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Population structure of root nodulating *Rhizobium leguminosarum* in *Vicia cracca* populations at local to regional geographic scales☆

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ABSTRACT

The genetic diversity and population structure of about 350 *Rhizobium leguminosarum* biovar *viciae* isolates from *Vicia cracca* were analysed. A hierarchical sampling design was used covering three regions, one region in Belgium and two in France, in which multiple local *V. cracca* populations were sampled. *Rhizobium* isolates were genotyped using RAPD and by sequencing two chromosomal housekeeping genes (*glnII* and *recA*) and one plasmid-borne gene (*nodC*). Twenty-six *nodC* types and sixty-seven chromosomal types were identified, many of which appeared to be regional or local endemics. We found strong genetic differentiation both among *V. cracca* populations that are separated by only a few kilometres, and among regions that are 50 to 350 km apart. Despite significant plasmid exchange, chromosomal and *nod* types were similarly structured among host populations and regions. We found two lineages of which one prevailed in the Belgian region while the other dominated the French regions. Although a significant correlation between genetic differentiation and geographic distance was found, it is deemed more likely that the observed biogeographic patterns are rather due to coevolutionary interactions and environmental pressures. Furthermore, the impact of recombination on the chromosomal differentiation was found to be considerable.

Introduction

Rhizobia are nitrogen fixing bacteria that are capable of establishing an endosymbiotic mutualism with legumes (Leguminosae), providing their legume hosts with a steady supply of plant usable nitrogen and a competitive advantage, especially in nitrogen poor soils. The mutualism between legumes and rhizobia is therefore of high agricultural and ecological importance.

Nitrogen fixation occurs in specialized organs, called root nodules, which are formed through the exchange of a series of molecular signals between the host plant and rhizobia. The key genes for nodule formation, the *nod* genes, are often located on transferable symbiotic plasmids [25]. The proteins encoded by *nod* genes are also involved in host recognition and specificity [37]. Although some rhizobia are able to nodulate a broad range of hosts [39], most strains show a certain degree of host specificity, likely acquired as a result of long-term coevolutionary interactions between the host and the endosymbiont [25,33]. This is possible because legume hosts are capable to select against unfavourable rhizobial strains through decreasing oxygen supply [57], or through discrimination prior to nodulation, also known as 'partner choice' [20].

Legumes are predicted to select for different rhizobial genotypes across different environmental conditions [57]. This could create geographic differences in the effectiveness of the mutualism, which in turn is expected to translate into genetic differentiation between the rhizobial populations. Several studies have already been conducted on the population structure of rhizobia nodulating legumes. Some have demonstrated population differentiation at a

☆ Note: Nucleotide sequence data reported are available in the GenBank database under the accession numbers: KJ923022 – KJ923048 (*nodC*), KJ923049 – KJ923115 (*glnII*), KJ923116 – KJ923182 (*recA*).

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continental scale [41,47,55], while others demonstrated regional population structuring [40,53]. Very few studies so far (e.g. [18]) have evaluated how the population structure of *Rhizobium* is changing from the within host plant population level, across different local host plant populations, to host plant populations from different geographical regions. This hierarchical approach, however, may provide important information regarding the scale of local adaptation and gene flow in rhizobia, especially when the population genetic structure is quantified based on both putatively neutral genetic loci and on genes interfering with the nodulation process. Furthermore, as far as we know, no studies have attempted to test whether plasmid geographic structuring differs from the geographic structuring of chromosomal genes. However, incongruence in the geographical structuring of chromosomal genes and plasmid genes can be expected to be important, since it is widely acknowledged that horizontal gene transfer (HGT) is frequent in bacteria [10].

The herbaceous grassland plant species *Vicia cracca* (tufted vetch or bird vetch) has been demonstrated to be one of the most *Rhizobium* selective legumes [25,33], with a strong preference for *Rhizobium leguminosarum* biovar *viciae* [5]. The strong specificity between both symbionts makes it an ideal study system to quantify the population genetic structuring of specific *Rhizobium* lineages across different geographical scales.

Here, we adopted a hierarchical sampling design covering one Belgian and two French regions, in which we sampled rhizobia from multiple *V. cracca* populations. *Rhizobium* isolates were genotyped for two chromosomal housekeeping genes (*glnII* and *recA*) and one plasmid-borne gene (*nodC*). Additionally, we used putatively neutral RAPD (Random Amplified Polymorphic DNA) markers to genotype the *Rhizobium* isolates. Our general aim was to infer the genetic diversity and population genetic structure of *R. leguminosarum* biovar *viciae* strains that nodulate *Vicia cracca* at various geographic scales, in order to acquire insight in the scale of local adaptation and the level of *Rhizobium* admixture. The specific aims were to:

(1) Partition rhizobial genetic diversity among and within different *Vicia cracca* populations and regions;
(2) Examine patterns of isolation by distance in *R. leguminosarum* biovar *viciae* across different spatial scales;
(3) Compare patterns of *Rhizobium* population genetic structure for housekeeping genes, a plasmid-borne gene involved in nodulation, and genome wide putatively neutral RAPDs;
(4) Assess the effect of recombination of chromosomal genes on genetic differentiation of *R. leguminosarum*.

