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

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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 paxilaei LMTR 21T and Bradyrhizobium icense LMTR 13T, 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. paxilaei (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. paxilaei and B. lablabi. Novel strain 5-10T 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 5-10T (LMG 28789, DSM 100300, NTCCM0017 (Windhoek)).

Smallholder farms widely dominate agricultural land use in the Kavango region of Namibia. Neither irrigation nor agrochemical 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...
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 5-10^4 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 RNA with primers Bac8uf and Univ1492r according to Grönemeyer and aligned for phylogenetic analyses: 16S rRNA with primers TSdnaK2 and TSdnaK4 [10, 11], 223 positions; gyrB with primers gyrB343F and gyrB1043R [14], 551 positions; rpoB with primers rpoB-454F and rpoB-1364R [12], 367 positions; recA with primers recA 41F and recA 640R [9], 375 positions; dnaK with primers rpoB-1364R [9], 375 positions; recA-atpD-glnII-dnaK-gyrB-rpoB, were used (Fig. 3 and Table S1). While strains 5-10^4 and 5-11 differed slightly only in their dnaK sequences, strains 5-10^4 and 30 3-2 showed differences especially in their atpD (97.7% identity), but also in their rpoB, recA and dnaK sequences. For the concatemers, 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. paxllaeri* (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 concamers (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. paxllaeri*, 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) indicating that it belongs to the same species. In contrast, the three reference strains of the sister clade, *B. paxllaeri, B. jicamae* or *B. lablabi*, respectively, showed less than 35% DNA–DNA hybridization (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 delineation [25]. A draft genome of strain 5-10³ 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. ANI values between strain 5-10³ and B. paxllaeri LMTR 21¹ (SAMDN05230119), B. icense LMTR 13¹ (SAMDN05230120), B. lablabi CCBAU 23086¹ (SAMDN02689497), B. jicamae PAC68¹ (SAMDN02689491), Bradyrhizobium retamae Ro19¹ (SAMDN02689496) and B. elkanii USDA 76¹ (SAMDN02261261) were calculated in JSpeciesWS [27] using Mummer for sequence alignment. Strain 5-10³ showed only low ANI values (maximum 88.1 %) with closely related type strains, well below the threshold for species differentiation (Table 1).

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 composition of strain 5-10³ 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 5-10³ were C₁₆ : 0 (14.3 %) and C₁₈ : 1ω7c/C₁₈ : 1ω6c (80.8 %), a typical feature of members of the genus Bradyrhizobium [28]. The other detected fatty acids were C₁₇:0cyd/C₁₈:0 C₁₈ : 1ω7c 11-methyl, C₁₉:0cydω8c and C₁₈ : 1ω7c/C₁₆ : 1ω6c, but all at low concentrations (0.5–1.7 %). Differences in the fatty acid profiles of strain 5-10³ and closely related reference strains are indicated especially by the low concentration of C₁₈ : 1ω7c 11-methyl found in strain 5-10³, and the high levels of 13–19 % reported for B. icense, B. retamae and B. valentinum [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 nodC and nifH in our study. Partial sequences of nodC and nifH were generated with primers NodCfor540/NodCrev [30] and PolR/GPH19 [31], respectively, and phylogenetically analysed as described above. The phylogenetic trees (Figs S1 and S2) showed that the novel isolates harboured symbiotic genes closely related to those of B. paxllaeri and B. lablabi, the latter being another natural
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). Growth rates were evaluated in YMB containing 1 g l⁻¹ of yeast extract with shaking at 200 r.p.m. at 28 °C. Cell morphology was analysed from these exponentially growing cultures extracts with shaking at 200 r.p.m. at 28 °C.

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 of 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 Pikovsky 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 3-2 could not be differentiated from strains 5-11 and 30 3-2, respectively, in the tests for carbon source utilization and enzymatic activities, but some characteristics varied between strains 5-10 and 30 3-2, such as resistance to chloramphenicol, growth on D-fructose, dextrin, D- and L-alanine, acetic acid, D-saccharic acid, D-leucine, D-pyrrolglutamic acid and glycerol, and acid phosphatase activity. Features that differentiate the novel genospecies from most related Bradyrhizobium species are higher temperature (1 % NaCl) and in MAG broth (0.5, 0.75 and 1.0 % NaCl).

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

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>ANI (%) with strain 5-10&lt;sup&gt;T&lt;/sup&gt;</th>
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<tr>
<td><em>B. paxillae</em> LMR 21&lt;sup&gt;T&lt;/sup&gt;</td>
<td>88.1</td>
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<tr>
<td><em>B. lablabi</em> CCBU 23086&lt;sup&gt;T&lt;/sup&gt;</td>
<td>88.1</td>
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<tr>
<td><em>B. incense</em> LMR 13&lt;sup&gt;T&lt;/sup&gt;</td>
<td>87.9</td>
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<tr>
<td><em>B. jicamae</em> PAC68&lt;sup&gt;T&lt;/sup&gt;</td>
<td>87.8</td>
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<tr>
<td><em>B. retamae</em> Ro19&lt;sup&gt;T&lt;/sup&gt;</td>
<td>87.7</td>
</tr>
<tr>
<td><em>B. elkanii</em> USDA 76&lt;sup&gt;T&lt;/sup&gt;</td>
<td>85.5</td>
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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 *BRADYRHIZOBIUM 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 35°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 1% NaCl. Produces an alkaline reaction 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 1% NaCl, or solubilize calcium phosphate on Pikovskaya agar.

<table>
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<tr>
<th>Carbon source utilization</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tr>
<td>l-Rhamnose</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<td>+/-</td>
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<tr>
<td>Citric acid</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>D-Galacturonic acid</td>
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<td>–</td>
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<td>–</td>
<td>(–)</td>
<td>+</td>
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<td>D-Glucuronic acid</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>±</td>
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<tr>
<td>Succinic acid</td>
<td>(–)</td>
<td>(–)</td>
<td>(–)</td>
<td>–</td>
<td>+</td>
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<tr>
<td>L-Alaninamide</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Urocanic acid</td>
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<th>Antibiotic resistance (µg ml⁻¹)</th>
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<th>2</th>
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<tr>
<td>Streptomycin (5)</td>
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<td>±</td>
<td>ND</td>
<td>–</td>
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<td>Kanamycin (20)</td>
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<td>±</td>
<td>ND</td>
<td>±</td>
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<td>+</td>
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<td>Chloramphenicol (50)</td>
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<td>+</td>
<td>ND</td>
<td>–</td>
<td>±</td>
<td>(–)</td>
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<tr>
<td>Ampicillin (50)</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>±</td>
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<td>35°C</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
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<td>ND</td>
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<tr>
<td>37°C</td>
<td>±</td>
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<td>ND</td>
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<td>pH10</td>
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<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>0.5% (w/v) NaCl in MAG</td>
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<td>ND</td>
<td>ND</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>Siderophore production</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NG</td>
<td>NG</td>
<td>+</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 5-10⁷ are C₁₆:₀ and C₁₈:₁ω₇c/C₁₈:₁ω₆c. Effective nodules are induced on Vigna unguiculata, Vigna subterranea and Arachis hypogaea, Lablab purpureus but not on Phaseolus vulgaris.

The type strain 5-10⁷ [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%.

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

References


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