

Bradyrhizobium ontarionense sp. nov. isolated from Aeschynomene indica (Indian jointvetch) harbours photosynthesis, nitrogen fixation and nitrous oxide reductase genes.

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Abstract

Bacterial strain A19^T was previously isolated from a root-nodule of *Aeschynomene indica* (Indian jointvetch) and assigned to a new lineage in the genus *Bradyrhizobium*.

Here data are presented for the detailed phylogenomic and taxonomic characterisation of strain A19^T.

Phylogenetic analysis of whole genome sequences as well as 51 concatenated core gene sequences placed strain A19^T in a highly supported lineage that was distinct from described *Bradyrhizobium* species; *B. oligotrophicum*, a symbiont of *A. indica*, was the most closely related species. The digital DNA-DNA hybridization and average nucleotide identity values for strain A19^T in pair-wise comparisons with close relatives were far lower than the respective threshold values of 70% and ~96% for definition of species boundaries.

The complete genome of strain A19^T consists of a single 8.44 Mbp chromosome (DNA G+C content, 64.9 mol%) and contains a photosynthesis gene cluster, nitrogen-fixation genes and genes encoding a complete denitrifying enzyme system including nitrous oxide reductase. Nodulation and type III secretion system genes, needed for nodulation by most rhizobia, were not detected in the genome of A19^T.

Data for multiple phenotypic tests complemented the sequence-based analyses. Strain A19^T elicits nitrogen-fixing nodules on stems and roots of *A. indica* plants but not on soybeans or *Macroptilium atropurpureum*. Based on the data presented, a new species named *Bradyrhizobium ontarionense* sp. nov. is proposed with strain A19^T (= LMG 32638^T = HAMBI 3761^T) as the type strain.

Introduction

Bacteria belonging to the genus *Bradyrhizobium* represent one of the most abundant taxa in soils globally and are considered a priority group for research on furthering the understanding of the contribution of soil microbes to ecosystem functioning (Delgado-Baquerizo et al. 2018). Moreover, the genus *Bradyrhizobium* includes economically important species that fix nitrogen in symbiotic association with agricultural plants such as soybean (*Glycine max*) and peanut (*Arachis hypogaea*) (Lindström and Mousavi), species that are capable of photosynthesis (Giraud et al. 2007), and, species that possess genes for nitrous oxide (N₂O) reductase and are able to reduce N₂O, a potent greenhouse gas, to nitrogen (Minamisawa, 2023).

Bacterial species possessing photosynthesis genes are distributed across the genus *Bradyrhizobium* (Avontuur et al. 2023). These include apparently free-living (non-symbiotic) species such as *B. betae* (Rivas et al. 2004; Cloutier and Bromfield, 2019), *B. amphicarpaeae* (Bromfield et al. 2019) and *B. cosmicum* (Wasai-Hara et al. 2020). Others include symbiotic species such as *B. oligotrophicum* (Okubo et al. 2013; Ramirez-Bahena et al. 2013), *B. aeschynomenes* (Sun et al. 2022), and, *B. denitrificans* (van Berkum et al. 2006) that lack the Type III Secretion System (T3SS) and nodulation (*nod*) genes needed for symbiosis by most rhizobia and yet are still capable of eliciting root- and stem-nodules on tropical plant species of the genus *Aeschynomene* in a nodulation (*nod*) factor-T3SS independent manner (Giraud et al. 2007; Camuel et al. 2023).

In previous work (unpublished) we grew plants of *Aeschynomene indica* (Indian jointvetch) in pots in the glasshouse to produce seed for our research. After several weeks of plant growth in the glasshouse, a few sporadic nodules were observed on plant roots that were apparently due to “volunteer” bacteria in the rooting medium. Analysis of *recA* house-keeping (core) gene sequences placed bacteria isolated from these root-nodules in several novel lineages in the genus *Bradyrhizobium*.

The objective of the current work was the phylogenetic, genomic and taxonomic description of one of these new lineages represented by strain A19^T. This strain possesses photosynthesis, nitrogen fixation and nitrous oxide reductase genes and is capable of eliciting nitrogen-fixing nodules on the stems and roots of *A. indica* plants. Based on the data presented, a new species is proposed with the name *Bradyrhizobium ontarionense* sp. nov..

Materials and methods

Bacterial strains

Bacterial strain A19^T was isolated from a nodule that formed on the root of an *Aeschynomene indica* plant grown in a pot in a glasshouse (16 hours, 25–28°C (day); 8 hours, 16–18°C, (night)). The rooting medium consisted of a mixture of Canadian peat moss and locally sourced (Ottawa, Ontario) black-earth that had been sterilized by steaming for 8 hours and then stored in bulk without aseptic precautions until use.

Bacteria employed in this work are listed in Table S1 or in the Tables and Figures of the main text and Appendix. Bacterial strains were grown on modified yeast extract-mannitol (YEM) agar medium having the following composition (g/l⁻¹): yeast-extract (Thermo Scientific™ Oxoid™), 1.5; mannitol, 1.0; NaCl, 0.1; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; Bacteriological agar (Thermo Scientific™ Oxoid™), 18.0. Bacterial cultures were maintained in 20% w/v glycerol at -80°C.

Phylogenetic analysis

Analysis of the core gene sequences (*atpD*, *glnI*, *gyrB*, *recA*, *rpoB* and 16S rRNA) of strain A19^T and *Bradyrhizobium* reference strains were done using sequences retrieved from whole genome sequences (where available). Analysis of photosynthetic reaction center (*puflM*) genes, nitrogen fixation (*nifHDK*) genes and the nitrous oxide reductase (*nosZ*) gene were done using full-length gene sequences retrieved from genome sequences. Alignment of nucleotide sequences was performed using MUSCLE (Edgar, 2004). Sequence accession numbers are listed in Table S1.

Further analysis was carried out using 51 core gene sequences encoding ribosome protein subunits (*rps*) of novel strain A19^T and reference (type) strains consisting of 72 *Bradyrhizobium* species (Jolley et al., 2012). Aligned and concatenated sequences of *rps* genes of A19^T and reference strains were obtained from genome sequences using the Genome Comparator program implemented in the domain genome database of the BIGSdb software platform (Jolley and Maiden 2010). To avoid confounding the phylogenetic analysis, the sequences *rpsU* and *rpmH* were excluded because they were either incomplete or paralogous in several *Bradyrhizobium* reference strains.

