

AUTHOR(S):

TITLE:

YEAR:

Publisher citation:

OpenAIR citation:

Publisher copyright statement:

This is the \_\_\_\_\_ version of an article originally published by \_\_\_\_\_  
in \_\_\_\_\_  
(ISSN \_\_\_\_\_; eISSN \_\_\_\_\_).

OpenAIR takedown statement:

Section 6 of the "Repository policy for OpenAIR @ RGU" (available from <http://www.rgu.ac.uk/staff-and-current-students/library/library-policies/repository-policies>) provides guidance on the criteria under which RGU will consider withdrawing material from OpenAIR. If you believe that this item is subject to any of these criteria, or for any other reason should not be held on OpenAIR, then please contact [openair-help@rgu.ac.uk](mailto:openair-help@rgu.ac.uk) with the details of the item and the nature of your complaint.

This publication is distributed under a CC \_\_\_\_\_ license.



# Evaluation of the microbiome of decaying alder nodules by next generation sequencing

Neil R. McEwan<sup>1,4\*</sup>, Toby Wilkinson<sup>1</sup>, Susan E. Girdwood<sup>1</sup>, Tim J. Snelling<sup>1</sup>, Tatiana Collins<sup>1</sup>, Kirsty Dougal<sup>1</sup>, David L. Jones<sup>2</sup> and Douglas L. Godbold<sup>3</sup>

<sup>1</sup>Aberystwyth University, Institute of Biological, Environmental and Rural Sciences, Aberystwyth, SY23 3DD, Wales, United Kingdom; <sup>2</sup>Bangor University, School of Environment, Natural Resources and Geography, Bangor, LL57 2UW, Wales, United Kingdom; <sup>3</sup>University of Natural Resources and Life Sciences, Institute of Forest Ecology, Peter-Jordan-Straße 82, A-1190 Vienna, Austria; <sup>4</sup>Robert Gordon University, School of Pharmacy and Life Sciences, Garthdee Campus, Aberdeen, AB10 7GJ, Scotland, United Kingdom; \*correspondence to: n.mcewan@rgu.ac.uk

**This work investigated the microbial content of decaying nodules from alders. The 16S rDNA composition of the microbiome of six senescent alder nodules was investigated by 454 sequencing. All nodules still had some Frankia sequences present, but in each case it was only detected at minor levels, with other organisms predominating. Although organisms from three different phyla (Bacteroidetes, Proteobacteria and Actinobacteria) constituted almost all (98% or more) of all sequences, Bacteroidetes were most abundant in four nodules with Proteobacteria being most abundant in the other two. In addition a few families were represented at a level of 10% or more of the total sequences: Sphingobacteriaceae (all 6 nodules); Chitinophagaceae (5 of 6); non-Frankia Actinomycetales (2 of 6); Caulobacteraceae (2 of 6); Flavobacteriaceae (2 of 6); Oxalobacteraceae (1 of 6); and Xanthomoadaceae (1 of 6). Analysis at the genus level showed a diverse range of organisms, with members of the genus Pedobacter being found at an abundant level within most nodules.**

Endocytobiosis and Cell Research (2017) 28(1):14–19

**Category:** Research article

**Keywords:** *Alnus glutinosa*, microbiome, Frankia, senescent nodules, 454 sequencing

Accepted: May 18, 2017

Published online: June 02, 2017

## Introduction

Members of the genus *Frankia* are actinomycetes which fix atmospheric nitrogen in symbiotic association with the roots of a range of trees and woody shrubs, including alders. In this partnership *Frankia* acts as the microsymbiont, with the host plants acting as the macrosymbiont. As a result of this relationship the trees show an improved growth due to the provision of fixed nitrogen. In addition, other organisms also present in the immediate environment may also gain benefits in terms of access to nitrogenous compounds, which is the reason that underpins the use of alder trees to help facilitate growth of other trees in forestry practices. By way of illustration, many species of alders (e.g. *Alnus glutinosa*, *A. rubra*) have been planted in forestry plots to facilitate growth of the trees planted with them (Wheeler and Miller 1990) due to fixation of atmospheric nitrogen taking place in their root systems.

