Bradyrhizobium namibiense sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of Lablab purpureus, hyacinth bean, in Namibia

Jann Lasse Grönemeyer, Wiebke Bünger and Barbara Reinhold-Hurek*

Abstract

Four strains of symbiotic bacteria from root nodules of hyacinth bean (Lablab purpureus (L.) Sweet) from Namibia were previously identified as a novel group within the genus Bradyrhizobium. To confirm their taxonomic status, these strains were further characterized by taking a polyphasic approach. The type strain possessed 16S rRNA gene sequences identical to Bradyrhizobium paxilaeri LMTR 21\(^1\) and Bradyrhizobium incense LMTR 13\(^3\); the full-length sequences were identical to those retrieved from SAMN05230119 and SAMN05230120, respectively. However, the intergenic spacer sequences of the novel group showed identities of less than 93.1\% to described Bradyrhizobium species and were placed in a well-supported separate lineage in the phylogenetic tree. Phylogenetic analyses of six concatenated housekeeping genes, recA, glnII, gyrB, dnaK, atpD and rpoB, corroborated that the novel strains belonged to a lineage distinct from named species of the genus Bradyrhizobium, with highest sequence identities to Bradyrhizobium jicamae and B. paxilaeri (below 93\%). The species status was validated by results of DNA–DNA hybridization and average nucleotide identity values of genome sequences. The combination of phenotypic characteristics from several tests, including carbon source utilization and antibiotic resistance, could be used to differentiate representative strains from recognized species of the genus Bradyrhizobium. Phylogenetic analysis of nodC and nifH genes placed the novel strains in a group with B. paxilaeri and B. lablabi. Novel strain 5-10\(^1\) induces effective nodules on Lablab purpureus, Vigna subterranea, Vigna unguiculata and Arachis hypogaea. Based on our results, we conclude that our strains represent a novel species for which the name Bradyrhizobium namibiense sp. nov. is proposed, with type strain 5-10\(^1\) [LMG 28789, DSM 100300, NTCCM0017 (Windhoek)].

Smallholder farms widely dominate agricultural land use in the Kavango region of Namibia. Neither irrigation nor agricultural inputs are common, thus local farmers face low yields and decreasing soil fertility [1]. The application of nitrogen-fixing legume crops might promote soil fertility with respect to soil nitrogen and organic matter. A common practice is intercropping of grain legumes with local cereals, interspersed in an irregular pattern [2]. Cowpea (Vigna unguiculata (L.) Walp.) is the main grain legume grown by farmers, but also Bambara groundnut (Vigna subterranea (L.) Verdc.) and peanuts (Arachis hypogaea L.) are planted, albeit to a lesser extent [2]. Hyacinth bean (Lablab purpureus (L.) Sweet) is not commonly used. Although its largest diversity and agricultural utilization occurs in South Asia, its origin appears to be Africa [3]. As it has been reported to have higher drought tolerance than common beans or cowpea, Lablab it is thought to offer opportunities for African agriculture [3]. Therefore, it had been introduced into test fields at the Mashare Agricultural Development Institute near Rundu, Namibia. Isolation of symbiotic bacteria may help to develop inoculants adapted to this crop and the harsh environmental conditions.

In a previous study [4], populations of symbiotic bacteria from nodules of pulses collected in the Kavango region of Namibia were sampled and pure cultures of Bradyrhizobium characterized. On the basis of multilocus sequence analysis (MLSA) of concatemers of three protein-coding genes (glnII-recA-rpoB) and intergenic spacer (ITS) sequences, phylogenetic analyses identified several novel lineages within the genus Bradyrhizobium [4]. One of these novel lineages consisted of a group of four strains. Here, we

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**Keywords:** Bradyrhizobium; lablab; nodulation; Namibia; Kavango.

**Abbreviations:** ANI, average nucleotide identity; CAS, chrome azurol S; ITS, 16S-23S rRNA internal transcribed spacer; MAG medium, modified arabinose gluconate medium; MLSA, multilocus sequence analysis.

The Genbank/EMBL/DDBJ accession numbers for the 16S rRNA gene, ITS, recA, atpD, glnII, dnaK, gyrB, rpoB, nodC and nifH of strain 5-10\(^1\) are KX661401, KM378502, KM378377, KX661387, KM378440, KP402058, KX661393, KM378306, KX661399 and KM378249, respectively.