The ModelTest-NG tool (Darriba et al. 2020) in the web-based CIPRES Science Gateway version 3.3 (Miller et al., 2010) was used to select best fit substitution models. Analyses done using MrBayes (software version 3.2.1) were done employing default priors as described previously (Yu et al. 2014). Maximum-likelihood analyses were performed using 1,000 non-parametric bootstrap replications (Guindon et al. 2010). Bayesian trees are only presented in this work as trees reconstructed from Bayesian and Maximum-likelihood methods exhibited closely similar topologies.

A whole genome sequence based phylogenetic tree was reconstructed using the online Type Strain Genome Server (TYGS) (Lefort et al. 2015; Meier-Kolthoff and Göker 2019).

Genome analysis

Genomic DNAs were isolated and purified from bacterial cells grown for 7 days at 28°C on YEM agar medium as detailed by Bromfield et al. (2023).

Sequencing of the genome of strain A19^T was done at the Genome Quebec Innovation Centre, Canada, employing Pacific Biosciences (PacBio) Sequel Single-Molecule Real-Time (SMRT) technology (Ardui et al. 2018). Flye software (version 2.9) (Kolmogorov et al. 2019) was used for genome sequence assembly.

The overall genome relatedness indices of digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) are routinely employed in bacterial taxonomic studies to facilitate species circumscription (Chun et al. 2018; Meier-Kolthoff and Göker 2019). Algorithms implemented in the TYGS were used to calculate dDDH values and associated confidence intervals (Meier-Kolthoff et al. 2013; Meier-Kolthoff and Göker 2019). The accepted dDDH threshold of 70% was used to define species boundaries (Meier-Kolthoff and Göker 2019). ANI values were calculated utilizing FastANI (Jain et al. 2018) performed in the K base web server (Arkin et al., 2018). The ANI threshold ~ 96% was used for delineation of bacterial species boundaries (Richter and Rosselló-Móra 2009; Lee et al. 2016; Ciufu et al. 2018).

Genome sequence comparisons were facilitated by utilizing the software, Geneious Prime 2023.0.4 (<https://www.geneious.com>) and GenomeMatcher (Ohtsubo et al. 2008).

Phenotypic characterisation

The Gram-stain reactivity of bacterial cells was assessed using the protocol outlined by Buck (1982).

Analysis of fatty acids, was done using bacteria grown at on at 28°C on YEM agar medium for 7 days. Fatty acids were extracted as detailed by Sasser (1990). Identification of fatty acids was performed using the Sherlock Microbial Identification System (MIDI) version 6.0 and RTSBA6 database.

Tests of chemical sensitivity and carbon source utilization were done using BIOLOG GEN III MicroPlates (Biolog™, United States) as detailed in the manufacturer's instructions.

Cells of strain A19^T were examined using a scanning electron microscope (model, Hitachi SU7000 FESEM) and a transmission electron microscope (model, H-7000; Hitachi). For microscopic examination of cells, strain A19^T was grown for 96 hrs in YEM broth at 28°C as outlined previously (Bromfield et al. 2023).

Tests of acid or alkali production by A19^T and reference strains grown for 21 days on YEM agar medium at 28°C were performed as detailed by Bromfield et al. (2010).

Plant tests of nodulation and nitrogen fixation were carried out using modified Leonard jars (Vincent 1970) (two plants per jar with three replicate jars for each inoculation treatment) as outlined by Bromfield et al. (2010). Seeds of *A. indica* were vernalized by immersion in liquid nitrogen (-196°C) for 20 seconds and surface sterilized by serial immersion in 70% ethanol (30 seconds), 10.5% sodium hypochlorite solution (90 seconds) followed by multiple washes in water over two hours. The seeds were left in sterile water overnight at 4°C to facilitate imbibition and then transferred to water agar plates (Bacteriological agar (Thermo Scientific™ Oxoid™) 15 g/l) at 25°C to germinate. For tests of *A. indica* stem nodulation, cell suspensions of *Bradyrhizobium* test strains (ca. 10⁹ cells/ ml in sterile water) were applied to stems with cotton-wool swabs. The inoculated portions of stems were kept moist for 48 hrs using wrappings of moistened tissue paper covered with plastic cling film.

Results and Discussion

Genomic and phylogenetic characterisation

A complete genome sequence of novel strain A19^T was generated in this work; genome coverage was 634-fold with 60448 polymerase reads and 91671 bp average read length. The genome of strain A19^T consists of a single chromosome of size 8435845 bp and has a DNA G + C content of 64.9 mol% (Table 1).

Table 1
Genome characteristics of *Bradyrhizobium ontarionense* sp. nov. A19^T and reference strains.

Characteristic	Strain							
	<i>B. ontarionense</i> sp. nov. A19 ^T	<i>B. oligotrophicum</i> S58 ^T	' <i>B. aescynomenes</i> ' 83002	<i>Bradyrhizobium</i> sp. BTAi1	<i>Bradyrhizobium</i> sp. ORS 278	<i>Bradyrhizobium</i> sp. ORS 285	' <i>B. guangzhouense</i> ' CCBAU 51670	<i>B. amp</i> 39S ^T
Genome assembly (no. contigs)	Complete (1)	Complete (1)	Draft (58)	Complete (2)	Complete (1)	Complete (1)	Complete (2)	Com
Genome size (bp)	8435845	8264165	7522254	8493513	7456587	7797098	8138177	7044
Plasmid no. (size, bp)	0	0	na	1 (228826)	0	0	1 (979291)*	0
Genes (total)	7456	7276	6808	7700	6624	6937	7683	6604
CDSs (total)	7390	7212	6739	7638	6672	6877	7629	6547
G + C content %	64.9	65.1	65	64.8	65.5	65.2	63.4	64.7
No. of rRNA operons (5S, 23S, 16S)	2	2	na	2	2	2	1	2
Photosynthetic gene cluster	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
<i>Nodulation</i> (<i>nodABC</i>) genes	No	No	No	No	No	Yes	Yes	No
<i>Nitrogen fixation</i> (<i>nifHDK</i>) genes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Type III Secretion System (T3SS) genes	No	No	No	No	No	Yes	Yes	No
Nitrous oxide reductase (<i>nosRZDFYL</i>) genes	Yes	Yes	Yes	Yes	No	Yes	No	No
tRNAs	56	54	51	52	52	50	47	50
* Symbiosis plasmid								
na, data not available								

The analysis of 16S rRNA gene sequences has traditionally been used as a taxonomic tool in species descriptions. However, this gene is highly conserved and different bacterial species may possess identical 16S rRNA sequences (Richter and Rosselló-Móra, 2009; de Lajudie et al. 2019). Nevertheless the analysis of 16S rRNA gene sequences is considered to be useful for verifying the genus level identity of bacteria (Young et al. 2023).

