpoB-dnaK placed the strains in a highly supported lineage distinct from species of the genus Bradyrhizobium with validly published names; they were most closely related to Bradyrhizobium subterraneum 58 2-1T. The status of the species was validated by results of DNA–DNA hybridization. The combination of phenotypic characteristics from several tests, including carbon source utilization and antibiotic resistance, could be used to differentiate representative strains of species of the genus Bradyrhizobium with validly published names. Novel strain 7-2T induced effective nodules on Vigna subterranea, Vigna unguiculata, Arachis hypogaea and on Lablab purpureus. The DNA G + C content of strain 7-2T was 65.4 mol% (Tm). Based on the data presented, we conclude that these strains represent a novel species for which the name Bradyrhizobium vignae sp. nov. is proposed, with strain 7-2T [LMG 28791T, DSMZ 100297T, NTCCM0018T (Windhoek)] as the type strain.

In the Kavango region of Namibia, agriculture is largely dominated by smallholder farms, without irrigation or agrochemical inputs. Local farmers are confronted with low yields and decreasing soil fertility (Pröpper et al., 2010). The intercropping of grain legumes is common practice with local cereals interspersed in an irregular pattern (Grönenmeyer et al., 2013). Cowpea (Vigna unguiculata (L.) Walp., local name in Kavango: makunde) is the main grain legume grown by farmers, but also Bambara groundnut [Vigna subterranea (L.) Verdc., local name: nongo-mene] and peanuts (Arachis hypogaea) L. are planted, albeit to a lesser extent (Grönenmeyer et al., 2013). Inoculants adapted to the crops and the harsh environmental conditions (long dry seasons and high temperatures) may help to increase yields for local smallholders.

In a previous study (Grönenmeyer et al., 2014), populations of symbiotic bacteria from nodules of pulses collected in the Kavango region of Namibia and in the Angolan province Bié were sampled. Pure cultures of Bradyrhizobium were isolated and characterized. On the basis of multilocus sequence analysis (MLSA) of concatenated three protein-coding genes (glnII-recA-rpoB) and intergenic spacer (ITS) sequences, several novel lineages within the genus Bradyrhizobium were suggested by phylogenetic analyses (Grönenmeyer et al., 2014). One of these novel lineages consisted of a large group of 21 strains. Here, we have characterized this group in depth, by using a combination of genotypic and phenotypic methods, and propose the novel species, Bradyrhizobium vignae sp. nov., to accommodate this group.

The bacteria were isolated and grown on MAG medium (van Berkum, 1990). Stock solution was stored at −80 °C in 10 %

Abbreviations: ITS, intergenic spacer; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 7-2T is KP899563. The GenBank/EMBL/DDBJ accession numbers for the ITS, glnII, recA, rpoB, dnaK, rnh and nodC sequences of strain 7-2T are KM378504, KM378443, KM378374, KM378308, KR259951, KM378251 and KT362339, respectively.

Three supplementary figures and three supplementary tables are available with the online Supplementary Material.
Multiple sequence alignments were generated using either MUSCLE or CLUSTAL W version 5.1 (Larkin et al., 1999), with phylogenetic analysis according to Willems et al. (2003) with 627 positions; nodC, 441 bp with primers NodCfor540 and NodCrev1160 (Sarita et al., 2005); glnII, 304 bp with primers glnII 12F and glnII 689R (Vinuesa et al., 2005); recA, 381 bp with primers recA 41F and recA 640R (Vinuesa et al., 2005); rpoB, 416 bp with primers rpoB-454F and rpoB-1364R (Vinuesa et al., 2008); dnaK, 239 bp with primers TSDnaK2 and TSDnaK4 (Stepkowski et al., 2005; Stepkowski et al., 2007) under conditions described previously (Gro¨nemeyer et al., 2014). Concatemers of glnII-recA-rpoB-dnaK contained 1540 positions. Multiple sequence alignments were generated using either CLUSTAL W version 5.1 (Larkin et al., 2007) or MUSCLE (Edgar, 2004) incorporated in MEGA 5.2 software (Tamura et al., 2011). The best-fit models of evolution were determined using MODELTEST (Posada & Crandall, 1998) integrated in MEGA5 software, and the substitution model with the lowest Bayesian information criterion (Schwarz, 1978) score was chosen for a maximum-likelihood-based phylogenetic analysis. The reliability of the tree topology was estimated by conducting a bootstrap test (Felsenstein, 1985) with 500 pseudoreplicates. GenBank accession numbers are listed in Table S1 (available in the online Supplementary Material).

The 16S rRNA gene sequence similarity of the representative strain 7-2 T ranged from 95.6 to 99.2 % to reference strains (Table S2) and was most similar to the sequence of Bradyrhizobium manausense BR 3351 T and Bradyrhizobium ganzhouense RITF806 T at 99.2 %, corroborating that the proposed species threshold value of 98.5 % (Stackebrandt & Ebers, 2006) is not sufficiently stringent within the genus Bradyrhizobium. Phylogenetic analysis of the 16S rRNA gene sequence indicated that the novel group was most closely related to the B. ganzhouense/Bradyrhizobium cytisi group, but this was not well-supported by bootstrap values (Fig. 1).

