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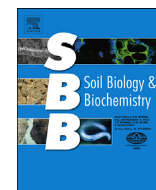

A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium)

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ABSTRACT

We analysed the genetic properties of non-rhizobial root nodule endophytes (NRE) isolated from indigenous legumes in Flanders. In total, 654 isolates were obtained from 30 different plant species within the Faboideae legume subfamily. Partial sequencing of the 16S rRNA gene revealed a large diversity of different taxa from the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmibacteria, Flavobacteria and Sphingobacteria. Many of the isolates belonged to the genera *Bacillus* (17.9%) and *Pseudomonas* (15.9%). No symbiosis (*nodC*) or nitrogen fixation related genes (*nifH*) could be detected amongst the isolates, which indicate the endophytic nature of the bacteria. Statistical analysis grouped the investigated plant species into six clusters according to the presence of particular NRE. However, no correlations could be found within these six clusters towards plant tribes or ecoregions the plants had been sampled from. Cluster analysis of the ecoregions according to the presence of NRE, revealed correlations between bacterial genera and those areas. However, groups present in the ecoregions did not correlate with the groups present in the different plant clusters. When combining our previous study on rhizobial diversity recovered from the same sampling campaign (De Meyer et al., 2011) with the current study, 84.1% of the isolates belonged to the traditional rhizobia groups and only 15.9% were NRE. The Loamy ecoregion yielded the lowest number of culturable NRE (8.04%) and the Campine ecoregion the highest number (24.19%). The present study highlights the frequent presence of these NRE in root nodules. The occurrence of certain rhizobia was correlated with the presence of particular NRE, suggesting their presence may not be accidental, however their functions remain unclear at this point.

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1. Introduction

Rhizobia are soil bacteria capable of symbiosis with legume plants where they can reside in root or stem nodules and perform nitrogen fixation for the host. While traditionally, rhizobia belonged to the genera, *Azorhizobium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* (Sawada et al., 2003), in recent years nitrogen fixing root nodule bacteria have also been described in other Alphaproteobacterial genera, including *Ochrobactrum* (Trujillo et al., 2005), *Methylobacterium* (Sy et al., 2001), *Microvirga* (Ardley et al., 2012; Radl et al., 2014), *Devosia* (Rivas et al., 2003) and *Phyllobacterium* (Zakhia et al., 2006). Furthermore, so-called Beta-rhizobia have in the last ten years been described in the Betaproteobacterial genera *Burkholderia* and *Cupriavidus* (Chen et al., 2001; Moulin et al., 2001; De Meyer et al., 2013a; De Meyer et al., 2013b; De Meyer et al., 2014). In addition to strains that can elicit nodules and belong to documented rhizobial species, several other bacterial species have been reported from legume nodules without a clear indication of their role within the host. In the absence of positive nodulation tests, they can be regarded as non-rhizobial endophytes (NRE). These include i.a., Alphaproteobacteria (*Aminobacter* (Estrella et al., 2009), *Ochrobactrum* (Zurdo-Pineiro et al., 2007; Imran et al., 2010), *Methylobacterium* (Palaniappan et al., 2010), *Devosia* (Bautista et al., 2010) and *Phyllobacterium* (Mantelin et al., 2006)), Betaproteobacteria (*Herbaspirillum* (Valverde et al., 2003) and *Shinella* (Lin et al., 2008)), Gammaproteobacteria (*Pantoea*, *Enterobacter* and *Pseudomonas* (Benhizia et al., 2004; Ibáñez et al.,

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Table 1
Genera recovered in this study with their rep-clusters, identification according to partial 16S rRNA gene sequencing and the number of isolates.

Genera	Representative strains	Rep-clusters	# isolates
Actinobacteria			
Actinoplanes sp.	R-45801	Alone	1
Aeromicrobium sp.	R-45950, R-45951	411	13
Arthrobacter sp.	R-45644, R-45645,R-45677, R-45678, R-45679, R-45733, R-46132, R-46278, R-46319	Alone, 317, 403, 443	24
Brevibacterium sp.	R-45585, R-45586	Alone	2
Corynebacterium sp.	R-45865, R-45902, R-45903, R-45927, R-46008	Alone, 172	5
Curtobacterium sp.	R-46162, R-46314	Alone, 69	10
Kocuria sp.	R-45655, R-45665, R-45689, R-45691, R-45692, R-46308	Alone, 257, 442, 448	20
Leifsonia sp.	R-45745, R-46062, R-46076, R-46167, R-46259	Alone, 427	7
Microbacterium sp.	R-45570, R-45573, R-45658, R-45659, R-45676, R-45694, R-45704, R-45758, R-45762, R-45772, R-45841, R-45861, R-45991, R-46024, R-46029, R-46031, R-46041	Alone, 79, 185, 275, 324, 325, 404, 415, 444	43
Microbispora sp.	R-45698	Alone	1
Micromonospora sp.	R-45554	Alone	1
Moraxella sp.	R-45536, R-45904	Alone, 24	10
Mycobacterium sp.	R-45620, R-46056, R-46330	Alone, 77, 180	5
Oerskovia sp.	R-45820	Alone	1
Plantibacter sp.	R-46164	Alone	1
Promicromonospora sp.	R-45862, R-45892, R-46030, R-46035	Alone, 326, 379	16
Rhodococcus sp.	R-45548, R-45551	Alone	2
Sphaerisporangium sp.	R-46174	Alone	1
Streptomyces sp.	R-45560, R-45795, R-45838, R-45839, R-45840, R-45852, R-45853, R-45856, R-45857, R-45858, R-45880, R-46032, R-46033, R-46034, R-46036, R-46037, R-46057, R-46058, R-46156, R-46264, R-46282, R-46320	Alone, 280, 321, 336	30
Alphaproteobacteria			
Ancylobacter sp.	R-45799, R-45800	Alone	2
Bosea sp.	R-45681, R-46060, R-46070, R-46073	Alone, 6	5
Caulobacter sp.	R-46323	Alone	1
Inquilinus sp.	R-45827, R-46318	Alone, 323	3
Novosphingobium sp.	R-45660	329	2
Paracoccus sp.	R-46302, R-46307	Alone	2
Phyllobacterium sp.	R-45564, R-45798, R-46124, R-46157	Alone, 129, 130, 181	9
Sphingomonas sp.	R-46285, R-45731, R-45732	Alone, 211	3
Sphingomonadaceae sp.	R-46192	Alone	1
Betaproteobacteria			
Herbaspirillum sp.	R-45723	Alone	1
Massilia sp.	R-45804, R-45805, R-45830	99, 100, 335	6
Roseateles sp.	R-45571	Alone	1
Variovorax sp.	R-46208	33	10
Firmibacteria			
Bacillus sp.	R-40421, R-45534, R-45535, R-45537, R-45540, R-45543, R-45549, R-45607, R-45608, R-45628, R-45640, R-45650, R-45656, R-45667, R-45669, R-45671, R-45672, R-45706, R-45708, R-45775, R-45785, R-45787, R-45792, R-45793, R-45794, R-45824, R-45833, R-45837, R-45842, R-45851, R-45859, R-45885, R-45890, R-45942, R-45943, R-45944, R-45945, R-45947, R-45997, R-46011, R-46013, R-46020, R-46025, R-46141, R-46144, R-46146, R-46152, R-46169, R-46176, R-46193, R-46216, R-46226, R-46228, R-46238, R-46245, R-46246, R-46263, R-46279, R-46280	Alone, 23, 30, 161, 170, 188, 303, 362, 375, 376, 377, 378, 380, 420, 421, 422, 423, 424, 425, 432, 434, 435, 439, 440	117
Brevibacillus sp.	R-45680	Alone	1
Cohnella sp.	R-45709	428	1
Exigobacterium sp.	R-45918	Alone	1
Lysinibacillus sp.	R-45541, R-45670, R-45748, R-46326	Alone	4
Paenibacillus sp.	R-45550, R-45610, R-45623, R-45647, R-45649, R-45664, R-45673, R-45674, R-45675, R-45701, R-45776, R-45786, R-45807, R-45812, R-45813, R-45814, R-45815, R-45816, R-45939, R-45993, R-46010, R-46038, R-46080, R-46203, R-46243, R-46244, R-46251, R-46252, R-46257, R-46269, R-46305	Alone, 2, 4, 5, 12, 101, 111, 169, 171, 238, 357, 370, 381, 431	84
Staphylococcus sp.	R-45577, R-45580, R-45641, R-45663, R-45688, R-45690, R-45693, R-45875, R-46012, R-46052, R-46142, R-46143	Alone, 7, 406, 412, 413, 419	20
Flavobacteria			
Chryseobacterium sp.	R-45581, R-46064	Alone, 429	7
Gammaproteobacteria			
Acinetobacter sp.	R-45867	313	4
Buttiauxella sp.	R-45774	110	5
Enhydrobacter sp.	R-45682, R-45683, R-45684, R-45685, R-45686, R-45687	Alone, 426, 449	11
Enterobacter sp.	R-45810	82	7
Erwinia sp.	R-45811	Alone	1
Pantoea sp.	R-45539, R-45717, R-45789, R-45806, R-46081, R-46239, R-46301	Alone, 16, 281, 333, 347	27