Material and methods

Study species and sampling

Vicia cracca is native to Europe and naturally occurs in disturbed grasslands and road verges. The species is used as fodder for cattle, green manure fertilizer and cover crop, and has therefore been introduced in many temperate ecosystems worldwide. *V. cracca* individuals were collected in three different regions (Fig. 1): Brabant (Belgium), the Northern Vosges (Lorraine, France) and the Southern Vosges (Lorraine, France). The distance between Brabant and Northern Vosges is c. 300 km whereas the distance between Northern and Southern Vosges is c. 50 km. The distances between regions and the average distance between populations within regions are given in Table S1 (supplementary information). In total 16 *V. cracca* populations were sampled: eight populations in Brabant, and four populations in both Northern Vosges and Southern Vosges (Fig. 1). Latitudinal and longitudinal coordinates of *V. cracca* populations are given in Table S2. From each *V. cracca* population, about five plant individuals were excavated.

Isolation of rhizobia and DNA extraction

On average, five root nodules per individual plant were collected. Nodules were surface-sterilized by soaking them in 70% ethanol for 3 min and subsequently 10 min in 15% sodium hypochlorite. Root nodules were then rinsed 6–7 times with sterile distilled water and stored at −20 °C in 30% glycerol. Prior to the isolation of the rhizobia, root nodules were rolled over a Tryptone Yeast extract (TY) agar plate to test the effectiveness of the surface sterilization. Isolation of rhizobia was done by cutting the nodule with a sterilized blade and inoculating it on a TY agar plate containing 100 mg/L amphotericin B. After an incubation period of three days at 30 °C, all rhizobia isolates from successfully surface-sterilized nodules were purified by subculturing on TY plates. Slow growing rhizobia were given an additional period of three days incubation. Isolates were stored at −80 °C in 50% glycerol for long-term storage. From each isolate, DNA was extracted by the phenol–chloroform DNA extraction method [27]. DNA concentration was determined with a nanodrop spectrophotometer and all DNA extracts were diluted to 5 ng/μL.

RAPD fingerprinting

The rationale behind the RAPD fingerprinting step was twofold: (1) reducing sequencing effort [32] and (2) quantify the population genetic structure at putatively neutral loci [4]. The suitability of the Operon primers (Operon Technologies, Alameda, CA, USA) OP B-12 (CCTTGACGCA) and OP B-19 (ACCCCCGAAG) were first tested for a subset of isolates, after which OP B-12 was selected for further fingerprinting of the whole collection. This primer resulted in highly reproducible fingerprints, consisting of clearly distinct and multiple bands. PCR was performed using a Bio-Rad T100 thermal cycler in a total volume of 20 μL containing 0.5 μM of the OP B-12 primer, 0.15 mM of each deoxynucleoside triphosphate, 1.0 U Titanium Taq DNA polymerase with Titanium Taq PCR buffer (Clontech Laboratories, Palo Alto, CA, USA) and 5 ng DNA. Thermal cycling conditions comprised an initial denaturation at 94 °C for 2 min, followed by a cycling protocol of 35 cycles of denaturation at 94 °C for 1 min, annealing at 35 °C for 1 min and elongation at 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. 8 μL of each PCR product were loaded on a 1% (w/v) agarose gel stained with ethidium bromide. DNA fragments were separated by 90 min gel electrophoresis at 100 V in Tris-acetate-EDTA (TAE) buffer. A 10 kb DNA ladder (Smartladder, Eurogentec, Seraing, Belgium) was used as a size marker for comparison. Fingerprint patterns were visualized with the BioChemi System (UVP, Upland, CA, USA).

Amplification and sequencing of glnII, recA and nodC genes

Two chromosomal housekeeping genes, *glnII* and *recA*, and the *nodC* gene located on the symbiotic plasmid (pSym) were partially amplified. *recA* encodes recombinase A, an enzyme that is involved in DNA recombination and DNA repair systems [14]. *glnII* encodes glutamine synthetase 2, which is involved in nitrogen assimilation and fixation [34]. The *nodC* gene encodes a protein involved in the synthesis of the chitin oligosaccharide backbone of the Nod factor [24]. The used primers and PCR protocols are described in Table S3. A preliminary test showed that the *nodC*for540 primer of *nodC* [42] performed weakly. It was therefore eventually replaced by the *nodC*for495 primer obtained from Moschetti et al. [32]. Amplified products were purified using the Exo/SAP enzyme cleaning protocol [56]. Purified products were sent to Macrogen for sequencing

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