Mature alder trees will commonly have several nodules associated with their root systems, and it is known that actinorhizal plants can have more than one strain of *Frankia* in the various nodules around their roots (Dobritsa and Stupar 1989). However the level of diversity within an alder single nodule is low (Kennedy et al. 2010), with each nodule containing one predominant strain (Dai et al. 2004; McEwan et al. 2015).

There has been considerable research carried out into the nodulation process (e.g. Berry et al. 1986; Van Ghelue et al. 1997) and also the events within the nodule during the process of nitrogen fixation (e.g. Vikman et al. 1990) including analysis of genes involved in nitrogen-fixation (e.g. Normand et al. 1988). However, although there has been some work done to examine what happens after nodule senescence, the level of information is relatively limited. To date, it is known that a loss of nitrogenase activity in the microsymbiont is one of the first signs of nodule senescence in both alders (Vikman et al. 1990) and legumes (Swaraj et al. 1993). In terms of the macrosymbionts it has been shown that the alder protein *ag13* may act as a plant marker for senescence (Guan et al. 1997) and that in the case of soybeans cystatins increase during the onset of senescence (van Wyk et al. 2014).

However there has been no work done to investigate the bacterial species which invade a senescing alder nodule, and presumably obtain the benefits of nutrients from a niche rich in fixed nitrogen. The work presented here is the first

application of next generation sequencing to investigate the microbiome of the bacterial community within senescing nodules from *A. glutinosa* roots by analysing amplicons produced from PCR using 16S *rDNA* primers.

## Materials and Methods

### Collection of nodules

Root nodules were collected from *A. glutinosa* plants which had been growing at the Henfaes Experimental Station, Abergwyngregyn, Gwynedd, Wales (53°14'N, 4°01'W) for 3 years. The nodules were harvested from approximately 5 cm below the soil surface and only those already showing early signs of senescence were used for the current analysis. Following collection, nodules were stored in a freezer at -80°C until ready for DNA analysis.

### Performing PCR on nodule material

Thawed nodules were surface-washed to remove the nodule's surface-associated bacteria, taking care to avoid damaging the internal regions of the nodular structure. The periderm was peeled to remove the outer layer from six nodules, each from a different tree. An individual lobe from each nodule was used for extraction of DNA and subsequent analysis.

Lobes were placed in sterile molecular-grade water and then ground in microfuge tubes with mini-pestles, followed by a 15 min incubation at 95°C and centrifugation at 13000 *g* for 10 min. Supernatants were removed and 1 µl of it was added directly to a FastStart high fidelity PCR system master mix. This approach of performing PCR directly on extracted material has been applied previously to *Frankia* analysis

(McEwan and Wheeler 1995; McEwan et al. 2015) with the 95°C incubation rupturing even relatively robust organisms. The following PCR conditions were performed in keeping with the guidelines of the manufacturer (Roche) for FastStart PCR for 454 sequencing of 16S *rDNA* amplicons: 95°C, 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min and a final extension of 72°C for 7 min. Primers used were barcoded prokaryote primers (Caporaso et al. 2010) and were used to target the V1-V3 region of the bacterial 16S *rRNA* genes. One primer was used as the forward primer in all reactions (CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCM TGGCTCAG), corresponding to *Escherichia coli* position 27. Details of the reverse primers used are contained in Table 1. By using a different reverse primer for each of the six samples allowed identification of the source nodule. In all cases these corresponded to *E. coli* position 357. A final primer concentration of 400 nM was used with a reaction volume of 25 µl. After PCR was completed, amplicons were checked by electrophoresis on a 1% TAE agarose gel to verify successful amplification and that amplicons were around the approximate size predicted when using these primers (i.e. 300 bp).

Sample quantification was carried out using the QuantiT<sup>™</sup> PicoGreen® dsDNA reagent (Invitrogen) and a CFX 96<sup>™</sup> real-time system (Bio-rad) to measure relative fluorescence. Concentrations were calculated from a standard curve and reactions were normalized and pooled. Samples were normalized by mixing the six PCR products in equimolar concentrations. All replicates within the mix went through a final concentration measurement and samples were stored at -20°C until ready for 454 sequencing. Unincorporated dNTPs and primer dimers were removed using Agencourt® AMPure® XP beads (Beckman Coulter). Following this step, the pooled and purified libraries were re-quantified and diluted to a final concentration of 10<sup>7</sup> molecules/µl.