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Table 1 (*continued*)

Genera	Representative strains	Rep-clusters	# isolates
Pseudomonas sp.	R-45532, R-45533, R-45538, R-45582, R-45716, R-45757, R-45808, R-45809, R-45822, R-45823, R-45850, R-45864, R-45972, R-46026, R-46089, R-46100, R-46111, R-46145, R-46171, R-46240, R-46241, R-46242	Alone, 3, 17, 18, 19, 20, 31, 32, 68, 90, 97, 112, 273, 293	108
Rahnella sp.	R-46079	91	5
Stenotrophomonas sp.	R-46069, R-46300	Alone	2
Xanthomonas sp.	R-45826, R-45893, R-46004, R-46065	Alone, 213	8
Sphingobacteria			
Dyadobacter sp.	R-45763	338	2

2009; Shiraishi et al., 2010; Aserse et al., 2013)), Actinobacteria (*Arthrobacter*, *Microbacterium* and *Curtobacterium* (Sturz et al., 1997; Palaniappan et al., 2010)) and Firmibacteria (*Bacillus*, *Paenibacillus* and *Staphylococcus*, (Rajendran et al., 2008; Palaniappan et al., 2010; Shiraishi et al., 2010; Deng et al., 2011)). They were in most cases isolated on the same yeast mannitol agar medium as rhizobia. The characteristics important for the interaction with legumes seem to be shared by a growing number of unrelated taxa (Sawada et al., 2003).

Certain NRE bacteria have beneficial effects on the host plants, including plant growth promotion (Vessey, 2003; Kuklinsky-Sobral et al., 2004; Ibáñez et al., 2009; El-Tarabily et al., 2010; Tariq et al., 2014), nitrogen fixation (Andrews et al., 2010), siderophore mediated interactions (Rajendran et al., 2008; Andrews et al., 2010), increased promotion of plant stress tolerance (Andrews et al., 2010) and biological control of plant pathogens (El-Tarabily et al., 2010). Most legumes are good pioneer species, adapted to low nutrient soils and/or environments with heavy metals (Gonzalez-Andres et al., 2005; Vidal et al., 2009; Azcon et al., 2010). Multiple studies report on the beneficial effect of co-inoculating rhizobia with other bacteria (Zhang et al., 1996; Parmar and Dadarwal, 1999; Rajendran et al., 2008; Egamberdieva et al., 2010). All previously mentioned studies suggest that besides rhizobia, NRE are present inside root nodules, which may have beneficial effects on the host plant.

While many studies in the past have focussed on plant species of economic importance, including soybean, common bean, cowpea, chickpea and red clover (Delorme et al., 2003; Laranjo et al., 2004; Kuklinsky-Sobral et al., 2005; Duodu et al., 2007; Hung et al., 2007; Laranjo et al., 2008; Ogutcu et al., 2008; Appunu et al., 2009; Chagas et al., 2010; Li et al., 2010; Pule-Meulenberg et al., 2010), native legume species have generally received less attention. In Belgium, legumes are restricted to the *Faboideae* subfamily and contain 102 plant species in 30 different genera (Lambinon et al., 1998). In a previous study, we assessed the genetic diversity of 3810 isolates belonging to traditional rhizobial genera including *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*, isolated from a large diversity of indigenous legumes in Flanders (Belgium) (De Meyer et al., 2011). The present study, is based on the same sampling campaigns, however, it focuses on the other bacteria recovered from those root nodules. Our aim was to analyse the diversity of non-rhizobial root nodule endophytes (NRE) isolated from various indigenous and exotic legume plants in Flanders (Belgium), to gain insights in their potential nitrogen fixation ability and to investigate possible connections with plant species and ecoregion. Additionally, the results on the rhizobia from our previous study (De Meyer et al., 2011) were compared with the diversity found in the present study.

2. Materials and methods

2.1. Nodule collection and isolation

Root nodules were collected as previously reported (De Meyer et al., 2011). Briefly, the sampling campaigns were performed over the summers of 2008 and 2009. Sampling plots were selected based on the diversity of legume species present (Van Landuyt et al., 2006) and the difference in ecoregion (campine, dune, polder, loamy and sandy-sandloamy region) (Van Landuyt et al., 2006; Van Landuyt et al., 2011). In most cases, whole plants were excavated and taken to the laboratory where the nodules were excised from roots in situ, brushed free of soil debris and preserved at 4 °C in tubes containing dried silica beads. Healthy, non-ruptured nodules were collected to maximise the isolation of root-nodule bacteria. Bacteria were isolated in 2009 and 2010 according to the protocol described previously (De Meyer et al., 2011) and the surface sterilized nodules were rolled over YMA agar plates (Vincent, 1970) to confirm adequate surface sterilisation. The control plates without bacterial growth were considered as successfully surface sterilized and isolates from these nodules were further used in the study. All YMA plates were incubated at 28 °C and regularly checked for growth up to 20 days. Two colonies of each morphological type were selected for isolation. The bacteria were purified by repeatedly streaking on YMA plates. All bacteria were stored in tubes with 15% glycerol and YMA broth at −20 °C.