Three supplementary figures and three supplementary tables are available with the online version of this article.
further characterize these strains using a combination of genotypic and phenotypic methods, and propose the novel species, *Bradyrhizobium namibiense* sp. nov., for this group. The bacteria were isolated and grown on modified arabinose gluconate (MAG) medium [5], and stocks were stored at −80°C in 10% DMSO. The four isolates were obtained from the root nodules of two individual plants of *Lablab purpureus*, grown on Kavango sands on test sites at the Mashare Agricultural Development Institute in the Mashare area of the Kavango region in Namibia. Strains 5-10⁷ and 5-11 originated from a field with coordinates 17° 53’ 44.79” S, 20° 12’ 44.68” E, while the other strains, 30 3-2 and 30 3-3, were isolated from a plant grown at an adjacent spot in the successive year. All strains were grouped according to their BOX-PCR fingerprints [4]. According to their BOX-PCR patterns, they consisted of three similar genotypes, with the patterns of strains 30 3-2 and 30 3-3 being almost identical. Representative strains 5-11 and 30 3-2 differed in concatenate glnII-recA-rpoB sequences [4].

This group was further analysed. The following sequences were amplified and sequenced from test or reference strains and aligned for phylogenetic analyses: 16S rRNA with primers Bac8uf and Univ1492r according to Grönemeyer et al. [6], aligned at 1227 positions; ITS with primers FGPS1490 and FGPS132 [7], used for phylogenetic analysis according to Willems et al. [8] with 644 positions; glnII with primer glnII 12F and glnII 689R [9], 480 positions; recA with primers recA 41F and recA 640R [9], 375 positions; dnaK with primers TSDnaK2 and TSDnaK4 [10, 11], 223 positions; rpoB with primers rpoB-454F and rpoB-1364R [12], 367 positions, under conditions described previously [4]; atpD with primers atpD352F and atpD871R [13], 428 positions; and gyrB with primers gyrB343F and gyrB1043R [14], 551 positions, according to [13]. The concatenates of recA-atpD-glnII-dnaK-gyrB-rpoB contained 2424 positions. Multiple sequence alignments were generated using either CLUSTALW version 5.1 [15] or MUSCLE [16] incorporated in the MEGA 5.2 software [17]. The best-fit models of evolution were determined using MODELLER [18] integrated in MEGA5 software, and the substitution model with the lowest Bayesian information criterion (BIC) [19] score was chosen for maximum-likelihood-based phylogenetic analysis. The reliability of the tree topology was estimated by conducting a bootstrap test [20] with 1000 pseudoreplicates.

The partial 16S RNA gene sequence of strain 5-10⁷ was identical to *Bradyrhizobium paxillaei* LMTR 21T and *Bradyrhizobium icense* LMTR 13T (Table S1, available in the online version of this article), represented on the *Bradyrhizobium elkanii* lineage in the phylogenetic tree (Fig. 1).

However, ITS sequences of the novel group were very dissimilar to named species, with highest identities of only 93.0% to *Bradyrhizobium jicamae* and *B. icense* (Table S1). These values are well below 95.5%, which is considered the threshold for assigning strains to the same genospecies within *Bradyrhizobium* [8], demonstrating again that the 16S RNA gene is not a suitable marker for species delineation inside this genus [21]. This was also reflected in the phylogenetic analysis of ITS sequences of the novel group, with deep branching from the *B. jicamae* lineage at high bootstrap support (Fig. 2).

MLSA of several housekeeping genes were used as a reliable method to define phylogenetic relationships and for identification of novel lineages within the genus *Bradyrhizobium* [13]. Six concatenated housekeeping genes, recA-atpD-glnII-dnaK-gyrB-rpoB, were used (Fig. 3 and Table S1). While strains 5-10⁷ and 5-11 differed slightly only in their dnaK sequences, strains 5-10⁷ and 30 3-2 showed differences especially in their atpD (97.7% identity), but also in their rpoB, recA and dnaK sequences. For the concateners, sequence identities of more than 99.3% were high among the novel strains (Table S1). Sequence identities were only 88.8–92.9% between the novel group and the reference strains, with highest identities to *B. jicamae* and *B. paxillaei* (Table S1). For the five-gene concatenate (recA-atpD-glnII-dnaK-gyrB), the sequence identities were, at maximum, 93.6% (Table S1) and thus well below the cut-off value of 97% recently proposed for species discrimination inside *Bradyrhizobium* for this set of housekeeping genes [22]. The phylogenetic tree inferred from recA-atpD-glnII-dnaK-gyrB-rpoB concateners (Fig. 3) displayed a topology similar to the one obtained for the ITS sequence. Again, the novel strains formed a distinct group with deep branching from the *B. jicamae* lineage at high bootstrap support.