**Table 1: Reverse primers used in the current work.** All primers corresponded to *E. coli* position 357.

Nodule	Primer sequence (5' to 3')
1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCCTGCTGCCTYCCGTA
2	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGCTGCTGCCTYCCGTA
3	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGCTGCTGCCTYCCGTA
4	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGCTGCTGCCTYCCGTA
5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTACTGCTGCCTYCCGTA
6	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCTGCTGCCTYCCGTA

### Sequencing and bioinformatical analysis

DNA sequencing was performed using a Genome Sequencer FLX, 454 Life Sciences (Roche) with subsequent data analysis being performed on a Dell PowerEdge T710 with Biolinux Ubuntu 10.04 base running QIIME version 1.6.0 (Caporaso et al. 2010). De-replication of the raw input sequences was performed according to the barcode identifiers. Quality control was carried out using the default setting for minimum quality score, length, ambiguous bases, homopolymer runs and primer mismatches. The various operational taxonomic units (OTUs) were selected using

UCLUST (Edgar 2010) at identity levels of 97% and the Ribosomal Database Project (RDP) (Cole et al. 2005) was used to assign taxonomy. For all samples, Good's coverage and rarefaction curves were determined using rich (a package run in R) for samples to assess if the number of sequences appeared to as an appropriate representation of the population (Rossi 2011).

The top three most abundant bacterial sequences for each nodule were identified using a BLASTn search against the NCBI nucleotide database (Altschul et al. 1990) at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

## Results

Sequences obtained were deposited in the Genbank database with the accession codes ERS1732949, ERS1732952, ERS1732953, ERS1732954, ERS1732955 and ERS1732956.

In all six nodules trace levels (<1% of all sequences) of Frankia were identified, with the majority of sequences being from other organisms.

The non-Frankia sequences were classified at the level of phylum, class and order. The percentage of each taxonomic sequence group relative to the total number of sequences per nodule is shown in Table 2. In four of the six nodules the most abundant phylum was Bacteroidetes, where organisms

from this phylum constituted more than half of the sequences detected, with sequences from the phylum Proteobacteria being second most abundant. In the other two nodules sequences belonging to the phylum Proteobacteria were most abundant, comprising more than half of the sequences in one example and almost half in the other, with sequences belonging the phylum Bacteroidetes being second most abundant. The only two other phyla detected in any of the nodules were Acidobacteria (present in all nodules and always third most abundant) and Firmicutes (five of six nodules and never more than 1.5% of the total number).