2.2. DNA extraction and genomic fingerprinting by (GTG)5 rep-PCR

Total genomic DNA of each isolate was extracted as described by Baele et al. (2000) and a repetitive extragenic palindromic (rep) PCR was performed with the $(GTG)_5$ primer in a total volume of 25 µl (Versalovic et al., 1994; Gevers et al., 2001). To allow normalization, a combined 500 bp and 100 bp DNA marker (Biorad) was included 4 times in each 1.5% (w/v) agarose gel during electrophoresis (960 min at 55 V (constant voltage)) in a 4 °C incubator. The electrophoresed gels were stained for 30 min in a solution of 1 µg ml^{-1} EtBr in Tris acetic acid EDTA buffer (1 x) and digital pictures were taken under UV light. The rep $(GTG)_5$ patterns were normalized and cluster analysis was performed using the software package BioNumerics v5.1 (Applied Maths). Representatives of each rep cluster analysed in this study are listed in Table 1 and Table S1.

2.3. Sequence analysis of 16S rRNA gene and ribosomal database project (RDP) classification

The 16S rRNA gene was sequenced using the protocol described previously by Vancanneyt et al. (2004). High quality partial sequences (262–490 bp) were submitted to a FASTA search using the EMBL nucleotide sequence database to find related species or genera (Pearson, 1990). The Ribosomal Database Project (RDP) Classifier, a naïve Bayesian classifier, was used to obtain identifications at genus level (Wang et al., 2007). The RDP classifier estimates the classification reliability using bootstrap confidence estimation values with a default threshold of 80% (Wang et al., 2007). Strains identified with confidence estimates lower than 80% were analysed together with reference strains using the MEGA 5 software package (Tamura et al., 2011). Phylogenetic trees were constructed using the Maximum Likelihood method, with the

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General time reversible model. Bootstrap analysis with 500 replicates was performed to assess the reliability of the clustering. Sequences of the 16S rRNA gene determined in this study have been deposited in the EMBL database under the accession numbers: FR774919 to FR775187. Nodulation (*nodC*) and nitrogen fixation (*nifH*) genes have also been investigated according to previously described protocols (De Meyer et al., 2011), however no amplicons were obtained.

2.4. Statistical analysis

All analyses were performed with the statistical computing environment R (R Core Team, 2013), version 3.0.1. A hierarchical cluster analysis based on Ward's method (Ward Jr, 1963) was used to cluster plant species with similar patterns of presence/absence of NRE, given the presence of various rhizobia. Next, a double hierarchical cluster analysis was performed to reveal with which these clusters showed similar co-occurrence patterns of NRE. The results are shown by means of a heatmap. The colours in the heatmap are indicative of the proportion of occurrence of a specific NRE in a certain plant species. More specifically, they are based on the column Z-score of this proportion, which is the normalized proportion within each NRE in this case. Two additional double hierarchical cluster analyses were performed to reveal ecoregions with similar co-occurrence patterns of NRE and to reveal rhizobia with similar co-occurrence patterns of NRE. Heatmaps were constructed as described above.

To assess the sample coverage of NRE and rhizobia in our study, several rarefaction curves were calculated using the online rarefaction calculator (http://biome.sdsu.edu/fastgroup/caltools.htm). Different intensively sampled plant species, including *Trifolium pratense*, *Trifolium repens*, *Medicago lupulina* and *Vicia cracca* and two less intensively sampled plant species harbouring a large diversity of bacteria in their root nodules were analysed. Similarly, rarefaction curves were calculated for each ecoregion sampled in this study.

3. Results

3.1. Sampling and isolation of bacteria

We obtained 654 bacterial isolates originating from 162 surface sterilised root nodules from 30 plant species in 14 genera (Table S1). These isolates were obtained as part of a larger sampling campaign covering different regions in Flanders (De Meyer et al., 2011): one or two representatives from each legume species present in selected sampling sites were excavated and nodules collected. One nodule per sampled plant was selected and used for isolation. The surface sterilization treatment was generally efficient, since in most cases no growth occurred on the YMA plates upon which the surface sterilized nodules were rolled. All colony types appearing on the YMA plates were purified and investigated. There was a large variation in colony morphology; differences were observed in colour, shape and size (data not shown). In total, 4464 isolates were obtained (De Meyer et al., 2011). Here we report on the bacteria not belonging to the traditional rhizobia genera. They were recovered from all reported plant genera except for *Galega*, *Securigera* and *Ulex*. Our previous report covered the 3810 isolates that were found to be traditional rhizobia (De Meyer et al., 2011).

The rarefaction curves indicate that only for *T. pratense* and *T. repens* host plants the diversity was covered since these curves are reaching a plateau (Fig. S1). For all other plant species rarefaction curves are rising indicating that the diversity was not yet covered completely. Only the rarefaction curve for the Loamy ecoregion reaches a plateau and as a consequence covers fairly well the bacterial diversity in that region (Fig. S2). For all other ecoregions, graphs are rising and additional samples should be investigated to fully cover the bacterial diversity.

3.2. Rep (GTG)5 PCR fingerprinting

High resolution fingerprinting patterns were obtained in the $(GTG)_5$-PCR analysis which allowed identification of duplicate isolates, to reduce the number of strains and to get a first insight on the extent of diversity sampled. The reproducibility of this technique was analysed by including one control strain in each PCR. This resulted in repeated fingerprinting patterns with correlation levels of 90,8% to 98,3%, in line with previously reported data (Gevers et al., 2001) and confirming the strength of this technique. A Pearson correlation/UPGMA analysis was performed and rep-clusters were delineated at 80% correlation. This revealed a high level of diversity with 106 rep-clusters and 155 strains occupying isolated positions (Table S1). With regard to the number of colonies selected for purification, two of each morphology type seems reasonable since these duplicates always grouped within the same rep-cluster. In total, 269 strains were selected based on their unique rep-profile for further genetic identification.

3.3. Phylogenetic analysis of 16S rRNA

The RDP classifications and phylogenetic trees obtained with the 16S rRNA gene sequences revealed an enormous diversity including different taxa in the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmibacteria, Flavobacteria and Sphingobacteria (Fig. S3). The genera recovered with their representative strains and rep-clusters are summarised in Table 1 and detailed information on representative strains is listed in Supplementary Table S1. Actinobacteria were related to *Actinoplanes*, *Aeromicrobium*, *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Kocuria*, *Leifsonia*, *Microbacterium*, *Moraxella*, *Mycobacterium*, *Oerskovia*, *Plantibacter*, *Promicromonospora*, *Rhodococcus*, *Schumanella* and *Streptomyces* (Fig. S4). The Firmibacteria were related to *Bacillus*, *Brevibacillus*, *Cohnella*, *Exigobacterium*, *Lysinibacillus*, *Paenibacillus* and *Staphylococcus* (Fig. S5). Alphaproteobacteria strains were related to *Ancylobacter*, *Bosea*, *Caulobacter*, *Inquilinus*, *Novosphingobium*, *Paracoccus*, *Phyllobacterium* and *Sphingomonas* (Fig. S6). The Betaproteobacteria strains were related to *Herbaspirillum*, *Massilia*, *Roseateles* and *Variovorax* (Fig. S6). Gammaproteobacteria strains were related to *Acinetobacter*, *Buttiauxella*, *Enhydrobacter*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Rahnella*, *Stenotrophomonas* and *Xanthomonas* (Fig. S6).