DNA–DNA hybridization studies of genomic DNA play an important role as the ‘gold standard’ for species delimitation [23]. Thus, DNA–DNA hybridization experiments were carried out with two representative strains of our novel group and three of the reference species found to be most closely related in the phylogenetic analyses. *B. jicamae* and *B. paxillaei*, showing the highest sequence similarities in the analyses of housekeeping genes, and *B. lablabi*, another natural symbiont of hyacinth bean, were selected. The assays were microtitre-plate-based with a biotinylated probe [24] derived from the type strain of our novel group, strain 5-10. Hybridizations were carried out in quadruplicate and readings taken after 45 min. The slightly deviating member of the novel group, strain 30 3-2, showed 74±5% DNA–DNA relatedness (Table S2). These data are well below the 70% DNA–DNA relatedness value that is considered the threshold for novel species definition [23]. Thus, our novel group can be regarded as a new genospecies in the genus *Bradyrhizobium*.

The average nucleotide identity (ANI) of genome sequences is an additional reliable means to estimate the genomic relatedness between prokaryotic strains and is increasingly used as an alternative to DNA–DNA hybridization. Since it is now widely accepted that ANI values of 95–96% correspond to 70% DNA–DNA hybridization, ANI is often regarded as
a potential next-generation ‘gold standard’ for species delin-
eation [25]. A draft genome of strain 5-10\textsuperscript{T} was obtained by
using the Illumina MiSeq platform and the A5-miseq pipeline
[26]. Because of material transfer agreements and
research permits it is currently not possible to make the
sequence public. Global statistics are provided in Table S3.

Similarities in the composition of cellular fatty acids usually
correlate well with genomic data, hence the comparison of
fatty acid profiles is an accurate and reproducible approach to
distinguish between most species [28]. The fatty acid composi-
tion of strain 5-10\textsuperscript{T} was analysed by the Identification Service

of the DSMZ, Braunschweig, Germany, together with its DNA
G+C content. The predominantly detected fatty acids of strain
5\textsuperscript{-10}\textsuperscript{T} were C\textsubscript{16:0} (14.3 %) and C\textsubscript{18:1}ω7c/ω6c (80.8 %), a
typical feature of members of the genus Bradyrhizobium [28].
The other detected fatty acids were C\textsubscript{17:0cyclo}, C\textsubscript{18:0}ω7c 11-methyl, C\textsubscript{19:0cyclo}ω4c, and C\textsubscript{16:1}ω7c/ω6c, but all at
low concentrations (0.5–1.7%). Differences in the fatty acid
profiles of strain 5\textsuperscript{-10}\textsuperscript{T} and closely related reference strains are
indicated especially by the low concentration of C\textsubscript{18:1}ω7c 11-
methyl found in strain 5\textsuperscript{-10}\textsuperscript{T}, and the high levels of 13–19% reported for B. \textit{incense}, B. \textit{retamae} and \textit{B. \textit{valentinnum}} [22, 29].

Phylogenies of symbiotic genes often cluster rhizobial
strains according to their host or location, due to lateral
gene transfer [9]. Thus, we included phylogenetic analyses of
\textit{nodC} and \textit{nfh} in our study. Partial sequences of \textit{nodC} and \textit{nfh} were generated with primers \textit{NodCfor540/Nod-
Cfor350} and \textit{PolR/FGPH19} [31], respectively, and
phylogenetically analysed as described above. The phyloge-
netic trees (Figs S1 and S2) showed that the novel isolates
harboured symbiotic genes closely related to those of \textit{B. \textit{paxllaeri}}
and \textit{B. \textit{lablabi}}, the latter being another natural

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**Fig. 1.** Neighbour-joining phylogram inferred from 16S rRNA gene sequences of species type strains of the genus \textit{Bradyrhizobium},
based on the maximum composite likelihood method. The type strain of \textit{B. \textit{namibiense}} sp. nov. is marked in bold. \textit{Bosea \textit{thiooxidans}}
DSM 9653 was used as an outgroup. Accession numbers are indicated within parentheses. A bootstrap value is indicated when a
given node appeared in ≥50% of 1000 pseudoreplicates. The scale bar indicates the number of substitutions per site.
symbiont of hyacinth bean, isolated in China. Interestingly, they were only distantly related to the *nodC* or *nifH* genes of the other species that we isolated from pulses in the same region, *B. subterraneum*, *B. kavangense* and *B. vignae* [32–34], even though they shared the same host range in our cross-inoculation experiments [4]. Therefore, the novel group and its natural host may have been co-introduced into this region. This is further supported by its lower temperature and polyethylene glycol (PEG) tolerance compared to the indigenous species and its apparent absence in nodules of local crops [4] or wild legumes (unpublished data).