**Table 2: The percentage of different phyla, classes and orders of sequences present in each nodule**

Classification	Nodule 1	Nodule 2	Nodule 3	Nodule 4	Nodule 5	Nodule 6
Acidobacteria	2.4	6.8	18.5	11.7	7.3	8.3
Acidobacteria_Gp1	-	0.1	-	-	-	0.5
Granulicella	-	0.1	-	-	-	0.5
Actinobacteria	2.4	6.8	18.5	11.7	7.3	7.8
Actinomycetales	2.4	6.8	17.3	11.3	7.3	7.8
Solirubrobacterales	-	-	1.2	0.4	<0.1	-
Bacteroidetes	85.5	73.8	38.1	23.0	59.5	60.2
Flavobacteriia	-	11.8	15.0	7.0	29.4	5.3
Flavobacteriaceae	-	11.8	15.0	7.0	29.4	5.3
Sphingobacteriia	85.5	62.0	23.1	16.0	30.1	54.9
Chitinophagaceae	0.1	4.7	0.7	0.5	7.5	7.3
Cytophagaceae	-	1.5	3.3	3.6	2.5	0.8
Sphingobacteriaceae	85.4	55.8	19.1	11.9	20.1	46.8
Firmicutes	0.1	0.1	-	0.9	1.5	0.1
Bacilli	0.1	0.1	-	0.9	1.5	0.1
Bacillaceae 2	0.1	-	-	-	-	-
Paenibacillaceae 1	-	0.1	-	0.4	1.5	0.1
Thermoactinomycetaceae 1	-	-	-	0.4	<0.1	-
Proteobacteria	12.0	19.3	43.4	64.4	31.7	31.5
Alphaproteobacteria	3.6	7.8	9.8	43.7	25.6	10.2
Acetobacteraceae	<0.1	-	-	-	-	0.1
Beijerinckiaceae	<0.1	<0.1	-	-	-	<0.1
Bradyrhizobiaceae	1.4	0.8	0.2	0.5	0.4	1.6
Brucellaceae	<0.1	-	-	-	-	-
Caulobacteraceae	-	0.2	0.7	25.7	12.5	0.1
Hyphomicrobiaceae	0.1	0.5	6.5	10.9	10.0	0.7
Methylocystaceae	<0.1	0.1	<0.1	-	0.1	0.1
Phyllobacteriaceae	1.0	1.0	0.3	3.9	0.8	1.1
Rhizobiaceae	0.8	0.8	0.8	1.8	0.2	1.7
Rhizomicrobium	-	0.2	-	-	-	0.3
Rhodobacteraceae	-	0.1	-	-	-	0.1
Sphingomonadaceae	0.2	4.1	1.3	0.9	1.6	4.4
Betaproteobacteria	1.4	9.1	4.8	14.9	3.2	14.0
Alcaligenaceae	-	-	-	0.6	0.4	-
Burkholderiaceae	0.2	<0.1	-	-	-	<0.1
Comamonadaceae	0.8	4.1	4.2	1.5	1.5	5.8
Methylophilaceae	-	0.1	-	-	-	2.4
Oxalobacteraceae	0.4	4.9	0.7	12.7	1.1	5.8
Rhodocyclaceae	-	-	<0.1	0.1	0.2	-
Gammaproteobacteria	7.1	2.3	28.8	5.9	2.8	7.3
Pseudomonadaceae	2.2	0.6	1.9	1.0	1.3	4.4
Xanthomonadaceae	4.8	1.7	26.9	4.9	1.5	2.9

Two bacterial classes were detected within the Bacteroidetes; Flavobacteriia and Sphingobacteriia, with Sphingobacteriia being the more abundant in five of the six nodules, with them being present in approximately equal numbers in the other. Of the sequences identified as being from the class Sphingobacteriia the majority (66% or more) were from the order Sphingobacteriiales.

Proteobacteria were represented by three different classes in all six nodules (Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria), with the major class varying from nodule to nodule. Nodules three and four had the highest relative abundance of Proteobacteria, with Alphaproteobacteria more abundant in nodule four (43.7% of all sequences) and

Gammaproteobacteria more abundant in nodule three (28.8% of all sequences).

In terms of non-Frankia members of the phylum Acidobacteria, the majority (>90%) of the sequences identified were members of the order Actinomycetales in all nodules.

In all cases the abundance of Firmicutes was relatively low with all sequences assigned as members of the class Bacilli. Table 3 shows the number of operational taxonomic units (OTUs) detected per nodule for each of the classification levels. It is clear that even at the level of different orders, there is representation by many different organisms.

**Table 3: The number of distinct sequences present for each phylum, class and order present in each nodule.** Names underlined denote the same single member of a taxon was the only one identified in any of these nodules.