The phylogenetic tree of 16S rRNA gene sequences (Fig. S1) of strain A19^T and 86 *Bradyrhizobium* species (type strains) confirms placement of A19^T in the genus *Bradyrhizobium*. The tree also shows that strain A19^T is placed in a novel lineage and *B. oligotrophicum* is the most closely related species.

Multiple Locus Sequence Analysis (MLSA) of single copy, protein encoding gene sequences is a widely used tool for species differentiation (Jolley et al. 2012; de Lajudie et al. 2019).

The Bayesian tree of concatenated core gene sequences (*atpD-glnI-gyrB-recA-rpoB*; alignment length, 2679 bp) of strain A19^T and reference strains (Fig. S2), corroborates the placement of A19^T in a new *Bradyrhizobium* lineage with *B. oligotrophicum* S58^T as the closest relative. Fig. S2 also shows that strain A19^T is placed in a clade (represented by *B. oligotrophicum*) containing only photosynthetic symbionts (*B. oligotrophicum*, *B. aescynomenes* and *B. denitrificans*) of the tropical legume *A. indica*. It should be noted that like these photosynthetic symbionts, novel strain A19^T was able to elicit nitrogen-fixing stem- and root-nodules on *A. indica* plants (see Phenotypic characterisation section, below).

The taxonomic status of strain A19^T was further investigated by MLSA of 51 concatenated bacterial ribosome protein subunit (*rps*) gene sequences (Jolley et al. 2012) as well as a phylogenomic analysis (based on whole genome sequences) implemented in the TYGS (Meier-Kolthoff and Göker 2019). The

phylogenetic tree of *rps* gene sequences (Fig. 1) and the TYGS tree based on whole genome sequences (Fig. 2) support the finding that strain A19^T is placed in the clade of photosynthetic symbionts represented by *B. oligotrophicum* and occupies a highly supported novel lineage; *B. oligotrophicum* is the closest relative.

The dDDH and ANI values from pair-wise comparison of the genome sequence of A19^T with the genome sequences of '*B. aescynomenes*' 83002 and *B. oligotrophicum* S58^T (i.e., the only species type strains in the *B. oligotrophicum* clade that have genome sequences available in public databases) are shown in Table S2. The largest values in these comparisons (33.4% (dDDH) and 88.8% (ANI)) are far lower than the threshold values (70% and ~96%, respectively) used for the definition of species boundaries. Based on these results, strain A19^T represents a new species of *Bradyrhizobium* and *B. oligotrophicum* represents the most closely related species.

Genome sequence analyses revealed that strain A19^T possesses a photosynthesis gene cluster (PGC) of size about 49 kb (co-ordinates 3,498,729–3,547,553 bp). The PGC contains genes encoding bacteriochlorophyll (*bchIDOCXYZGPFNBHLM* and *acsF*), reaction centre L, M and H subunits (*puflM* and *puhA*), light-harvesting protein alpha and beta subunits (*pufBA*), carotenoid (*crtIBCDEF*), bacteriophytochrome (*bphP*) and photosynthesis repressor (*ppsR1* and *ppsR2*) proteins.

A Bayesian phylogenetic tree of concatenated photosynthetic reaction centre, *puflM*, genes (Fig. 3A) shows that strain A19 is placed in a clade with other symbionts of *A. indica*; the closest relative of A19^T is *B. oligotrophicum* S58^T. The tree also shows that nonsymbiotic species (*B. cosmicum*, *B. amphicarpeae* and *B. betae*) are placed in a clade separate from the *A. indica* symbionts, *B. denitrificans*, *B. oligotrophicum*, '*B. aescynomenes*' and *Bradyrhizobium* sp BTAi1 and novel strain A19^T. It is notable that the phylogenetic division of symbiotic and nonsymbiotic bradyrhizobia corresponds to the two types of photosynthetic gene clusters (PGC-1 and PGC-2, respectively) defined by Avontuur et al. (2023) on the basis of the organization of photosynthetic genes. The organization of genes in the PGC of strain A19^T is similar to close relative *B. oligotrophicum* (both with type-1 PGCs) but differs from non-symbiotic bacteria such as *B. amphicarpeae* with type-2 PGCs (Fig. 3B).

Further analyses show that novel strain A19^T lacks key nodulation (*nodABC*) and Type III Secretion System (T3SS) genes (Table 1) indicating that its symbiotic association with *A. indica* plants is initiated in a nod-factor and T3SS independent manner like the well characterised photosynthetic symbionts, *Bradyrhizobium* spp. BTAi1 and ORS278 (Giraud et al. 2007; Camuel et al. 2023).

In contrast to the absence of *nod* genes, the following key nitrogen fixation genes were found in the genome of strain A19: *nifDKEN*, *nifH*, *nifA* and *fixABCX* (co-ordinates 7,793,281–7,842,331 bp). The phylogenetic tree of concatenated full length *nifHDK* gene sequences (Fig. S3) shows that strain A19^T occupies a lineage that is well separated from other *Bradyrhizobium* species; the type strains of '*B. aescynomenes*' and *B. oligotrophicum* are closest relatives.

Agricultural soils are a major source of N₂O, a highly potent greenhouse gas and accelerant of ozone layer depletion (Montzka et al. 2011; Tian et al. 2020). The majority of N₂O released from soils originates as a byproduct from the respiratory activity of nitrifying and denitrifying microorganisms (Thompson et al. 2012). While N₂O can be generated by multiple mechanisms, the only known biological sink for N₂O is the reduction of N₂O to dinitrogen by the enzyme N₂O reductase (encoded by the *nosZ* gene) typically found in denitrifying bacteria (Torres et al. 2016; Minamisawa, 2023). Some symbiotic nitrogen fixing *Bradyrhizobium* species (e.g., *B. ottawaense* and *B. diazoefficiens*) possess denitrifying enzyme systems that include N₂O reductase and the use of these species has been suggested as a strategy for mitigating N₂O emissions from agricultural soils (Akiyama et al. 2016; Wasai-Hara et al. 2020; Wasai-Hara et al. 2023). In the current study we detected key genes encoding enzymes required for the complete denitrification of nitrate or nitrite to dinitrogen gas in the genome sequence of strain A19 as follows: *napEDABC* (nitrate reductase); *nirK* (nitrite reductase); *norCBQDE* (nitric oxide reductase) and *nosRZDFYLX* (nitrous oxide reductase). Based on these findings, novel strain A19 provides potential for further studies on the reduction of N₂O to inert gaseous nitrogen by members of the genus *Bradyrhizobium*.

