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Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium)

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ABSTRACT

We investigated the diversity of rhizobia isolated from different indigenous legumes in Flanders (Belgium). A total of 3810 bacterial strains were analysed originating from 43 plant species. Based on rep-PCR clustering, 16S rRNA gene and *recA* gene sequence analysis, these isolates belonged to *Bradyrhizobium*, *Ensifer (Sinorhizobium)*, *Mesorhizobium* and *Rhizobium*. Of the genera encountered, *Rhizobium* was the most abundant (62%) and especially the species *Rhizobium leguminosarum*, followed by *Ensifer* (19%), *Bradyrhizobium* (14%) and finally *Mesorhizobium* (5%). For two rep-clusters only low similarity values with other genera were found for both the 16S rRNA and *recA* genes, suggesting that these may represent a new genus with close relationship to *Rhodopseudomonas* and *Bradyrhizobium*. Primers for the symbiotic genes *nodC* and *nifH* were optimized and a phylogenetic sequence analysis revealed the presence of different symbiovars including genistearum, glycinearum, loti, meliloti, officinalis, trifolii and viciae. Moreover, three new *nodC* types were assigned to strains originating from *Ononis*, *Robinia* and *Wisteria*, respectively. Discriminant and MANOVA analysis confirmed the correlation of symbiosis genes with certain bacterial genera and less with the host plant. Multiple symbiovars can be present within the same host plant, suggesting the promiscuity of these plants. Moreover, the ecoregion did not contribute to the separation of the bacterial endosymbionts. Our results reveal a large diversity of rhizobia associated with indigenous legumes in Flanders. Most of the legumes harboured more than one rhizobial endosymbiont in their root nodules indicating the importance of including sufficient isolates per plant in diversity studies.

1. Introduction

Since the 19th century, the symbiosis between leguminous plants and bacteria collectively called rhizobia, is well known (As reviewed in: Dresler-Nurmi et al., 2007; Willems, 2006). Traditionally, most rhizobia species have been allocated to six genera in the Alphaproteobacteria: the fast to moderately fast growing genera *Rhizobium*, *Allorhizobium*, *Ensifer* (*Sinorhizobium*) and *Mesorhizobium*, the slow-growing genus *Bradyrhizobium* and the stem nodulating genus *Azorhizobium*. However, recent studies have reported the presence of other non-classical rhizobia belonging to the Betaproteobacteria (Chen et al., 2005; Vandamme et al., 2002), Gammaproteobacteria (Ibáñez et al., 2009; Muresu et al., 2010) and Actinobacteria (Palaniappan et al., 2010; Trujillo et al., 2010).

Nodulation and nitrogen fixation capacity of rhizobia are very important factors in understanding the symbiosis with legumes. The mechanisms behind this molecular interaction between the plant and the bacteria have been intensively studied (as reviewed in: Cooper, 2007; Downie, 2010; Masson-Boivin et al., 2009). Different genes are involved in the nodulation process, where the early nodulation genes *nodABC* are responsible for the core structure of the Nod factors. Therefore these genes are structurally and functionally conserved and found in most rhizobia studied to date (Dresler-Nurmi et al., 2007). The term biovar has been used in bacterial taxonomy to group strains of the same species with distinctive physiological characters. In rhizobia research it refers to strains symbiotic with the same host species. Recently Rogel et al. (2011) proposed to use the term symbiovar for strains distinguishable by their symbiotic capabilities and host plant. *Nif* and *fix* genes are responsible for the nitrogen fixation. N*ifH* is one of the most studied nitrogen fixing genes to date (Deng et al., 2011; Diouf et al., 2010; Mierzwa et al., 2010a; Rogel et al., 2011; Wdowiak-Wrobel and Malek, 2010). It encodes the two identical subunits of

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dinitrogenase reductase and has proven to be useful for phylogenetic analysis (Chen et al., 2003; Dobert et al., 1994).

The family of leguminous plants is one of the major plant families in the world, comprising more than 17,000 species (APGIII, 2009). This family is divided into three subfamilies, the *Mimosoideae*, *Caesalpinioideae* and *Faboideae* (APGIII, 2009). The first two subfamilies consist of woody species with a more tropical distribution. The *Faboideae* is the largest subfamily and comprises woody and herbaceous species with a cosmopolitan distribution. The legume species in Belgium are restricted to this third subfamily and contain 102 plant species in 30 different genera (Lambinon et al., 1998). The flora of Flanders, the northern part of Belgium, is dominated by Atlantic and Mid-European species although some sub-boreal and sub-mediterranean species can be found (Van Landuyt et al., 2006a). Since Flanders is situated in the Atlantic biogeographical region, limited climate variation is present (Van Landuyt et al., 2011). The small-scale distribution of plant species is thus mainly dictated by geological and landscape elements, such as soil type and land-use system. Several characteristics were used to designate ecoregions as areas with a more or less uniform landscape, they include soil type, landscape morphology, land-use system, climate, topography and hydrology (Van Landuyt et al., 2006a). In Flanders, six ecoregions can thus be observed: dunes, polders, sandy-sandloamy region, campine, loamy region and the region of the Valley of the River Meuse (Van Landuyt et al., 2006a).