Classification	Nodule 1	Nodule 2	Nodule 3	Nodule 4	Nodule 5	Nodule 6
Acidobacteria	12	25	29	23	24	23
<u>Acidobacteria Gp1</u>	0	1	0	0	0	1
<u>Granulicella</u>	0	1	0	0	0	1
Actinobacteria	12	24	29	23	24	22
Actinomycetales	12	24	26	20	23	22
Solirubrobacterales	0	0	3	3	1	0
Bacteroidetes	10	27	21	23	25	30
Flavobacteriia	0	5	5	8	7	6
Flavobacteriaceae	0	5	5	8	7	6
Sphingobacteriia	10	22	16	15	18	24
Chitinophagaceae	1	7	5	5	4	7
Cytophagaceae	0	1	1	1	1	1
Sphingobacteriaceae	9	14	10	9	13	16
Firmicutes	1	2	0	3	3	1
Bacilli	1	2	0	3	3	1
<u>Bacillaceae 2</u>	1	0	0	0	0	0
Paenibacillaceae 1	0	2	0	2	2	1
<u>Thermoactinomycetaceae 1</u>	0	0	0	1	1	0
Proteobacteria	29	47	34	38	39	50
Alphaproteobacteria	20	32	21	21	23	34
<u>Acetobacteraceae</u>	1	0	0	0	0	1
<u>Beijerinckiaceae</u>	1	1	0	0	0	1
Bradyrhizobiaceae	5	7	3	4	4	6
Brucellaceae	1	0	0	0	0	0
Caulobacteraceae	0	4	3	3	4	2
Hyphomicrobiaceae	2	3	4	4	3	3
<u>Methylocystaceae</u>	1	1	1	0	1	1
Phyllobacteriaceae	4	3	2	2	3	4
Rhizobiaceae	3	3	1	2	1	3
<u>Rhizomicrobium</u>	0	1	0	0	0	1
<u>Rhodobacteraceae</u>	0	1	0	0	0	1
Sphingomonadaceae	2	8	7	6	7	11
Betaproteobacteria	4	7	5	8	9	8
<u>Alcaligenaceae</u>	0	0	0	1	1	0
<u>Burkholderiaceae</u>	1	1	0	0	0	1
Comamonadaceae	2	1	1	4	3	2
<u>Methylophilaceae</u>	0	1	0	0	0	1
Oxalobacteraceae	1	4	3	2	4	4
<u>Rhodocyclaceae</u>	0	0	1	1	1	0
Gammaproteobacteria	5	8	8	9	7	8
Pseudomonadaceae	2	1	2	2	2	1
Xanthomonadaceae	3	7	6	7	5	7

The three most abundant taxa in each nodule are listed in Table 4. *Pedobacter* spp. were among the top three species in four of the six nodules, and in two of these nodules, all three of the most abundant sequences were associated with different organisms from this single genus. Other than this,

the most abundant species were identified as *Brevundimonas* sp., *Chryseobacterium* sp., *Devosia* riboflavin, *Flavobacterium* sp., *Massilia* sp., *Rhodanobacter* sp., *Stenotrophomonas rhizophila* and *Streptomyces prunicolor*.

**Table 4: The identity of the three most abundant sequences in each of the nodules**

Nodule	Most abundant	2 <sup>nd</sup> most abundant	3 <sup>rd</sup> most abundant
1	<i>Pedobacter panaciterrae</i>	<i>Pedobacter humicola</i>	<i>Stenotrophomonas rhizophila</i>
2	<i>Pedobacter</i> sp.	<i>Pedobacter panaciterrae</i>	<i>Pedobacter</i> sp.
3	<i>Rhodanobacter</i> sp.	<i>Streptomyces prunicolor</i>	<i>Flavobacterium</i> sp.
4	<i>Brevundimonas</i> sp.	<i>Massilia</i> sp.	<i>Devosia riboflavina</i>
5	<i>Chryseobacterium</i> sp.	<i>Brevundimonas</i> sp.	<i>Pedobacter</i> sp.
6	<i>Pedobacter panaciterrae</i>	<i>Pedobacter</i> sp.	<i>Pedobacter</i> sp.

## Discussion

The first point to make is that although these nodules were all identified as showing signs of early senescence, they were not selected as being at the same stage in all cases. Therefore the current work does not necessarily identify the initial colonisers, but rather serves as a guide to the types of organisms which are opportunistic in terms of invading the nodules at an early stage after their physical appearance has changed from the form shown by those which are actively fixing nitrogen.

In all cases low levels (<1% of all sequences) were identified as being *Frankia*. This is in contrast to the situation reported in functional nodules (McEwan et al. 2015) where the majority of the sequences were from a single strain of *Frankia*. Likewise in most cases a low level of chloroplast sequences from *A. glutinosa* (the macrosymbiont plant in this relationship) were also detected (data not presented). Similar to the *Frankia* content, this was always less abundant than that detected in the functional nodules.