Overall, Firmibacteria (34.9%) were the most abundantly recovered, followed by Actinobacteria (29.5%), Gammaproteobacteria (27.2%), Alphaproteobacteria (4.3%) and Betaproteobacteria (2.8%) (Table 1, Fig. S3). A limited number of isolates were related to Flavobacteria (1%) and Sphingobacteria (0.3%). *Bacillus* (17.9%) and *Pseudomonas* (15.9%) were the most frequently recovered bacterial genera within this NRE population.

3.4. Host plant – NRE association

The legumes sampled were identified as 30 species divided over 14 genera: *Anthyllis*, *Colutea*, *Cytisus*, *Lathyrus*, *Lotus*, *Lupinus*, *Medicago*, *Melilotus*, *Ononis*, *Ornithopus*, *Robinia*, *Trifolium*, *Vicia* and *Wisteria*. An overview of the host plant species with their NRE is shown in Table 2.

Double cluster analysis was performed to reveal plants with similar co-occurrence patterns of NRE (Fig. 1). These data group the host plants in six clusters according to the presence of particular NRE species. Cluster 1 contains *Cytisus scoparius*, *Lathyrus pratensis*,

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Table 2
Host plants with the number of non-rhizobial endophytes found per plant species.[a]

Plant genera	*Cytisus*	*Lathyrus*			*Lotus*		*Lupinus*	*Medicago*		*Melilotus*			*Ononis*	*Ornithopus*	*Robinia*	*Trifolium*					*Vicia*		
Plant species[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Actinobacteria																							
Actinoplanes sp.	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aeromicrobium sp.	–	–	–	–	–	12	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–
Arthrobacter sp.	–	–	–	–	1	–	11	–	–	–	–	5	–	–	4	–	–	–	–	2	–	–	1
Brevibacterium sp.	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–
Corynebacterium sp.	–	–	–	–	1	–	–	2	–	–	–	–	–	–	–	–	1	1	–	–	–	–	–
Curtobacterium sp.	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	4	–	3	–	1	–	–
Kocuria sp.	1	–	–	–	–	–	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	14	–
Leifsonia sp.	1	–	–	–	–	–	–	–	–	–	–	–	–	1	2	–	–	–	–	–	3	–	–
Microbacterium sp.	2	14	–	–	–	–	12	4	–	1	–	–	1	6	–	2	–	–	–	1	–	–	–
Microbispora sp.	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Micromonospora sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–
Moraxella sp.	1	–	1	–	–	–	–	2	2	1	–	1	–	–	1	–	–	–	–	–	1	–	–
Mycobacterium sp.	–	2	1	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Plantibacter sp.	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Promicromonospora sp.	–	13	–	–	–	–	–	1	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–
Rhodococcus sp.	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sphaerisporangium sp.	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Streptomyces sp.	1	15	1	–	–	–	–	–	3	–	1	1	3	–	2	2	–	–	–	1	–	–	–
Alphaproteobacteria																							
Ancylobacter sp.	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bosea sp.	–	2	–	–	–	–	1	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–
Caulobacter sp.	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Inquilinus sp.	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Novosphingobium sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	2	–	–	–
Paracoccus sp.	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Phyllobacterium sp.	5	–	–	–	3	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–
Sphingomonas sp.	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xanthobacteraceae sp.	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Betaproteobacteria																							
Herbaspirillum sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–
Massilia sp.	–	–	–	–	–	–	–	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Roseateles sp.	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Variovorax sp.	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	4	–	1	–	–	–	3
Firmibacteria																							
Bacillus sp.	13	–	5	–	6	20	14	20	1	4	2	8	1	1	–	1	–	2	1	–	6	5	–
Brevibacillus sp.	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cohnella sp.	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Exigobacterium sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–
Lysinibacillus sp.	–	–	–	–	–	–	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–
Paenibacillus sp.	8	–	2	2	–	18	6	–	–	8	–	–	–	1	8	–	–	5	5	5	5	9	2
Staphylococcus sp.	–	–	1	–	2	6	5	3	–	1	–	–	–	–	–	–	–	–	–	–	1	–	–
Flavobacteria																							
Chryseobacterium sp.	1	–	–	–	–	5	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–
Gammaproteobacteria																							
Acinetobacter sp.	–	–	–	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Enhydrobacter sp.	–	–	–	–	–	–	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Enterobacter sp.	–	–	–	3	–	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Erwinia sp.	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pantoea sp.	–	–	5	–	–	1	–	1	–	–	–	1	–	–	12	5	–	–	–	–	–	2	–
Pseudomonas sp.	–	7	6	4	17	7	1	11	–	8	–	4	–	–	20	2	–	–	2	4	14	–	–
Rahnella sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–
Stenotrophomonas sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	1	–
Xanthomonas sp.	–	–	–	–	1	2	–	–	–	–	–	–	–	–	3	–	–	–	2	–	–	–	–
Sphingobacteria																							
Dyadobacter sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–

[a] Plant species that were sampled less than three times and that harbour only one endosymbiont are not included in the table, but mentioned hereafter. *Anthyllis vulneraria*: *Bacillus* sp.(3), *Colutea arborescens*: *Buttiauxella* sp. (5), *Medicago falcata*: *Paracoccus* sp. (1), *Melilotus indicus*: *Bacillus* sp. (1), *Vicia lathyroides*: *Lysinibacillus* sp.(1), *Vicia sepium*: *Pseudomonas* sp.(1), *Vicia tetrasperma*: *Lysinibacillus* sp.(1), *Wisteria sinensis*: *Bacillus* sp. (3).

[b] Plant species: 1, *Cytisus scoparius*; 2, *Lathyrus latifolius*; 3, *Lathyrus pratensis*; 4, *Lathyrus sylvestris*; 5, *Lotus corniculatus*; 6, *Lotus pedunculatus*; 7, *Lupinus polyphyllus*; 8, *Medicago lupulina*; 9, *Medicago sativa*; 10, *Melilotus albus*; 11, *Melilotus altissimus*; 12, *Melilotus officinalis*; 13, *Ononis repens*; 14. *Ornithopus perpusillus*; 15, *Robinia pseudoacacia*; 16, *Trifolium arvense*; 17, *Trifolium dubium*; 18, *Trifolium hybridum*; 19, *Trifolium pretense*; 20, *Trifolium repens*; 21 *Vicia cracca*; 22, *Vicia hirsuta*; 23, *Vicia sativa*.