ITS sequences were generated for 16 Namibian strains as representatives of different genotypes and plant hosts. Sequence identity was high within this group, ranging from 99.5–100 % (Table S2). Together with reference sequences, phylogenetic analysis indicated that this group was most closely related to Bradyrhizobium yuanmingense CCBAU 10071 T (Fig. 2, Table S2) with 96.4–96.7 % sequence identity. However, ‘Bradyrhizobium arachidis’ CCBAU 051107 T also showed 96.3–96.7 % sequence identity. For subsequent analysis, only the representative strains, 7-2 T, 28 T, 36 3-2, and 9-5, were used because of the high sequence identity of many Namibian strains.

Within the genus Bradyrhizobium, phylogenetic MLSA of several housekeeping genes is used as a reliable method to define relationships and for the identification of novel lineages (Rivas et al., 2009). Four concatenated housekeeping genes, glnII-recA-rpoB-dnaK, were used (Fig. 3, Table S2). For the concatenators, sequence identities were high among the strains of the novel group (98.9–99.9), but only 89.0–95.7 % between the novel group and the reference strains (Table S2). Representative strains of this novel group clustered monophyletically with high bootstrap support, indicating that they might belong to the same species (Fig. 3). Two of the four housekeeping genes as well as the concateners showed highest identity (Table S2) to Bradyrhizobium subterraneum 58 2-1 T, representing a novel group of Bradyrhizobium from the same region, Kavango (Gro¨nemeyer et al., 2015). This was also corroborated by phylogenetic analysis with high confidence (Fig. 3, bootstrap value 75 %). For the reference species with the highest 16S rRNA gene sequence similarities, B. ganzhouense RITF806 T, only few reference sequences were available. Therefore, a phylogenetic analysis of concatameric glnII-recA was carried out (Fig. S1). It revealed that this species is only distantly related to our novel group.

A fragment of the nifH gene, encoding the iron protein of the key enzyme for nitrogen fixation, nitrogenase, is commonly used as a marker for diazotrophic bacteria (Burbano et al., 2011). Therefore, phylogenetic analysis of nifH was carried out. The phylogenetic tree (Fig. S2), based on 216 positions, showed that nifH fragments of the representative isolates were highly related to each other, and most closely related to novel bradyrhizobium from the Kavango region (Bradyrhizobium kavangense 14-3 T, B. subterraneum 58 2-1 T) (Gro¨nemeyer et al., 2014) and ‘B. arachidis’ CCBAU 051107 T. ‘Bradyrhizobium kavangense’ was recently described to encompass strains isolated from various pulses (Gro¨nemeyer et al., in press). As previously observed (Vinuesa et al., 2005), nifH phylogeny may indicate lateral gene transfer relating to the host, as our strains were isolated from African pulses as with the other species. In order to characterize better the symbiovar, phylogenetic analysis was carried out for another symbiosis-related gene, nodC (Fig. S3), which encodes one of the proteins for the synthesis of Nod-factor. This corroborated the grouping with ‘B. kavangense’ 14-3 T and ‘B. arachidis’ CCBAU 051107 T.

DNA–DNA hybridization studies of genomic DNA are commonly used to differentiate bacterial species from each other (Stackebrandt & Ebers, 2006). Therefore, DNA–DNA hybridization experiments were carried out
with strains of our novel group and the reference species; they were found to be most closely related in phylogenetic analyses of housekeeping genes, marked in bold in Fig. 3. The assays were microtitre-plate based with a biotinylated probe (Willems et al., 2001) derived from the type strain of our novel group, 7-2\textsuperscript{T}. Hybridizations were carried out in quadruplicate, with readings taken after 45 min. The other representative members of our group, 28 2-1 and 9-5, showed 71±3 % or 69±4 % DNA–DNA relatedness with strain 7-2\textsuperscript{T} (Table S3), indicating they belong to the same species. In contrast, all reference strains showed less than 38 % DNA–DNA relatedness (Table S3). This is well below the value of 70 % DNA–DNA relatedness that is considered the threshold for delineation of a novel species.

### Table S3: DNA–DNA relatedness percentages

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<th>Species</th>
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<td>Bosea thiooxidans DSM 9653\textsuperscript{T} (A250796)</td>
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### Fig. 1. Neighbour-joining phylogram inferred from 16S rRNA sequences with 1228 positions. Distances were calculated using the maximum composite likelihood method. Bosea thiooxidans DSM 9653\textsuperscript{T} was included as an outgroup strain. A bootstrap value is indicated when a given node appeared in $\geq$50 % out of 1000 pseudoreplicates. Bar, number of substitutions per site.
For phenotypic characterization, a variety of tests were carried out for three members of our genospecies described here, type strain 7-2T, strain 28 2-1, and strain 9-5, and for the reference species. As commonly applied to bradyrhizobia (Lu et al., 2014), carbon source oxidation was tested with Biolog GN2 microplates (Biolog) by following the manufacturer’s instructions, with some modifications. Tested with Biolog GN2 microplates (Biolog) by following the manufacturer’s instructions, with some modifications.