In the case of the other sequences detected, the composition varied from nodule to nodule, suggesting that there is no distinct pattern to be seen, and that decaying nodules can be invaded by whatever opportunistic organisms may be in a particular environment at any one time. However, it is interesting to note that members of the genus *Pedobacter* were detected among the three most abundant OTUs in four of the nodules (see Table 3), and in two cases all three of the most abundant OTUs were *Pedobacter* sequences. Moreover, even the two which did not have *Pedobacter* sequences among the three most abundant OTUs still had sequences which were identified at low levels in the nodules (data not presented). In total four different OTUs corresponding to this genus were identified; *Pedobacter humicola* (Dahal and Kim 2016), *Pedobacter panaciterrae* (Yoon et al. 2007) and two unclassified members of the genus *Pedobacter*. Members of the genus *Pedobacter* (phylum Bacteroidetes, class Sphingobacteriia, order Sphingobacteriaceae) are Gram-negative rods which

are often detected in soil samples (Margesin and Shivaji 2015). Members of this genus have been implicated as having a role to play in terms of microbial mediation of components of the nitrogen cycle such as nitrification and denitrification (e.g. Harter et al. 2016).

Other relatively abundant organisms identified in Table 3 were: *Stenotrophomonas rhizophila* (Wolf et al. 2002); *Streptomyces prunicolor* (Shirling and Gottlieb 1969); *Rhodanobacter* sp. (Nalin et al. 2015); *Flavobacterium* sp. (Bernardet and Bowman 2015); *Brevundimonas* sp. (Vancanneyt et al. 2015); *Massilia* sp. (Editorial Board 2015); *Devosia riboflavin* (Nakagawa et al. 1996) and *Chryseobacterium* sp. (Bernardet et al. 2015). All of these have been described as being organisms which are found in general environmental organisms found in many ecosystems, with most of them being regarded as being found in either soil or silt samples.

Thus it is clear that there is no obvious single pattern regarding the species associated with early colonising of decaying nodules, it appears that members of the genus *Pedobacter* are among the more abundant organisms present, with others being representative of those likely to be found in the general rhizosphere at that time.

## Conclusion

As alder nodules start to decay, there is no one group of bacteria which are first to colonise the nodules.

## Acknowledgement

This work was supported by the award of a grant from the Centre for Integrated Research in the Rural Environment (CIRRE).