Legumes, including important crops such as beans, soybean, peanuts and clover are ecologically important plant species that can often grow on nutrient deficient soils, as a result of their associations with rhizobia that fix nitrogen. When the plants or the nodules decay, the fixed nitrogen is released in the soil and becomes available for other plants. This process improves soil structure and enables other plants to settle in this environment. Many studies have proven the usefulness of legumes in revegetation of arable lands (Freitas et al., 2010; Howieson et al., 2000; Rincon et al., 2008). However, because of this ecologic and economic importance, most studies have focused on cultivated legumes. Only a small portion of the rhizobial diversity present in wild legumes has been investigated, especially in indigenous legumes in Western Europe.

The aim of this study was to perform a systematic exploration of endosymbionts present in indigenous legumes in Flanders. Isolates obtained in several sampling campaigns were grouped and identified by using rep-PCR analysis, 16S rDNA and *recA* gene sequencing. Additionally, the phylogenies of the symbiosis genes *nodC* and *nifH* were investigated to assess the symbiotic capacity and the symbiovar type of the isolates. The focus, however, was on the typical rhizobia present in these indigenous legumes in Flanders, other isolates representing non-typical rhizobia will be reported in a separate study.

2. Materials and methods

2.1. Sampling

To organise the sampling campaigns we used the INBO grid (Research Institute for Nature and Forest) that divides Flanders into plots of 1 km^2 and is linked to a database that documents the plant species reported in each plot (Van Landuyt et al., 2006a). Plots were selected for sampling on the basis of the diversity of legume species and difference in ecoregion (campine, dune, polder, loamy and sandy-sandloamy region; Fig. 1). The sampling campaigns were performed over the summers of 2008 and 2009. In each selected plot one or two representative plants were sampled for each recognizable legume species. In most cases, whole plants were excavated and taken to the laboratory where the nodules were removed and preserved at 4 °C in tubes containing dried silica beads.

2.2. Isolation of rhizobia

The bacteria were isolated from surface-sterilized root nodules as follows: nodules were rehydrated for 30 min in sterile distilled

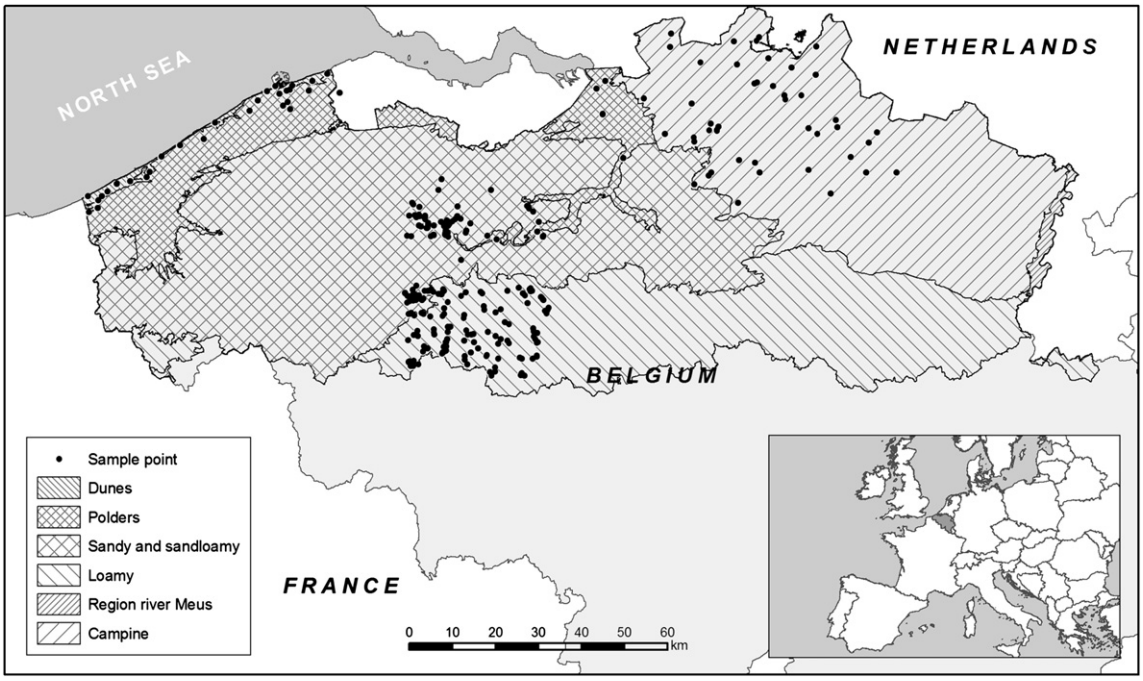

Fig. 1. Map of Belgium showing the sampling sites, marked as black dots, and the different ecoregions in Flanders, the northern part of Belgium.