Resistance to antibiotics was tested on agar plates (Gao et al., 1994) on YMA containing 1 g l\(^{-1}\) yeast extract. An
### Table 1. Phenotypic features of three strains of B. vignae and type strains of closely related species of the genus Bradyrhizobium

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*Data from Gröneneyer et al. (2014).
growth on D-psicose, vary between strains of the novel genospecies, such as published names. Within this novel group, some characteristics from species of the genus Table 1 and can be used for differentiating the novel species characteristics in comparison to reference species are listed in (Hewlett Packard). The fatty acid profile of the type strain 7-2T was: C₁₈:₂ 14.7 %; C₁₈:₃ω7c 78.2 %; C₁₈:ω1 0.9 %; C₁₉:₂ω8c 5.7 %; C₁₆:ω7cC₁₆:ω2O₉H 0.43 %; C₁₆ω2O₈H/C₁₆: γω7c 0.43 %. The phenotypic characteristics in comparison to reference species are listed in Table 1 and can be used for differentiating the novel species from the genus Bradyrhizobium with validly published names. Within this novel group, some characteristics vary between strains of the novel genospecies, such as growth on D-psicose, α-keto-butyric acid and glycy1-L-glutamic acid, and resistance to erythromycin (Table 1). Features that differentiate the novel genospecies from most related species of the genus Bradyrhizobium tested are the unique ability to oxidize dextrin, the ability to oxidize d-fructose, cis-aconitic acid, d-glucoronic acid, malonic acid, d-alanine, l-proline and l-threonine, but not l-alaninamide or l-asparagine. One of the most striking features is the extraordinary temperature tolerance, as this novel species was able to grow at 40 °C.

Strains which might be related to this novel species, according to phylogenetic analyses of glnIII-recA concatemers and/ or ITS sequences, have been isolated from Senegalese pulses or trees; for example strains ORS3257 and ORS3258 isolated from cowpea (Wade et al., 2014) and ORS3656 and ORS3642 isolated from Macroptilium atropurpureum or Acacia seyal (Sene et al., 2013).

On the basis of our genotypic and phenotypic analyses, we propose that the novel group of 21 strains represent a novel species, named Bradyrhizobium vignae sp. nov.

**Description of Bradyrhizobium vignae sp. nov.**

Bradyrhizobium vignae (vig’nae. N.L. gen. n. vignae of the plant Vigna unguiculata referring to the origin of isolation, cowpea). Cells are motile, Gram-stain-negative, aerobic, non-spor-forming rods (approximately 2–3 µm long × 0.7 µm wide). Colonies are circular, convex, translucent, beige-whitish, 0.2–1 mm in diameter after 8 days of growth at 28 °C on YMA. The mean generation time is approximately 8 h in YMB at 28 °C. Produces an alkaline reaction on YMA. Growth occurs at pH 5–9 and at 40 °C, but not in the presence of 1 % (w/v) NaCl. Oxidizes L-arginine, D-arginine, L-fucose, D-galactose, α-D-glucose, D-mannitol, D-mannose, methylpyruvate, monomethylsuccinate, cis-aconitate, citrate, formiate, D-galactonic acid lactone, D-galacturonate, D-glucuronic acid, D-galactosaminic β-hydroxybutyrate, γ-hydroxybutyrate, α-ketoglutarate, α-ketovalerate, β-lactate, malonate, propionate, quinate, D-saccharate, sebacate, succinate, bromo-succinate, succinamidic, glycuronamide, L-aspartate, L-glutamate, L-leucine, L-pyroglutamate, urocanate, glycerol, Tween 40 and Tween 80. No oxidation of α-cyclodextrin, gyocigen, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine, adonitol, cellobiose, erythritol, gentobiose, m-inositol, α-lactose, lactulose, malose, D-melibiose, β-methyl-D-glucose, D-psicose, raffinose, D-sorbitol, sucrose, trehalose, turanose, xylitol, L-alaninamide, L-alanylglucine, L-asparagine, L-histidine, hydroxyl-L-proline, L-ornithine, L-proline, D-serine, L-serine, L-threonine, DL-carnitine, γ-amino-butyric acid, inosine, uridine, thymidine, phenylalanine, putrescine, 2-aminoo ethanol, 2,3-butanediol, DL-glycerolphosphate, glucose-1-phosphate, or glucose-6-phosphate. Resistant to: chloramphenicol, streptomycin and tetracycline. Effective nodules are induced on Vigna unguiculata, Vigna subterranea, Arachis hypogaea and Lablab purpureus.

The type strain, 7-2T [DSM 100297 T = LMG 28791 T = NTCCM0018 T (Windhoek)], was isolated from an effective nodule of Vigna unguiculata in the Kavango region of Namibia. The DNA G+C content of the type strain is 65.4 mol%.

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**References**