Trifolium arvense, *Trifolium hybridum*, *Vicia cracca*, *Vicia hirsuta* and *Wisteria sinensis* host plants and is characterised by the dominance of *Streptomyces* and *Rhodococcus* bacteria. Cluster 2 contains *Lotus corniculatus*, *Lotus pedunculatus*, *Medicago sativa*, *Melilotus officinalis*, *Ononis repens*, *Trifolium pratense*, *Trifolium repens* and *Vicia sativa* host plants and has a dominance of *Xanthomonas*, *Variovorax*, *Promicromonospora*, *Inquilinus* and *Novosphingobium* bacteria. Cluster 3 only consists of *Lupinus polyphyllus* and has *Staphylococcus*, *Microbacterium*, *Kocuria*, *Brevibacillus* and *Enhydrobacter* as dominant NRE. Cluster 4 contains *Lathyrus latifolius*, *Lathyrus sylvestris*, *Medicago lupulina*, *Melilotus alba* and *Vicia sepium* host plants and is characterised by the dominance of *Enterobacter*, *Brevibacterium* and *Paenibacillus* NRE. Cluster 5 only consists of *Ornithopus perpusillus* and has a dominance of *Leifsonia*, *Herbaspirillum*

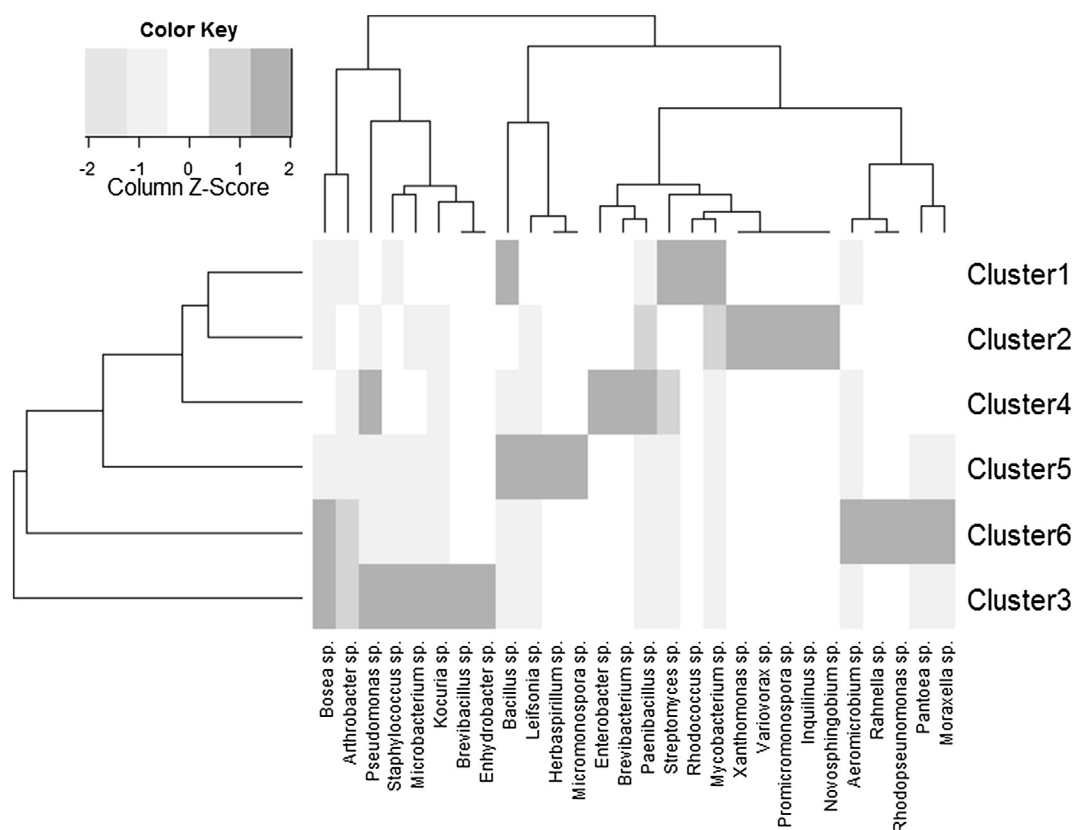

Fig. 1. Double cluster analysis of host plants with their NRE. Cluster 1: *Cytisus scoparius*, *Lathyrus pratensis*, *Trifolium arvense*, *Trifolium hybridum*, *Vicia cracca*, *Vicia hirsuta* and *Wisteria sinensis*; Cluster 2: *Lotus corniculatus*, *Lotus pedunculatus*, *Medicago sativa*, *Melilotus officinalis*, *Ononis repens*, *Trifolium pratense*, *Trifolium repens* and *Vicia sativa*; Cluster 3: *Lupinus polyphyllus*; Cluster 4: *Lathyrus latifolius*, *Lathyrus sylvestris*, *Medicago lupulina*, *Melilotus alba* and *Vicia sepium*; Cluster 5: *Ornithopus perpusillus* and Cluster 6: *Robinia pseudoacacia*. NRE that appear less than two times are not included and plants in which no NRE were found are not included.

and *Micromonospora* bacteria. Finally, cluster 6 contains only *Robinia pseudoacacia* as host plant and is characterised by the dominance of *Rahnella*, *Aeromicrobium*, *Pantoea* and *Moraxella* species.

Of the plant genus *Cytisus*, only the species *Cytisus scoparius* was sampled and its NRE were dominated by *Bacillus* (34%) and *Paenibacillus* (21%) species, accounting for more than 50% of all NRE found in this plants species. All other isolated bacterial species were recovered in less prominent amounts (<13%). The genus *Lathyrus* was represented by *Lathyrus latifolius*, *Lathyrus pratensis* and *Lathyrus sylvestris*. Remarkably, each of the *Lathyrus* species harboured different NRE in their root nodules, in addition to *Pseudomonas* species accounting for respectively 13%, 27% and 40% of the total NRE isolates. *Lathyrus latifolius* root nodule samples were dominated by *Streptomyces* (28%), *Microbacterium* (26%) and *Promicromonospora* (25%) species. The *Lathyrus pratensis* endophytes were dominated by *Pseudomonas* (27%) and *Bacillus* (23%) species. Finally, *Lathyrus sylvestris* contained a limited diversity of NRE mostly represented by *Pseudomonas* (40%), *Enterobacter* (30%) and *Bacillus* (20%) species. The *Lotus* species investigated in this study, *Lotus corniculatus* and *Lotus pedunculatus*, shared in common the following NRE: *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Xanthomonas*, although relative abundances differ in both plant species (Table 2). Within the genus *Lupinus*, only one species was sampled, *Lupinus polyphyllus*, and the predominant NRE isolated were *Bacillus* (20%) and *Microbacterium* (17%) species. Between *Medicago lupulina* and *Medicago sativa*, large differences were found in the presence of NRE, however both plant species were not equally sampled, as was also the case for the *Melilotus* plant species. Nevertheless, they all harboured *Bacillus* species in their root nodules. Plant genera *Ononis* and *Ornithopus* are both represented in this study by a single species, *Ononis repens* and *Ornithopus perpusillus*, and they harboured respectively *Streptomyces* (43%) and *Microbacterium* (46%) as dominant NRE. *Robinia pseudoacacia*, is a remarkably promiscuous host harbouring 13 different NRE genera. More than 50% belonged to the Gammaproteobacteria genera *Pantoea* and *Pseudomonas*. Furthermore, five Actinobacteria genera were present accounting for 16% of the isolates. Five *Trifolium* species were analysed in this study, *Trifolium arvense*, *Trifolium dubium*, *Trifolium hybridum*, *T. pratense* and *T. repens*. In three of the five plant species *Bacillus*, *Paenibacillus* and *Pseudomonas* NRE were present; all other bacteria differ greatly in presence and abundance (Table 2). The *Vicia* plant species investigated in this study include *V. cracca*, *Vicia hirsuta*, *Vicia lathyroides*, *V. sativa*, *V. sepium* and *Vicia tetrasperma*. *Paenibacillus* was the dominant NRE in half of these plant species (Table 2). However, not all *Vicia* species were sampled equally.