## References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990) Basic local alignment search tool. *J Mol Biol.* 215:403–410.
- Bernardet J-F, Bowman JP. (2015) Flavobacterium. Bergey's Manual of Systematics of Archaea and Bacteria. Ed. WB Whitman. Published by John Wiley & Sons, Inc. 1–75.
- Bernardet J-F, Hugo CJ, Bruun B. (2015) Chryseobacterium. Bergey's Manual of Systematics of Archaea and Bacteria. Ed. WB Whitman. Published by John Wiley & Sons, Inc. 1–35.
- Berry AM, McIntyre L, McCully ME. (1986) Fine structure of root hair infection leading to nodulation in Frankia-Alnus symbioses. *Can J Bot.* 64:292–305.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JL, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* 7:335–336.
- Cole JR, Chai B, Farris RJ, Wang Q, Kulam S A, McGarrell DM, Garrity GM, Tiedje JM. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33:D294–D296.
- Dahal RM, Kim J (2016) *Pedobacter humicola* sp. nov., a member of the genus *Pedobacter* isolated from soil. *Int J Syst Evol Microbiol.* 66:2205–2211.
- Dai YM, He XY, Zhang CG, Zhang ZZ. (2004) Characterization of genetic diversity of Frankia strains in nodules of *Alnus nepalensis* (D. Don) from the Hengduan Mountains on the basis of PCR-RFLP analysis of the *nifD-nifK* IGS. *Plant Soil.* 267:207–212.
- Dobritsa S V and Stupar O S (1989) Genetic-heterogeneity among *Frankia* isolates from root-nodules of individual actinorhizal plants. *FEMS Microbiol Lett.* 58:287–292.
- Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* 26:2460–2461.
- Editorial Board (2015) *Massilia*. Bergey's Manual of Systematics of Archaea and Bacteria. Ed. WB Whitman. Published by John Wiley & Sons, Inc. 1–2.
- Guan C, Akkermans ADL, van Kammen A, Bisseling T, Pawlowski K. (1997) *ag13* is expressed in *Alnus glutinosa* nodules in infected cells during endosymbiont degradation and in the nodule pericycle. *Physiol Plant.* 99:601–607.
- Harter J, Weigold P, El-Hadidi M, Huson DH, Kapplera A, Behrens S. (2016) Soil biochar amendment shapes the composition of N<sub>2</sub>O-reducing microbial communities. *Sci Total Environ.* 562:379–390.
- Kennedy PG, Schouboe JL, Rogers RH, Weber MG, Nadkaminorm NM. (2010) Frankia and *Alnus rubra* canopy roots: An assessment of genetic diversity, propagule availability, and effects on soil nitrogen. *Microb Ecol.* 59:214–220.
- Margesin R, Shivaji S. (2015) *Pedobacter*. Bergey's Manual of Systematics of Archaea and Bacteria. Ed. WB Whitman. Published by John Wiley & Sons, Inc. 1–17.
- McEwan NR, Wheeler CT. (1995) PCR on the Gram-positive organism Frankia without prior DNA extraction. *Trends Gen.* 11:168.
- McEwan NR, Snelling TJ, Collins T, Girdwood SE, Wilkinson T, Dougal K, Jones DL, Godbold DL. (2015) Lobes on *Alnus glutinosa* nodules contain a single major ribotype of Frankia. *Endocytobiosis Cell Res.* 26:83–86.
- Nakagawa Y, Sakane T and Yokota A (1996) Transfer of "*Pseudomonas riboflavina*" (Foster 1944), a Gram-Negative, Motile Rod with Long-Chain 3-Hydroxy Fatty Acids, to *Devosia riboflavina* gen. nov., sp. nov., nom. rev. *Int J Syst Evol Microbiol.* 46:16–22.
- Nalin R, Simonet P, Vogel TM, Normand P. (2015) Rhodanobacter. Bergey's Manual of Systematics of Archaea and Bacteria. Ed. W B Whitman. Published by John Wiley & Sons, Inc. 1–22.
- Normand P, Simonet P, Bardin R. (1988) Conservation of *nif* sequences in Frankia. *Mol Gen.* 213:238–246.
- Rossi J-P. (2011) rich: An R package to analyse species richness. *Diversity.* 3:112–120.
- Shirling EB, Gottlieb D. (1969) Cooperative description of type cultures of Streptomyces. IV. Species descriptions from the second, third and fourth studies. *Int J Syst Evol Microbiol.* 19:391–512.
- Swaraj K, Laura JS, Bishnoi NR. (1993) Nitrate induced nodule senescence and changes in activities of enzymes scavenging hydrogen peroxide in clusterbean (*Cyamopsis tetragonoloba* Taub.) *J Plant Physiol.* 141:202–205.
- Van Ghelue M, Lovaas E, Ringo E, Solheim B (1997) Early interactions between *Alnus glutinosa* and Frankia strain Ar13. Production and specificity of root hair deformation factor(s). *Physiol Plant.* 99:579–587.
- van Wyk S G, Du Plessis M, Cullis CA, Kunert KJ, Vorster BJ. (2014) Cysteine protease and cystatin expression and activity during soybean nodule development and senescence. *BMC Plant Biol.* 14:294.
- Vancanneyt M, Segers P, Abraham W-R, Vos PD. (2015) Rhodanobacter. Bergey's Manual of Systematics of Archaea and Bacteria. Ed. WB Whitman. Published by John Wiley & Sons, Inc. 1–14.
- Vikman P-A, Lundquist P-O, Huss-Danell K. (1990) Respiratory capacity, nitrogenase activity and structural changes of *Frankia*, in symbiosis with *Alnus incana*, in response to prolonged darkness. *Planta.* 182:617–625.
- Wheeler CT, Miller IM. (1990) Current and potential uses of actinorhizal plants in Europe. In "The Biology of Frankia and actinorhizal plants". Ed. CR Schwintzer and JD Tjepkema, Academic Press, Toronto pp 365–389.
- Wolf A, Fritze A, Hagemann M, Berg G. (2002) *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. *Int J Syst Evol Microbiol.* 52:1937–1944.
- Yoon MH, Ten LN, Im WT, Lee ST. (2007) *Pedobacter panaciterrae* sp. nov., isolated from soil in South Korea. *Int J Syst Evol Microbiol.* 57:381–386.