A Bayesian phylogenetic tree of the *nosZ* gene of strain A19 and reference strains of the genus *Bradyrhizobium* is presented in Fig. 4. The tree showing the placement of strain A19 in a distinct lineage (closest neighbours, *B. xenodulans*, *B. lablabi* and *B. zhenyangense*) is incongruent with its placement in the trees based on core genes (Fig. 1) and whole genome sequences (Fig. 2) (closest neighbours *B. oligotrophicum* and '*B. aescynomenes*'), suggesting that the *nosZ* gene was acquired by horizontal gene transfer.

We carried out further analyses to assess the frequency of occurrence of the *nosZ* gene (encoding N₂O reductase) in the genus *Bradyrhizobium* by screening the genome sequences of type strains of named species. The results (Table S3) show that a substantial minority (i.e., 21 of 73 *Bradyrhizobium* species type strains) possess the *nosZ* gene. It is noteworthy that most symbionts of the aquatic legume *A. indica* (e.g. *B. oligotrophicum* S58^T, '*B. aescynomenes*' 83002, novel strain A19 (Table 1 and Table S3) and *Bradyrhizobium* spp. strains BTAi1 and ORS 285 (Table 1) possess the *nosZ* gene, suggesting that reduction of N₂O to nitrogen (where nitrate rather than oxygen is used as a terminal electron acceptor during respiration) might be an adaptation to oxygen limitation in environments subject to periodic waterlogging.

Phenotypic analyses

Colonies of strain A19^T are circular, cream coloured, raised and with diameters ~0.5 mm after growth on YEM agar medium for 7 days at 28°C. Cells of strain A19^T are Gram-stain-negative, rod shaped, and based on examination by electron microscopy, possess at least one flagellum (Fig. S4). Growth on YEM agar medium at 28°C is accompanied by an alkaline reaction (Table S4), which is characteristic of the genus *Bradyrhizobium*. Strain A19^T, like close relative *B. oligotrophicum* S58^T, shows growth at pH 5, but does not grow at pH 10, at 10°C, or, in the presence of 0.5% NaCl, after 7 days incubation on YEM agar medium. However, strain A19^T differed from *B. oligotrophicum* S58^T, in that it did not grow at 37°C (Table S4).

Results for fatty acid profiles of strain A19^T and four reference strains are presented in Table S5. Fatty acids C16:0, 18:1 ω 7c 11-methyl and C18:1 ω 6c/C18:1 ω 7c (summed feature 8), were detected in A19^T and all four reference strains. The dominance of fatty acids C16:0 and C18:1 ω 6c/C18:1 ω 7c (summed feature 8) in strain A19^T is typical of the genus *Bradyrhizobium* (Tighe et al. 2000).

Table S6 shows the results for assays of carbon source utilization and chemical sensitivity utilizing Biolog[™] phenotype microarrays. The data show that strain A19^T can be readily differentiated from photosynthetic symbionts of *A. indica* (*B. oligotrophicum* S58^T, *B. denitrificans* IFAM 1005^T and *B. aeschynomenes* 83002^T), as well as from the (non-photosynthetic) genus type strain (*B. japonicum* USDA6^T) based on multiple tests.

Plant tests showed that strain A19^T was able to elicit efficient nitrogen fixing nodules on the stems and roots of *A. indica* plants (Fig. 5) but did not form nodules on ‘Glengarry’ soybeans or *Macroptilium atropurpureum* ‘siratro’.

Description of *Bradyrhizobium ontarionense* sp. nov.

Bradyrhizobium ontarionense (on.ta.ri.o.nen'se. N.L. neut. adj. *ontarionense*, of or belonging to the province of Ontario). Bacterial cells are aerobic, non-spore-forming rods, Gram-stain-negative and possess one or more flagella. Colonies on YEM agar medium are cream colored, raised and circular with diameters ~ 0.5 mm after growth for 7 days at 28°C. Produces an alkaline reaction on YEM agar medium. Grows at pH 5 but not at pH 10 (optimum ~ pH 7.0). Does not grow at 10°C or 37°C (optimal at ~ 28°C) or in the presence of 0.5% (w/v) NaCl. Dominant fatty acids are C16:0 and C18:1 ω 6c/C18:1 ω 7c (summed feature 8). The type strain is able to utilize 17 carbon sources including α -D-Glucose, D-Galactose, D-Sorbitol, D-Mannitol, D-Arabitol, D-Gluconic Acid, D-Malic Acid, L-Malic Acid, Tween 40, Propionic Acid, Acetic Acid and Formic Acid. The type strain does not utilize 53 carbon sources including D-Fructose, L-Fucose, myo-Inositol, D-Glucose- 6-PO₄, D-Fructose- 6-PO₄, D-Aspartic Acid, Gelatin, Pectin, D-Galacturonic Acid, L-Galactonic Acid Lactone, Mucic Acid, L-Lactic Acid, Citric Acid, and γ -Amino-Butyric Acid. The type strain is resistant to 1% Sodium Lactate, Troleandomycin, Rifamycin SV, Minocycline, Lincomycin, Tetrazolium Violet, Tetrazolium Blue, Nalidixic Acid and Aztreonam. Susceptible to Fusidic Acid, Niaproof 4, D-Serine, Guanidine HCl, Vancomycin, Potassium Tellurite, Lithium Chloride, Sodium Butyrate and Sodium Bromate.

Elicits efficient nitrogen fixing root- and stem-nodules on plants of the aquatic legume *Aeschynomene indica*. Does not elicit nodules soybeans or *Macroptilium atropurpureum*.

The type strain, A19^T (= LMG 32638^T = HAMBI 3761^T) was isolated from a root-nodule of an *Aeschynomene indica* plant grown in a greenhouse. The size of the genome is 8.44 Mbp and the DNA G + C content is 64.9 mol%. The type strain does not possess nodulation or type III secretion system genes but contains photosynthesis genes, nitrogen-fixation genes and genes encoding a complete denitrifying enzyme system including nitrous oxide reductase.

Abbreviations

ANI
Average Nucleotide Identity
dDDH
digital DNA DNA Hybridization
GBDP
Genome Blast Distance Phylogeny
MLSA
Multiple Locus Sequence Analysis
TYGS
Type Strain Genome Server
YEM
Yeast-extract mannitol medium.

Declarations

Statements and Declarations

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Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Ethical statement

Not applicable.

Author contributions

ESPB, conceptualization, funding acquisition and draft manuscript writing. SC and ESPB, experimentation, data analyses, writing-review and editing. Both authors read and approved the final manuscript.