3.5. Ecoregion – NRE association

As previously described, plants were collected from different ecoregions in Flanders (De Meyer et al., 2011). These ecoregions are based on climatologic conditions, geology, geomorphology, soil type, groundwater and surface water content (Van Landuyt et al., 2006) and thus reflects soil texture, water content and nutrient availability. Double cluster analysis on the presence of NRE in certain ecoregions was performed using Ward's method in R and the results are given in a heatmap (Fig. 2). For each ecoregion characteristic patters of present and/or absent NRE genera is

visualised. *Paenibacillus*, *Kocuria* and *Leifsonia* NRE dominate the Campine ecoregion. The Dunes ecoregions is characterized by the dominance of *Promicromonospora*. *Moraxella*, *Aeromicrobium*, *Microbacterium*, *Rahnella*, *Bosea* and *Rhodopseudomonas* NRE dominate the Loamy ecoregions, whereas *Xanthomonas*, *Inquilinus*, *Enterobacter*, *Pantoea* and *Streptomyces* dominate the Polders ecoregions. The Sandy and sandloamy ecoregion is characterized by a dominance of *Brevibacterium*, *Novosphingobium*, *Variovorax*, *Brevibacillus*, *Enhydrobacter*, *Mycobacterium*, *Micromonospora*, *Rhodococcus*, *Herbaspirillum*, *Staphylococcus* and *Arthrobacter* NRE.

3.6. Rhizobia – NRE association

When combining the data obtained from our previous study (De Meyer et al., 2011) and the current study we see that on average across plant species 84.1% of the isolates were traditional rhizobia (*Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*) ranging between 77.7% and 90.5% at a confidence interval of 95%. A detailed comparison of the investigated host plants shows that for the majority of the plant species far more rhizobia than NRE were recovered from the nodules (Fig. 3). However, *Anthyllis vulneraria* and *Medicago falcata* yielded a 50% ratio of both. In general, plant genera *Trifolium* and *Vicia* seem to be restrictive hosts with low numbers of NRE (4%), whereas *Lathyrus* and *Lotus* genera yielded a larger number of NRE (41% and 31%, respectively see Fig. 3). Ratio analysis of rhizobia versus NRE in the different ecoregions revealed 75.87% rhizobia in the Campine ecoregion, 76.96% in the Dunes ecoregion, 87.17% in the Polders ecoregion, 88.53% in the Sandy and sandloamy ecoregion and 91.96% in the Loamy ecoregion. Additionally, a double cluster analysis was performed for rhizobia and NRE, to understand the correlation between the two groups. The results are shown in a heat map (Fig. 4) and highlight the correlation of rhizobia species occurrence with certain NRE in root nodules. Within the genus *Bradyrhizobium* we found a co-occurrence of *B. canariense* with *Bacillus*, *Rhodococcus* and *Streptomyces* and *Bradyrhizobium japonicum* with *Pantoea*, *Bacillus*, *Streptomyces*, *Staphylococcus* and *Arthrobacter*. All *Ensifer* species had *Pseudomonas* and *Moraxella* as dominant NRE. Additionally, *E. kummerowiae* had *Mycobacterium*, *Brevibacterium*, *Staphylococcus* and *Paenibacillus* as NRE. *E. meliloti* co-occurred with *Pantoea*, *Bacillus* and *Paenibacillus*, whereas *E. medicae* only had *Pantoea* as additional NRE. Within the genus *Mesorhizobium* we found co-occurrence of *Mesorhizobium loti* with *Rahnella*, *Aeromicrobium*, *Bosea*, *Pantoea*, *Moraxella* and *Rhodopseudomonas*. For *Rhizobium*, correlations were found for *Robinia giardinii* with *Staphylococcus*, *Inquilinus* and *Arthrobacter*, for *Robinia cellulosilyticum* and *Robinia radiobacter* with *Enterobacter*, *Pantoea* and *Pseudomonas*, for *Robinia alamii* with *Microbacterium* and *Moraxella*, and for *Rhizobium leguminosarum* with *Bacillus*, *Moraxella*, *Pseudomonas* and *Pantoea*. Given that rarefaction curves for most host species indicate not all diversity was covered, further studies are needed to assess the significance of these observations.

4. Discussion

Previously, we reported on traditional rhizobia from native legume species in Flanders that were collected over the summers of 2008 and 2009 (De Meyer et al., 2011). In addition to 3810 rhizobia isolates characterised previously (De Meyer et al., 2011), these campaigns also yielded 654 NRE that were studied using 16S rRNA, *nodC* and *nifH* gene sequencing. Only nodules that appeared healthy (presence of red colour) were harvested and analysed further.