A variety of tests for phenotypic characterization were carried out for members of the novel genospecies and reference species. Carbon source utilization was tested by using BIOLOG GN2 microplates (Biolog, Hayward CA) following the manufacturer’s instructions, with some modifications. Cells were precultured on MAG medium, the cell density in the inoculation fluid was at an OD$_{600nm}$ of 0.25–0.30, and plates were incubated at 28 °C in a moist atmosphere. Duplicates were evaluated after 1 week of incubation. Carbon source utilization was further tested by substituting mannitol in yeast mannitol broth (YMB) with selected...
carbohydrates (L-arabinose, α-D-glucose, L-rhamnose, D-xylose or melizitose) and inspecting growth capacities in YMB [35] over a period of 8 days. Enzyme activities were tested by using the API ZYM system (bioMérieux [35]) over a period of 8 days. Enzyme activities were tested by using the API ZYM system (bioMérieux [35]) and inspecting growth capacities in YMB containing 1 g l⁻¹ yeast extract with shaking at 200 r.p.m. at 28 °C. Cell morphology was analysed from these exponentially growing cultures by phase contrast microscopy. Type of motility and rotation of cells when sticking with one cell pole to the slide indicated polar flagellation. Colony morphology was evaluated after 8 days of growth at 28 °C on yeast mannitol agar (YMA). Alkalization was tested with 25 mg l⁻¹ Bromothymol blue. Tolerance against acidic or alkaline conditions was tested on YMA at pH 4.0, pH 4.5 and pH 5.0 with a 25 mM Homo-PIPES (homopiperazine-N,N'-bis-2-ethanesulphonic acid) buffer, or at pH 9.0 and pH 10.0 with 20 mM CAPSO (N-cyclohexyl-2-hydroxy-3-aminopropanesulfonic acid) buffer. Salt tolerance was tested in YMB containing 1 % NaCl and in MAG broth (0.5, 0.75 and 1.0 % NaCl). Optimal growth conditions and temperature tolerance were tested in MAG broth at pH 5.5, pH 6.0, pH 6.6, pH 7.5 and pH 8.0 and at temperatures of 28, 32, 35 and 37 °C. The host range was analysed as previously described [4]. Resistance to antibiotics was tested on agar plates [36] on YMA containing 1 g l⁻¹ yeast extract. Ten microlitres of a cell suspension at OD₆₀₀=0.1 was spotted, and readings from duplicates taken after incubation for 7–10 days at 28 °C. Concentrations were (in µg ml⁻¹) ampicillin 50, chloramphenicol 50, kanamycin 20, streptomycin 5, tetracycline 15 and erythromycin 50. Siderophore production on chrome azurol S (CAS) agar and inorganic phosphate solubilization on Pikovskaya agar were tested as previously described [6], with prolonged incubation of 21 days. Indole-3-acetic acid production was assayed according to [37], with the exception that cultures were grown on MAG medium for 7 days. The phenotypic characteristics in comparison to reference species are listed in Table 2 and can be used for differentiation of the novel species from validly published Bradyrhizobium species. Within this novel group, strains 5-10 and 30-3 could not be differentiated between strains 5-11 and 30-3, respectively, in the tests for carbon source utilization and enzymatic activities, but some characteristics varied between strains 5-10 and 30-3, such as resistance to chloramphenicol, growth on D-fructose, dextrin, D- and L-alanine, acetic acid, D-saccharic acid, L-lysine, L-pyroglutamic acid and glyceral, and acid phosphatase activity. Features that differentiate the novel genospecies from most related Bradyrhizobium species are higher temperature.

Table 1. ANI values between genome sequences of strain 5-10T and related type strains

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>ANI (%) with strain 5-10T</th>
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<tbody>
<tr>
<td>B. paxilliari LMTR 21T</td>
<td>88.1</td>
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<tr>
<td>B. lablabi CCBAU 23086T</td>
<td>88.1</td>
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<tr>
<td>B. icense LMTR 13T</td>
<td>87.9</td>
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<tr>
<td>B. jicamae PAC68T</td>
<td>87.8</td>
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<tr>
<td>B. retanae Ro19T</td>
<td>87.7</td>
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<tr>
<td>B. elkanii USDA 76T</td>
<td>85.5</td>
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</table>
tolerance (growth at 35°C on MAG broth), and a lower desiccation tolerance (no tolerance to 20% (w/v) PEG).