Data availability

All data required for this study are included in this paper and Supplementary Information. The whole genome shotgun project for *Bradyrhizobium ontarionense* sp. nov. strain A19^T was deposited at DDBJ/ENA/ GenBank as accession number CP088156. Raw PacBio data was deposited in the NCBI Sequence Read Archive as BioProject accession number PRJNA783021. A culture of *Bradyrhizobium ontarionense* sp. nov. strain A19^T was deposited in the Culture Collection of Bacteria (BCCM/LMG), University of Ghent, Belgium as LMG 32638^T and in the Microbial Culture Collection (HAMBI), University of Helsinki, Finland as HAMBI 3761^T.

References

1. Akiyama H, Hoshino Y, Itakura M, Shimomura Y, Wang Y, Yamamoto A et al (2016) Mitigation of soil N₂O emission by inoculation with a mixed culture of indigenous *Bradyrhizobium diazoefficiens*. *Sci Rep* 6:32869. <https://doi.org/10.1038/srep32869>
2. Ardui S, Ameur A, Vermeesch JR, Hestand MS (2018) Single molecule real-time (SMRT) sequencing comes of age: applications and utilities for medical diagnostics. *Nucleic Acids Res* 46:2159–2168. <https://doi.org/10.1093/nar/gky066>
3. Arkin AP, Cottingham RW, Henry CS, Harris NL, Stevens RL, Maslov S, et al (2018) KBase: the United States Department of Energy Systems Biology Knowledgebase. *Nat Biotechnol* 36:566–569. <https://doi.org/10.1038/nbt.4163>
4. Avontuur JR, Wilken PM, Palmer M, Coetzee MPA, Stępkowski T, Venter SN, Steenkamp ET (2023) Complex evolutionary history of photosynthesis in *Bradyrhizobium*. *Microb Genomics* 9:001105. <https://doi.org/10.1099/mgen.0.001105>
5. Bromfield ESP, Tambong JT, Cloutier S, Prévost D, Laguerre G, van Berkum P et al (2010) *Ensifer*, *Phyllobacterium* and *Rhizobium* species occupy nodules of *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover) grown at a Canadian site without a history of cultivation. *Microbiology* 156:505–520. <https://doi.org/10.1099/mic.0.034058-0>
6. Bromfield ESP, Cloutier S, Nguyen HDT (2019) Description and complete genome sequence of *Bradyrhizobium amphicarpaceae* sp. nov., harbouring photosystem and nitrogen-fixation genes. *Int J Syst Evol Microbiol* 69:2841–2848. <https://doi.org/10.1099/ijsem.0.003569>
7. Bromfield ESP, Cloutier S, Hynes MF (2023) *Ensifer canadensis* sp. nov. strain T173^T isolated from *Melilotus albus* (sweet clover) in Canada possesses recombinant plasmid pT173b harbouring symbiosis and type IV secretion system genes apparently acquired from *Ensifer medicae*. *Front Microbiol* 14:1195755. <https://doi.org/10.3389/fmicb.2023.1195755>
8. Camuel A, Teulet A, Carcagno M, Haq F, Pacquit V, Gully D et al (2023) Widespread *Bradyrhizobium* distribution of diverse Type III effectors that trigger legume nodulation in the absence of Nod factor. *ISME J* 17:1416–1429. <https://doi.org/10.1038/s41396-023-01458-1>
9. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS et al (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 68:461–466. <https://doi.org/10.1099/ijsem.0.002516>
10. Ciufo S, Kannan S, Sharma S, Badretin A, Clark K, Turner S et al (2018) Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes at the NCBI. *Int J Syst Evol Microbiol* 68:2386–2392. <https://doi.org/10.1099/ijsem.0.002809>
11. Cloutier S, Bromfield ESP (2019) Analysis of the complete genome sequence of the widely studied strain *Bradyrhizobium betae* PL7HG1^T reveals the presence of photosynthesis genes and a putative plasmid. *Microbiol Resour Announc* 8:e01282–19. <https://doi.org/10.1128/MRA.01282-19>
12. Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T (2020) Model test-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Mol Biol Evol* 37:291–294. <https://doi.org/10.1093/molbev/msz189>
13. Delgado-Baquerizo M, Oliverio AM, Brewer TE, Benavent-González A, Eldridge DJ, Bardgett RD et al (2018) A global atlas of the dominant bacteria found in soil. *Science* 359:320–325. <https://doi.org/10.1126/science.aap9516>
14. de Lajudie PM, Andrews M, Ardley J, Eardly B, Jumas-Bilak E, Kuzmanović N et al (2019) Minimal standards for the description of new genera and species of rhizobia and agrobacteria. *Int J Syst Evol Microbiol* 69:1852–1863. <https://doi.org/10.1099/ijsem.0.003426>
15. Edgar RC (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform* 5:113. <https://doi.org/10.1186/1471-2105-5-113>
16. Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre J-C et al (2007) Legumes symbioses: absence of *Nod* genes in photosynthetic bradyrhizobia. *Science* 316:1307–1312. <https://doi.org/10.1126/science.1139548>
17. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol* 59:307–321. <https://doi.org/10.1093/sysbio/syq010>
18. Holland BR, Huber KT, Dress A, Moulton V (2002) Delta plots: a tool for analyzing phylogenetic distance data. *Mol Biol Evol* 19:2051–2059. <https://doi.org/10.1093/oxfordjournals.molbev.a004030>
19. Itakura M, Uchida Y, Akiyama H, Hoshino YT, Shimomura Y, Morimoto S et al (2013) Mitigation of nitrous oxide emissions from soils by *Bradyrhizobium japonicum* inoculation. *Nat Clim Change* 3:208–212. <https://doi.org/10.1038/nclimate1734>
20. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S (2018) High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat Commun* 9:5114. <https://doi.org/10.1038/s41467-018-07641-9>
21. Jolley KA, Maiden MCJ (2010) BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinform* 11:595. <https://doi.org/10.1186/1471-2105-11-595>
22. Jolley KA, Bliss CM, Bennett JS, Bratcher HB, Brehony C, Colles FM et al (2012) Ribosomal multilocus sequence typing: universal characterisation of bacteria from domain to strain. *Microbiology* 158:1005–1015. <https://doi.org/10.1099/mic.0.055459-0>