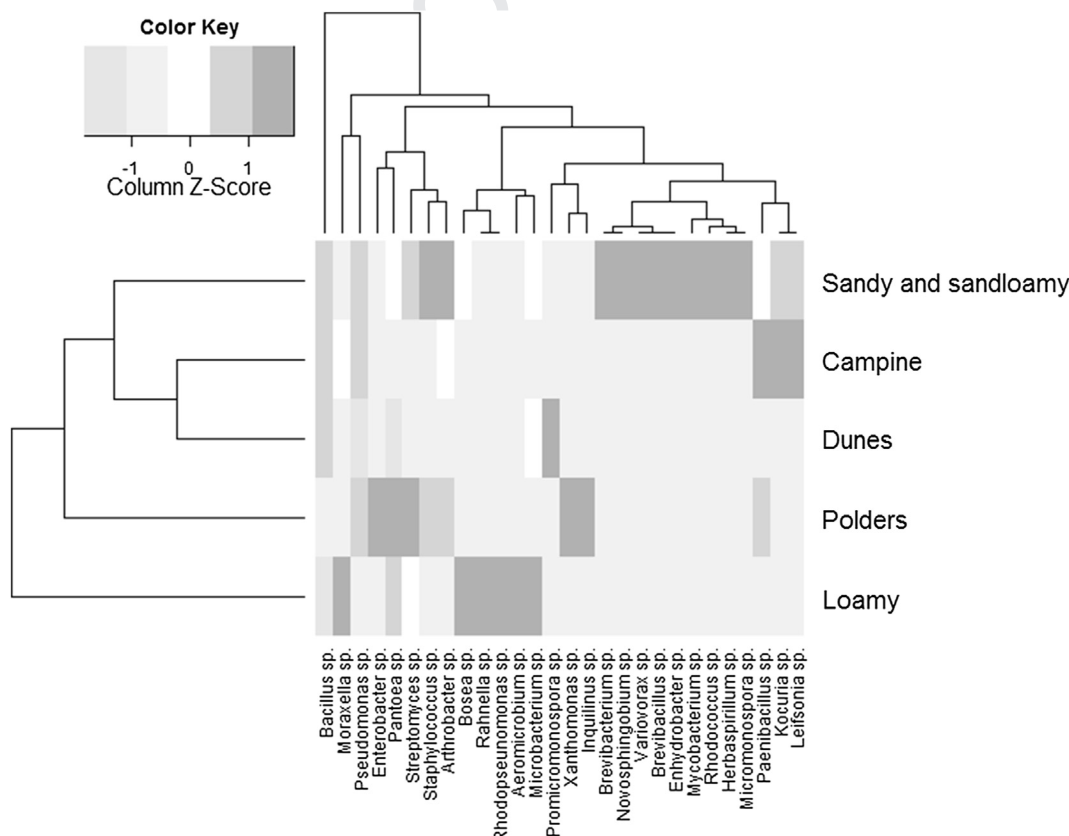

Fig. 2. Double cluster analysis of the ecoregions with their NRE. NRE that appear less than two times are not included.

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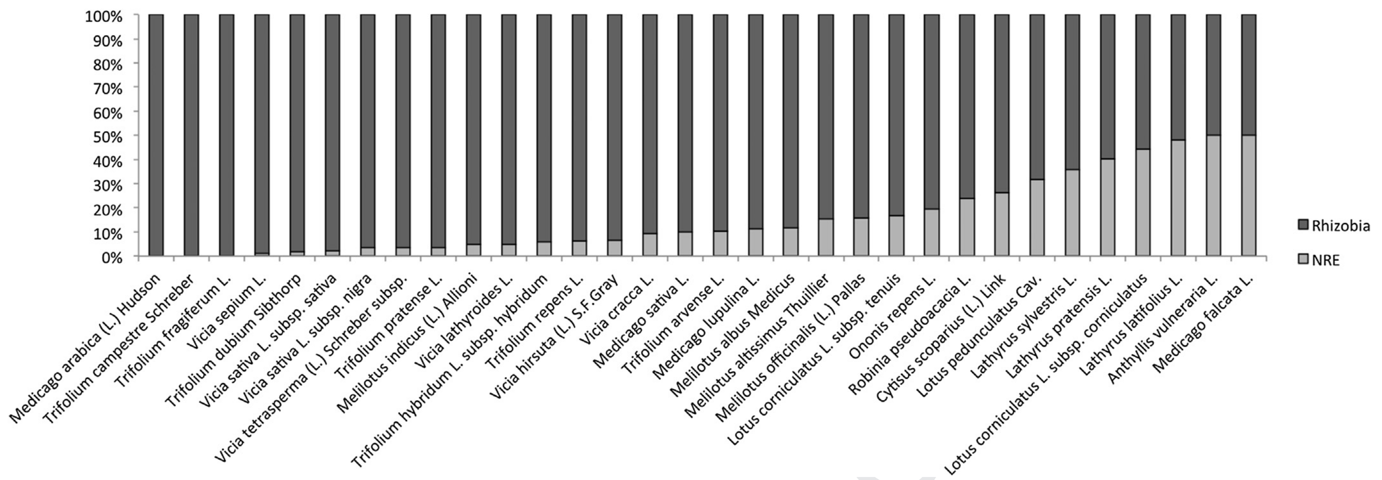

Fig. 3. Bar chart representing the percentage rhizobia and NRE recovered from the different host plants.

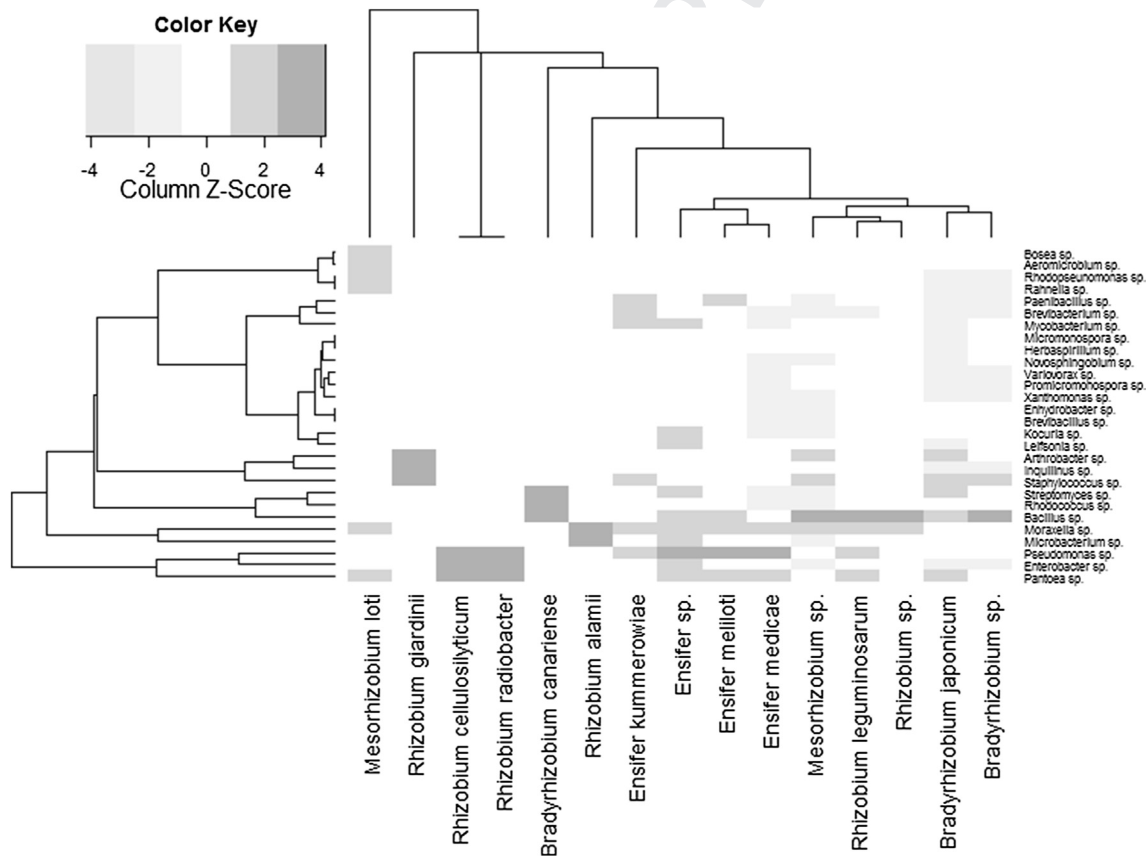

Fig. 4. Double cluster analysis of rhizobia and NRE. Bacteria that appear less than two times are not included. Q2