Therefore, based on our genotypic and phenotypic analyses, we propose that the novel group of four strains represent a novel species, named Bradyrhizobium namibiense sp. nov.

**DESCRIPTION OF BRADYRHIZOBUM NAMIBIENSE SP. NOV.**

Bradyrhizobium namibiense (na.mi.bi.en.se. N.L. neut. adj. namibiense pertaining to Namibia, where this novel group was first detected).

Cells are motile, Gram-stain-negative, aerobic, non-spore-forming rods (approx. 2–3 μm long and 0.7 μm wide). Colonies are circular, convex, translucent, beige-whitish and very small at <0.2 mm in diameter after 8 days of growth at 28°C on YMA. Mean generation time is 10.7±1.1 h in YMB at pH 6.6. Growth still occurs at pH 5.5 and 28°C on YMA. Mean generation time is 10.7±1.1 h in YMB at pH 6.6. Growth still occurs at 35°C and weakly at 37°C, with optimum growth at 28–32°C. Does not grow in the presence of 1% (w/v) NaCl. Produces an alkaline reaction on YMA. Does not produce siderophores on CAS plates, indole-3-acetic acid in MAG medium supplemented with l-tryptophan, or solubilize calcium phosphate on Pikovskaya agar.

Table 2. Phenotypic features of *B. namibiense* and closely related *Bradyrhizobium* type strains

<table>
<thead>
<tr>
<th>Strains: 1, <em>B. namibiense</em> 5-10; 2, <em>B. namibiense</em> 30 3-2; 3, <em>B. namibiense</em> 5-11, 4, <em>B. jicamae</em> PAC68; 5, <em>B. lablabi</em> CCBAU 23086; 6, <em>B. paxllaeri</em> LMTR 21†</th>
<th>1</th>
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<td>Carbon source utilization</td>
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<td>l-Rhamnose</td>
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<td>Citric acid</td>
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<tr>
<td>d-Galacturonic acid</td>
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<td>d-Glucuronic acid</td>
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<td>Succinic acid</td>
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<td>l-Alaninamide</td>
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<td>Uronic acid</td>
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<td>Antibiotic resistance (µg ml−1)</td>
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<td>Streptomycin (5)</td>
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<td>ND</td>
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<td>Kanamycin (20)</td>
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<td>ND</td>
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<td>Chloramphenicol (50)</td>
<td>±</td>
<td>+</td>
<td>ND</td>
<td>–</td>
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<td>Ampicillin (50)</td>
<td>+</td>
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<td>ND</td>
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<td>Growth at</td>
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<td>35°C†</td>
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<td>+</td>
<td>ND</td>
<td>–</td>
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<td>ND</td>
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<tr>
<td>37°C</td>
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<td>ND</td>
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<td>pH10</td>
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<td>ND</td>
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<td>Tolerance to</td>
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<td>20% (w/v) PEG†</td>
<td>–</td>
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<td>ND</td>
<td>+</td>
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<td>ND</td>
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<td>0.5% (w/v) NaCl in MAG</td>
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<td>ND</td>
<td>ND</td>
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<td>Siderophore production</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NG</td>
<td>NG</td>
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*Dependent on test format (BIOLOG GN2/utilization for growth).
†Data from Grönemeyer et al. [4].
streptomycin and kanamycin. The predominant fatty acids of strain 5-10 \(^T\) are C\(_{16:0}\) and C\(_{18:1} \omega 7c/C_{18:1} \omega 6c\). Effective nodules are induced on Vigna unguiculata, Vigna subterranea and Arachis hypogaea, Lablab purpureus but not on Phaseolus vulgaris.

The type strain 5-10 \(^T\) [LMG 28789=DSM 100300 =NTCCM0017 (Windhoek)] was isolated from an effective nodule of Lablab purpureus in the Kavango region of Namibia near Mashare. The DNA G+C content of the type strain is 63.3 mol%.

Funding information
This work was supported by a grant by the BMBF (Federal Ministry of Education and Research) in the framework of the project 'SASSCAL: Southern African Science Service Centre for Climate Change and Adaptive Land Management' (01 LG 1201D) to BRH and TH.

Acknowledgements
Samples were collected under the Research and collection permit 1569/2011 and 1635/2011 and export permit 83786, 90409. We would like to thank Bruce Kasoana (MADI, Rundu, Namibia) for providing lab-lab plants.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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