23. Kolmogorov M, Yuan J, Lin Y, Pevzner PA (2019) Assembly of long, error-prone reads using repeat graphs. *Nat Biotechnol* 37:540–546. <https://doi.org/10.1038/s41587-019-0072-8>
24. Lee I, Ouk Kim Y, Park SC, Chun J (2016) OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* 66:1100–1103. <https://doi.org/10.1099/ijsem.0.000760>
25. Lefort V, Desper R, Gascuel O (2015) Fast ME 2.0: a comprehensive, accurate, and fast distance-based phylogeny inference program. *Mol Biol Evol* 32:2798–2800. <https://doi.org/10.1093/molbev/msv150>
26. Lindström K, Mousavi SA (2019) Effectiveness of nitrogen fixation in rhizobia.
27. *Microb Biotechnol* 13:1751–7915. <https://doi.org/10.1111/1751-7915.13517>
28. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinform* 14:60. <https://doi.org/10.1186/1471-2105-14-60>
29. Meier-Kolthoff JP, Göker M (2019) TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat Commun* 10:2182. <https://doi.org/10.1038/s41467-019-10210-3>
30. Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES science gateway for inference of large phylogenetic trees. In: *Proceedings of the Gateway Computing Environments Workshop*. New Orleans, LA.
31. Minamisawa K (2023) Mitigation of greenhouse gas emission by nitrogen-fixing bacteria. *Biosci, Biotechnol, Biochem* 87:7–12. <https://doi.org/10.1093/bbb/zbac177>
32. Montzka SA, Dlugokencky EJ, Butler JH (2011) Non-CO₂ greenhouse gases and climate change. *Nature* 476:43–50. <https://doi.org/10.1038/nature10322>
33. Ohtsubo Y, Ikeda-Ohtsubo W, Nagata Y, Tsuda M (2008) Genome matcher: a graphical user interface for DNA sequence comparison. *BMC Bioinform* 9:376. <https://doi.org/10.1186/1471-2105-9-376>
34. Okubo T, Fukushima S, Itakura M, Oshima K, Longtonglang A, Teamroong N, et al (2013) Genome analysis suggests that the soil oligotrophic bacterium *Agromonas oligotrophica* (*Bradyrhizobium oligotrophicum*) is a nitrogen-fixing symbiont of *Aeschynomene indica*. *Appl Environ Microbiol* 79:2542–2551. <https://doi.org/10.1128/AEM.00009-13>
35. Ramirez-Bahena MH, Chahboune R, Peix A, Velazquez E (2013) Reclassification of *Agromonas oligotrophica* into the genus *Bradyrhizobium* as *Bradyrhizobium oligotrophicum* comb. nov. *Int J Syst Evol Microbiol* 63:1013–1016. <https://doi.org/10.1099/ijms.0.041897-0>
36. Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U. S. A.* 106:19126–19131. <https://doi.org/10.1073/pnas.0906412106>
37. Rivas R, Willems A, Palomo JL, García-Benavides P, Mateos PF, Martínez-Molina E et al (2004) *Bradyrhizobium betae* sp. nov., isolated from roots of *Beta vulgaris* affected by tumour-like deformations. *Int J Syst Evol Microbiol* 54:1271–1275. <https://doi.org/10.1099/ijms.0.02971-0>
38. Sasser M (1990) Identification of Bacteria by gas Chromatography of Cellular Fatty acids *MIDI Technical Note 101*. Newark, DE: MIDI Inc.
39. Sun L, Zhang Z, Dong X, Tang Z, Ju B, Zongjun D et al (2022) *Bradyrhizobium aeschynomenes* sp. nov., a root and stem nodule microsymbiont of *Aeschynomene indica*. *Syst Appl Microbiol* 45:126337. <https://doi.org/10.1016/j.syapm.2022.126337>
40. Thomson AJ, Giannopoulos G, Pretty J, Baggs EM, Richardson DJ (2012) Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Phil Trans R Soc* 12:B3671157–1168. <https://doi.org/10.1098/rstb.2011.0415>
41. Tian H, Xu R, Canadell JG, Thompson RL, Winiwarter W, Suntharalingam P et al (2020) A comprehensive quantification of global nitrous oxide sources and sinks. *Nature* 586:248–256. <https://doi.org/10.1038/s41586-020-2780-0>
42. Tighe SW, de Lajudie P, Di Pietro K, Lindström K, Nick G, Jarvis BD (2000) Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the sherlock microbial identification system. *Int J Syst Evol Microbiol* 50:787–801. <https://doi.org/10.1099/00207713-50-2-787>
43. Torres MJ, Simon J, Rowley G, Bedmar EJ, Richardson DJ, Gates AJ, Delgado MJ (2016) Chapter Seven - Nitrous Oxide Metabolism in Nitrate-Reducing Bacteria: Physiology and Regulatory Mechanisms. In R K. Poole (ed) *Advances in Microbial Physiology*, Elsevier 68:353–432. <https://doi.org/10.1016/bs.ampbs.2016.02.007>
44. van Berkum P, Leibold JM, Eardly BD (2006) Proposal for combining *Bradyrhizobium* spp. (*Aeschynomene indica*) with *Blastobacter denitrificans* and to transfer *Blastobacter denitrificans* (Hirsch and Muller, 1985) to the genus *Bradyrhizobium* as *Bradyrhizobium denitrificans* (comb. nov.). *Syst Appl Microbiol* 29:207215. <https://doi.org/10.1099/ijsem.0.004380>
45. Vincent JM (1970) *A Manual for the Practical Study of Root-Nodule Bacteria*. Blackwell Scientific, Oxford
46. Wasai-Hara S, Hara S, Morikawa T, Sugawara M, Takami H, Yoneda J et al (2020) Diversity of *Bradyrhizobium* in non-leguminous sorghum plants: *B. ottawaense* isolates unique in genes for N₂O reductase and lack of the type VI secretion system. *Microbes Environ* 35:1–6. <https://doi.org/10.1264/jsme2.ME19102>
47. Wasai-Hara S, Minamisawa K, Cloutier S, Bromfield ESP (2020) Strains of *Bradyrhizobium cosmicum* sp. nov., isolated from contrasting habitats in Japan and Canada possess photosynthesis gene clusters with the hallmark of genomic islands. *Int J Syst Evol Microbiol* 70:5063–5074. <https://doi.org/10.1099/ijsem.0.004380>
48. Wasai-Hara S, Itakura M, Siqueira AF, Takemoto D, Sugawara M, Mitsui H et al (2023) *Bradyrhizobium ottawaense* efficiently reduces nitrous oxide through high *nosZ* gene expression. *Sci Rep* 13:18862. <https://doi.org/10.1038/s41598-023-46019-w>
49. Young JPW, Jorin B, Moeskjær S, James EK (2023) *Rhizobium brockwellii* sp. nov., *Rhizobium johnstonii* sp. nov. and *Rhizobium beringeri* sp. nov., three genospecies within the *Rhizobium leguminosarum* species complex. *Int J Syst Evol Microbiol* 73:005979. <https://doi.org/10.1099/ijsem.0.005979>