The data presented in this study suggest the presence of a large diversity of bacteria belonging to 50 genera inside nodules of indigenous legumes in Flanders. Plants house several endophytes within their tissues and multiple studies have reported the presence of endophytes in legumes (Muresu et al., 2008; Ibáñez et al., 2009; Muresu et al., 2010). One of the genera in our study, *Phyllobacterium* (Table 1) is a known nodule endosymbiont and is capable of fixing nitrogen (Valverde et al., 2005; Baimiev et al., 2007). However, in our study no symbiosis or nitrogen fixation related genes were detected for the isolates of this genus. Some genera including, *Arthrobacter*, *Microbacterium*, *Rhodococcus*, *Sphingomonas*, *Bacillus*, *Cohnella*, *Pseudomonas* and *Herbaspirillum*, were previously reported from root nodules of several legume species (Sturz et al., 1997; Mantelin et al., 2006; Zakhia et al., 2006; Putnam and Miller, 2007; Zurdo-Pineiro et al., 2007; Palaniappan et al., 2010). Other bacterial genera encountered, including *Pantoea*, *Corynebacterium*, *Chryseobacterium*, *Sphingomonas* and *Xanthomonas*, were described previously as endophytes in certain plant tissues including legume plant tissue (Burch and Sarathchandra, 2006). However, 33 of the 50 bacterial genera found in this study were to

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our knowledge, not previously reported from legume root nodules (Table 1). The majority of the investigated nodules contained *Bacillus* (17.9%), *Paenibacillus* (12.5%) and *Pseudomonas* (15.9%) NRE species, together with small numbers of other NRE bacterial genera from the classes Actinobacteria, Alpha- and Betaproteobacteria.

Nodulation and nitrogen fixation capacity are characters usually studied in rhizobia research, since they give an indication of the host specificity and nitrogen fixing ability (Perret et al., 2000; Moulin et al., 2004; Diouf et al., 2010). No *nodC* or *nifH* gene sequences could be detected among the isolates, indicating an endophytic lifestyle rather than a rhizobial lifestyle. However, this needs to be confirmed using authentication experiments (Howieson et al., 1995). Previous studies indicate that endophytes can have three main functions inside root nodules, firstly as true rhizobial endosymbionts capable of inducing nodulation and performing nitrogen fixation (Valverde et al., 2005; Ardley et al., 2012). Secondly, as helper bacteria increasing plant health and/or yield when co-inoculated with rhizobia (Annapurna et al., 2013; Tariq et al., 2014), and finally, as opportunistic endophytes thriving in this nitrogen riche nodule environment (Dudeja et al., 2012). Therefore, nodules seem to be an excellent environment for a wide variety of bacteria.

The presence of the NRE was analysed in function of the host plant and this indicates that certain plant groups prefer certain NRE in their root nodules (Fig. 1). However, these observations need to be confirmed, given that rarefaction curves for most host species were still rising slightly (Fig. S1). As Dudeja et al. (2012) mentioned, most endophytes originate from the rhizosphere or phyllosphere and only a small number may be transmitted via seed. We postulate that the host plant may select the appropriate NRE from the environment and hence characteristic NRE for certain legume species were found. Interestingly *Trifolium* and *Viciae* plant species harboured a small diversity of bacteria in their root nodules, with a dominance of *Rhizobium* species and more specific *R. leguminosarum*. If NRE can enter the root nodule as opportunists then *Rhizobium* bacteria might have defence mechanisms in place to prevent this from happening, as previously shown against arbuscular mycorrhizal fungi (Franzini et al., 2013) and against soil bacterial populations (Robleto et al., 1998; Wilson et al., 1998). Alternatively, the native *Rhizobium* strains could be highly competitive root nodule bacteria, quickly colonising the available root nodule space and therefore preventing other bacteria from getting established. This has been shown previously in studies where commercial inoculum strains were applied and out competed by native *Rhizobium* strains (Nangul et al., 2013). Remarkable is also the tentative correlation we found between certain NRE and traditional rhizobia. Previous studies have found co-occurrence of *Bacillus* species together with *Bradyrhizobium japonicum* in soybean nodules (Bai et al., 2002) and with *M. gobiense* in *Sphaerophysa salsula* root nodules (Deng et al., 2011). *Paenibacillus* and *Pseudomonas* species have also been found to co-occur with *Mesorhizobium* species (Deng et al., 2011). However, our study reveals an even more complex interaction between traditional rhizobia and NRE bacteria for legumes growing in the temperate climate in Western Europe. Most of the plants harboured *Bacillus* and *Pseudomonas* species in their root nodules together with traditional rhizobia, which is becoming a common observation (Dudeja et al., 2012). The function of these NRE as helpers still needs to be confirmed but the fact that these specific co-occurrence patterns exist, might suggest that not only the plant selects certain NRE but also the rhizobia inside the root nodules prefer certain NRE.

The NRE were also analysed in function of the biogeographical ecoregion they were isolated from. Flanders, the northern part of Belgium, is situated in the Atlantic biogeographical region and has limited climate variation (Van Landuyt et al., 2011). Geological and landscape elements, such as soil type and land use system are the driving forces in the small-scale distribution of plant species. Several characteristics, including soil type, landscape morphology, land-use system, climate, topography and hydrology were used by Van Landuyt et al. (2006) to designate six ecoregions (campine, dunes, loamy, polders, sandy-sandloamy region and the region of the Valley of the River Meuse) as areas with a more or less uniform landscape. A double cluster analysis was preformed using Ward's method in R and revealed a correlation between NRE species and the ecoregion where the plants were collected (Fig. 2). Noticeable is also the fact that ecoregions with similar environmental factors have more similar NRE, as is the case for the Dunes and Campine ecoregions (Fig. 2). The significance of these observations remains to be confirmed given that rarefaction curves were still rising. However, such biogeographical correlation of NRE has been reported before and our study confirms these findings (Deng et al., 2011). However, the NRE groups from the plant analysis are not the same as those in the ecoregion analysis, indicating that different environmental parameters drive the selection of NRE. It is not clear at this point what these factors might be. However, firstly Flanders is a densely populated area and also serves as crossroads for goods and people in Western Europe. Secondly, most of the sampled legumes occur everywhere in Flanders and have thus no specific niche with respect to the ecoregion. Both considerations may contribute to the limited correlation of host plants with ecoregions and available NRE. Small-scale differences in ecoregions may affect bacterial distribution by enabling speciation, permitting certain NRE adapted to these conditions to thrive in these environments and as such explaining the preference of certain NRE for certain ecoregions.

In conclusion, this study investigated a large diversity of legume plant species and their associated NRE. The detection of bacteria other than traditional rhizobia in legume plants has been reported before, however, to our knowledge, this is the first report of such an extensive bacterial diversity analysis in root nodules of indigenous legumes in Western Europe.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.01.002.

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