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Crotalarieae and Genisteae of the South African Great Escarpment are nodulated by novel *Bradyrhizobium* species with unique and diverse symbiotic loci

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ABSTRACT

The genus *Bradyrhizobium* contains predominantly nitrogen-fixing legume symbionts. Phylogenetic analysis of the genes responsible for their symbiotic abilities (i.e., those encoded on the nodulation [*nod*] and nitrogen-fixation [*nif*] loci) has facilitated the development of an extensive phylogeographic framework for the genus. This framework however contains only a few nodulating isolates from Africa. Here we focused on nodulating *Bradyrhizobium* isolates associated with native southern African legumes in the tribes Genisteae and Crotalarieae found along the Great Escarpment in the Mpumalanga Province of South Africa. The aims of this study were to: (1) obtain rhizobial isolates from legumes in the Genisteae and Crotalarieae; (2) verify their nodulation ability; (3) characterize them to species level based on phylogenetic analyses of several protein coding gene regions (*atpD*, *dnaK*, *glnII*, *recA*, *rpoB* and *gyrB*) and (4) determine their placement in the phylogeographic framework inferred from the sequences of the symbiotic loci *nodA* and *nifD*. Twenty of the 21 *Bradyrhizobium* isolates belonged to six novel species, while one was conspecific with the recently described *B. arachidis*. Among these isolates, the *nodA* phylogeny revealed several new clades, with 18 of our isolates found in Clades XIV and XV, and only three forming part of the cosmopolitan Clade III. These strains formed predominantly the same groups in the *nifD* phylogeny although with slight differences; indicating that both vertical and horizontal inheritance of the symbiotic loci occurred. These findings suggest that the largely unexplored diversity of indigenous African rhizobia are characterized by unique ancestries that might mirror the distribution of their hosts and the environmental factors driving their evolution.

1. Introduction

Plants in the family Leguminosae are capable of forming nitrogen-fixing symbioses with certain Gram-negative soil bacteria (Held et al., 2010). Although these bacteria do not represent a monophyletic assemblage, they are collectively referred to as rhizobia (Kereszt et al., 2011) to recognize their role in the symbiosis. The most obvious outcome of the legume-rhizobium symbiosis is the formation of root or stem nodules within which atmospheric nitrogen is fixed to more usable forms such as ammonia (Santi et al., 2013). The symbiosis is initiated by molecular cross-talk between the two partners and is facilitated by specific signaling molecules (Moscatiello et al., 2010). The plant partner releases root exudates such as flavonoids, which attracts the rhizobia and initiates transcription of the genes involved in formation of the rhizobial signaling molecule (Ferguson et al., 2010; Wang et al., 2012). This molecule is known as the Nod factor and its recognition initiates morphological changes in the legume root hair, which leads to nodule formation (Abdel-Lateif et al., 2012; Hassan and Mathesius, 2012; Oldroyd et al., 2011; Udvardi and Poole, 2013). The basic

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structure and production of the Nod factor depend on the products of the common nodulation genes (*nodABC* and *nodIJ*) (Noel, 2009) encoded by almost all rhizobia (Aoki et al., 2013).

The genus *Bradyrhizobium* (class Alphaproteobacteria) was established approximately a century after Beijerinck's discovery of rhizobia (Jordan, 1982; Laranjo et al., 2014). It currently includes 27 species (Durán et al., 2014; Euzéby, 1997; Parte, 2013; Wang et al., 2013), as well as numerous additional groups that are yet to be formally described as distinct species (Aserse et al., 2012; Horn et al., 2014; Koppell and Parker, 2012; Parker and Rousteau, 2014; Steenkamp et al., 2008; Stępkowski et al., 2012). *Bradyrhizobium* has a world-wide distribution and its members nodulate diverse legumes, i.e., some species in the subfamily Caesalpinioideae (Fonseca et al., 2012; Parker, 2008), various species in the Mimosoideae (Rodríguez-Echeverría, 2010), and many species in the Papilionoideae, including the tribes Crotalarieae and Genisteae (Aserse et al., 2012; Stępkowski et al., 2005, 2007, 2012; Vinuesa et al., 2005a). Indeed, the genus *Bradyrhizobium* forms nodules on legumes belonging to at least 24 legume tribes (out of the 33 nodule-forming legumes tribes) thus showing the broadest host range among all rhizobial genera (Parker, 2015).

Horizontal gene transfer (HGT) is thought to be an important driver of rhizobial evolution and *Bradyrhizobium* species are no exception (Bailly et al., 2007; Crossman et al., 2008; Steenkamp et al., 2008; Tian et al., 2010). This is because the symbiotic loci responsible for nodulation (*nod*) and nitrogen-fixation (*nif*) form part of mobile elements such as plasmids or symbiosis islands (Aoki et al., 2013; Barcellos et al., 2007; Ormeño-Orrillo et al., 2013). During the last decade, numerous studies have documented the occurrence of HGT of symbiotic loci among rhizobial species as well as among rhizobial genera (e.g., Barcellos et al., 2007; Ormeño-Orrillo et al., 2013; Parker, 2012; Vinuesa et al., 2005b). HGT is generally thought to facilitate the dispersal of symbiotic loci (Menna and Hungria, 2011; Rogel et al., 2011) and also explains the polyphyletic nature of rhizobia (Aoki et al., 2013; Bontemps et al., 2010).