Figures

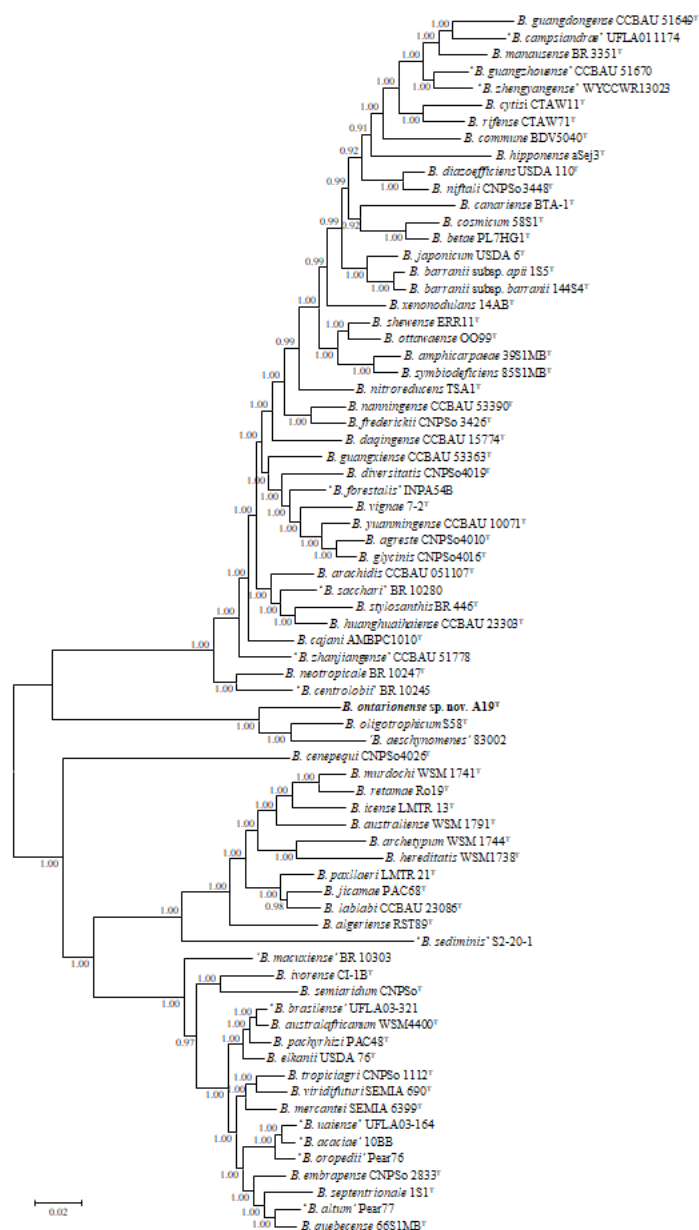


Figure 1

Bayesian tree (GTR+G+I substitution model) inferred from 51 full-length concatenated ribosome protein subunit (*rps*) gene sequences of *Bradyrhizobium ontariense* sp. nov. strain A19T and reference taxa. Alignment length, 22598bp. Posterior probabilities ≥ 0.90 are indicated. Bar represents expected substitutions per site.

Fig. 2. Phylogenomic tree based on TYGS implementation showing *Bradyrhizobium ontarioense* sp. nov. A19^T and reference taxa (species type strains) of the genus *Bradyrhizobium*. The tree was inferred with FastME 2.1.6.1 (Ciufo et al. 2018) from Genome Blast Distance Phylogeny (GBDP) distances calculated from genome sequences. Branch lengths are scaled according to GBDP distance formula d5. The numbers above branches represent GBDP pseudo-bootstrap support values ($\geq 70\%$) from 100 replications, with an average branch support of (97%). The tree is rooted at the mid-point. Leaf labels are annotated by affiliation to species (1), genomic G+C content (2), delta values (3), overall genome sequence length (4) and number of proteins (5). Delta statistics permit assessment of accuracy in terms of tree-likeness; the lower the delta value, the greater the accuracy (Holland et al. 2002).

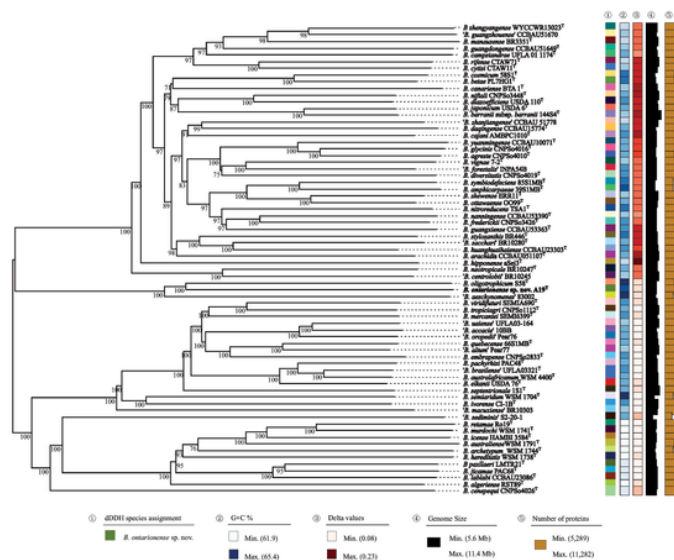
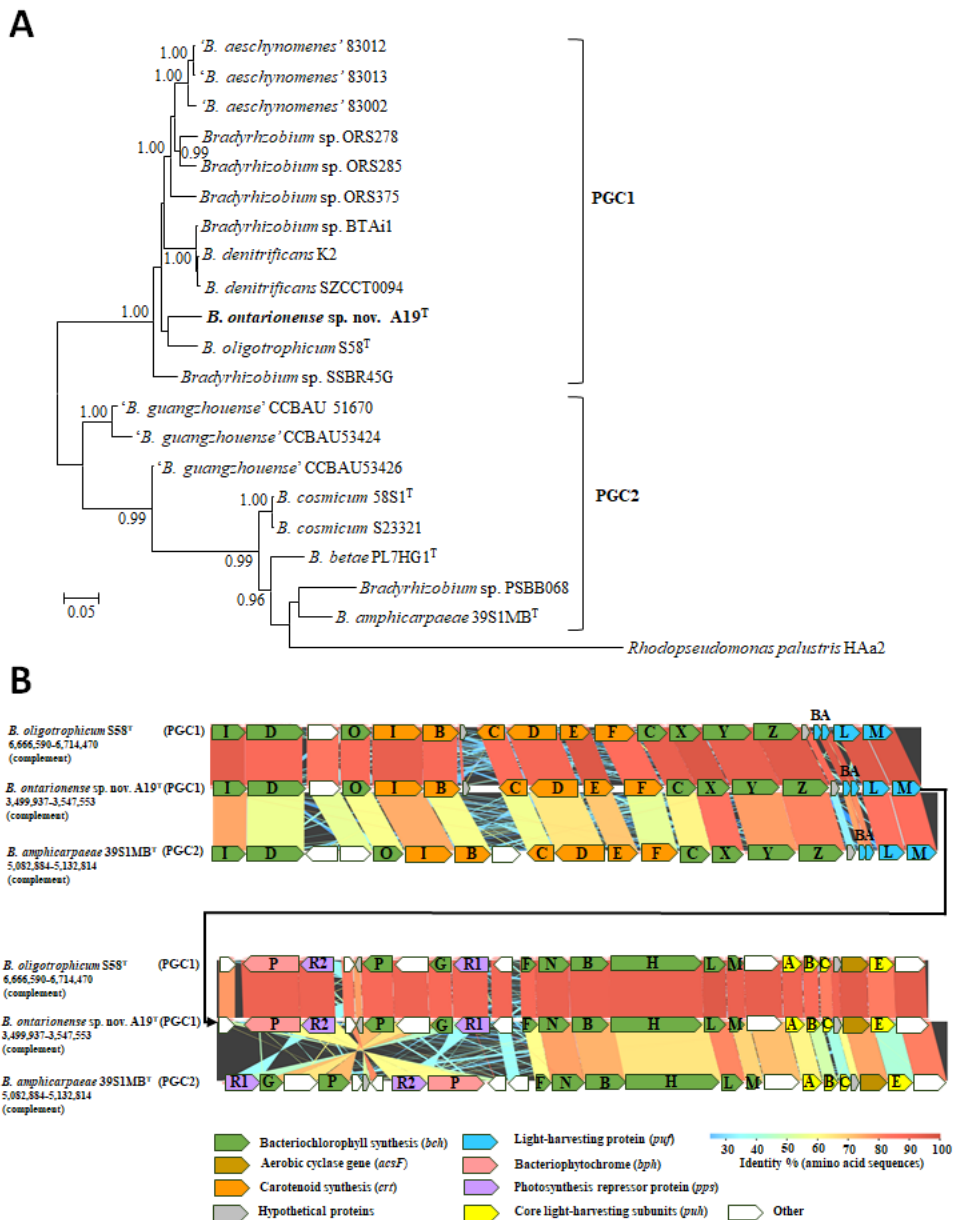


Figure 2

See image above for figure legend.



(A) Bayesian phylogenetic tree (GTR+G+I substitution model) of concatenated photosynthetic reaction centre, *pufLM*, genes (1739 bp) for *Bradyrhizobium ontarionense* sp. nov. strain A19T and reference taxa showing division of *Bradyrhizobium* strains into two clades corresponding to photosynthetic gene cluster type 1 (PGC1) and type 2 (PGC2) as defined by Avontuur et al. (2023). (B) Comparative arrangement of the photosynthesis gene cluster of *Bradyrhizobium ontarionense* sp. nov. strain A19T (PGC1) relative to *Bradyrhizobium oligotrophicum* (PGC1) and *Bradyrhizobium amphicarpaceae* (PGC2). Color coding represents % identity based on amino acid sequences of PGC genes calculated by GenomeMatcher software (Ohtsubo et al. 2008).

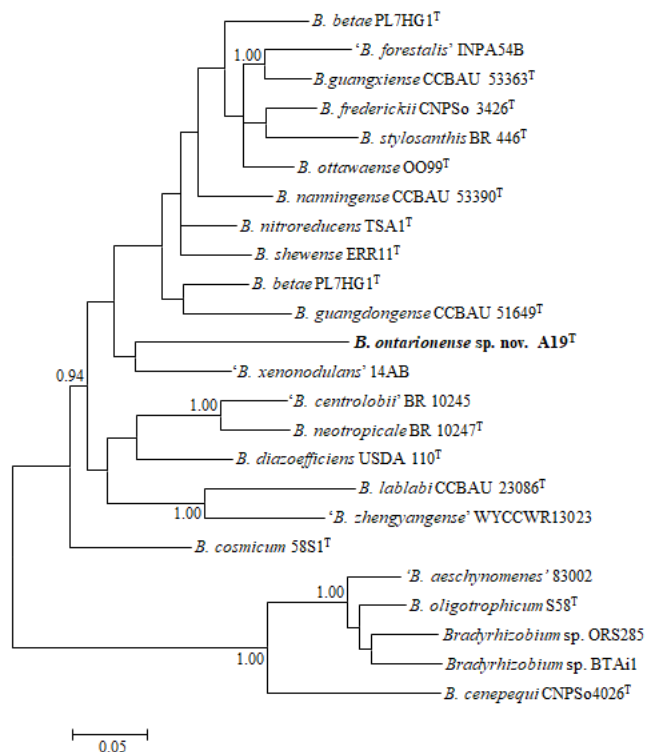


Figure 4

Bayesian phylogenetic tree (GTR+G+I substitution model) of nitrous oxide reductase (*nosZ*) gene sequences (1748 bp) for *Bradyrhizobium ontarionense* sp. nov. strain A19^T and reference taxa consisting of type strains of described species of the genus *Bradyrhizobium*. Posterior probabilities ≥ 0.90 are indicated. Bar represents expected substitutions per site.

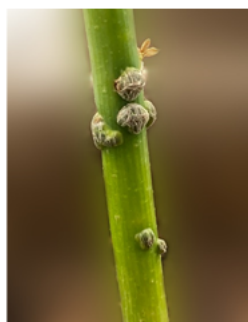


Figure 5

Effective nitrogen-fixing root (A) and stem (B and C) nodules on *Aeschynomene indica* plants inoculated with *Bradyrhizobium ontarionense* sp. nov. A19^T. Panel (C) shows the cut surface of an effective stem nodule exhibiting red colored pigment characteristic of leghemoglobin that is required for nitrogen fixation.

Supplementary Files

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- [SupplementaryMaterialsB.ontarionensev8.pdf](#)