In recent years, many studies have investigated the biogeography of rhizobia where the broad focus has been on understanding how specific groups or species are distributed in geographic space and through geological time (Han et al., 2009; Tian et al., 2010; Vinuesa et al., 2008). In fact, prokaryotic biogeography, specifically studies involving pathogens and symbionts, has received much attention in the scientific literature (Martiny et al., 2006; Ramette and Tiedje, 2007; Ranjard et al., 2010). This is because a focus on the distribution and evolution of the ability to nodulate legumes (a property that is prone to HGT) allows circumvention of the complexities associated with prokaryotic species definitions (Green et al., 2008; Stępkowski et al., 2012). For example, an extensive phylogeographic framework has been developed for the genus *Bradyrhizobium* that is based on *nodA* gene information (Muñoz et al., 2011; Steenkamp et al., 2008; Stępkowski et al., 2011, 2012) and to some extent *nifD* sequence data (Crisóstomo et al., 2013; Parker and Rousteau, 2014). As legume symbionts, these bacteria are thus particularly well-suited for bio- and phylogeographic studies aimed at resolving the evolution of their biological properties.

The picture that emerges from previous rhizobial work on indigenous legumes in specific locations is that they may be nodulated by any of a number of known or new rhizobial species, while their symbiotic abilities likely originate from ancestors common to the rhizobial groups associated with the specific host in its habitat range (Beukes et al., 2013; Parker, 2012; Steenkamp et al., 2008; Stępkowski et al., 2012; Tian et al., 2010). Here, we intended to test this hypothesis by focussing on the *Bradyrhizobium* symbionts of indigenous Crotalarieae and Genisteae (subfamily Papilionoideae) occurring in native environments in South Africa. Although both tribes have their centres of origin in southern Africa (Lewis et al., 2005), Genisteae is mostly found in the Mediterranean with some species occurring in Africa and certain mountainous areas of the western hemisphere (Sprent, 2009). Crotalarieae, on the other hand, is the largest papilionoid tribe in Africa (Ardley et al., 2013; Boatwright et al., 2008) with a limited number of species occurring in Asia, Australia and the Americas (Sprent, 2009).

The overall goal of this study was to investigate whether evolutionary processes inherent to the diversification of the *Bradyrhizobium* symbionts associating with indigenous Crotalarieae and Genisteae could have been responsible for the geographic distribution of these bacteria. For this purpose, we specifically targeted plants occurring along the Great Escarpment in the Mpumalanga Province of South Africa. This geological formation is a remnant of the break-up of Gondwana (Thuiller et al., 2006) and is thought to be species-rich with high levels of endemism due to its topographical and climatic diversity (Thuiller et al., 2006). Our specific aims were as follows: (i) to isolate *Bradyrhizobium* strains from the root nodules of indigenous members of the Crotalarieae and Genisteae occurring in this region; (ii) to confirm the symbiotic ability of the isolated bacteria using nodulation tests on the promiscuous hosts siratro (*Macroptilium atropurpureum*) and cowpea (*Vigna unguiculata*); (iii) to identify the bacteria using a multilocus sequence analysis (MLSA) approach; and (iv) to determine the position of the bacteria within the existing phylogeographic framework for *Bradyrhizobium* and finally (v) to verify a hypothesis concerning the putative South-African origin of bradyrhizobia nodulating Genesiteae legumes – specifically Clade II members (Moulin et al., 2004; Stępkowski et al., 2007). For MLSA, the DNA sequences of six housekeeping genes were used (i.e., *recA* encoding recombinase A, *glnII* encoding glutamine synthetase isoform II, *atpD* encoding ATP synthase subunit beta, *dnaK* encoding the Hsp70 chaperone, *gyrB* encoding DNA gyrase subunit B and *rpoB* encoding RNA polymerase beta subunit). To study the phylogeography of these rhizobia, we inferred genealogies based on the *nodA* and *nifD* genes, which respectively encodes an acyltransferase and the alpha subunit of dinitrogenase.

2. Materials and methods

2.1. Legumes and sampling locations

Rhizobia were isolated directly from legume nodules collected in the field at sites in the Badplaas, Barberton and Dullstroom regions (Mpumalanga Province, South Africa) (Table 1). The Barberton and Badplaas sampling sites were situated in the Lowveld where the elevation ranges from approximately 835 to 1104 meters above sea (MAS) level, while the Dullstroom site was located on the Escarpment at an elevation of approximately 2100 MAS level. These two regions differ extensively in terms of geography and climate (Zunckel, 1999).

For the tribe Genisteae nodules were collected from *Argyrolobium sericeum* in the Badplaas area and *A. rupestre* in the Barberton area. Members of the tribe Crotalarieae from which nodules were collected were *Leobordea pulchra*, *L. divaricata* and *L. lanceolata* in the Barberton and Dullstroom areas, as well as *Pearsonia obovata* collected in the Dullstroom area. For comparative purposes, we also collected nodules from an unrelated caesalpinioid legume in the genus *Chamaecrista* (tribe Cassieae) growing in the Gauteng Province of South Africa.

2.2. Rhizobial isolation and nodulation tests

Rhizobia were isolated within 3–5 days following the collection of nodule-bearing plant tissue. Each nodule was surface sterilized