Certification

The genetic diversity of *Bradyrhizobium japonicum* in soils of the soybean growing regions of western Canada and the northern United States

by

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A Thesis Submitted to Saint Mary's University, Halifax, Nova Scotia,
in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Applied Science

July 24, 2007

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ISBN: 978-0-494-35766-8
Our file Notre référence
ISBN: 978-0-494-35766-8

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Genetic diversity of *Bradyrhizobium japonicum* in the soils of soybean growing regions of western Canada and the northern United States.

By

Faisal Tariq Farooq

Abstract

Introduction of early maturing cultivars of soybean (*Glycine max* [L.] Merr.) in western Canada has dramatically increased soybean production in the region. Soybean grows in symbiotic association with rhizobia which carry out biological nitrogen fixation in the plant. Previous studies have shown that rhizobium populations evolve quickly in the soil. In this study, we have examined the genetic diversity of 107 *Bradyrhizobium japonicum* isolates from the soybean growing areas in western Canada and the northern United States (North Dakota, South Dakota and Minnesota) by rep-PCR genomic fingerprinting techniques – (REP-PCR) and (ERIC-PCR). Results of our study shows that the *B. japonicum* isolates are genetically diverse. Our results also point towards the influence of agricultural practices and geographical origin of isolates on genetic diversity of *B. japonicum* populations. Our results also suggest that sandy soil texture could have negative influence on genetic diversity of *B. japonicum* populations at site Elm Creek in Manitoba.

[July 24th, 2007]

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Introduction

In recent years, growing international concerns regarding the issues of environmental degradation and loss of natural resources have renewed the scientific interest towards biological nitrogen fixation for the development of more sustainable and effective agricultural system. In biological nitrogen fixation (BNF), nitrogen-fixing soil bacteria (rhizobia) induce the formation of root or stem nodules on leguminous plants in which atmospheric nitrogen is reduced to ammonia for the benefit of the plant (Vanrhijn and Vanderleyden, 1995). The symbiotic association between rhizobia and legumes plays a significant role in world agricultural productivity by an estimated annual conversion of approximately 120 million tonnes of atmospheric nitrogen into ammonia (Freiberg *et al.*, 1997).

Rhizobium inoculation is a less expensive and usually more effective agronomic practice for ensuring adequate nitrogen (N) nutrition of legumes, compared with the application of N fertilizers. In western Canada, soybean production has dramatically increased in the last decade with the introduction of low heat requiring cultivars along with climate change in the region (Miller *et al.*, 2002). Soybean is a relatively new crop to the region. According to 2002 estimates, total soybean production in Manitoba was 109,000 tonnes representing of only 4.7% of total soybean production in Canada in the year 2002 (Statistics Canada, 2002). Inoculation of soybean crop in western Canada is extremely important in order to optimize current soybean production in the region.

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In screening and selection for strains that could be potentially developed into commercial inoculants, it is important for the inoculant companies to distinguish strains of rhizobia. Currently used genetic fingerprinting techniques, including rep-PCR genomic fingerprinting, are useful tools for strain identification (Van Belkum *et al.,* 1994; Louws *et al.,* 1996). The most common method of strain selection for legume involves isolation of rhizobial strains from fields where they have been introduced previously, usually as commercial inoculants (Santos *et al.*, 1999). In order to maximize biological nitrogen fixation in legume crops, efficient and infective strains should be sampled from the agricultural fields. Collected strains of rhizobia should be screened for effectiveness and infectiveness under controlled environmental and field conditions before they could be potentially used by inoculant companies for developing them into commercial inoculants.

The ability of inoculant strains to compete with very diverse indigenous rhizobial strains is an important requirement for an agronomically useful rhizobium-soybean association. Currently, soybean inoculants used in western Canada contain soybean rhizobial strains isolated and tested for soybean production in other regions and it is found that the current commercial inoculants of *Bradyrhizobium japonicum* used in western Canada are not as effective and are not ideal for this region (Vessey, 2007 pers. comm.). Strains within these inoculants are either not competitive in the rhizosphere or are not well suited for the edaphic conditions of western Canada. Selection of rhizobia that are well adapted to the environmental conditions of the agricultural region is important to make best use of efficiency of inoculants strains.

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The aim of the present study is to provide an assessment on genetic diversity of *Bradyrhizobium japonicum* natural field populations isolated from the soybean growing areas in western Canada and the northern US. I hypothesize that that due to genetic recombination among commercial inoculant strains and indigenous populations of soil rhizobia including native *Bradyrhizobium japonicum* populations and due to the influence of selective environmental pressure, the indigenous populations of *Bradyrhizobium japonicum* in western Canada are genetically diverse relative to the diversity of soybean rhizobia acknowledged in other parts of the world (Chen *et al.*, 2000; Sikora & Redzepovic, 2003; Prakash &Annapurna, 2006). Influence of important environmental, ecological and edaphic conditions of the region were also considered in this study to correlate the genetic diversity of *B. japonicum* isolates to the site characteristics. The information on previous crop grown in field, year since last soybean grown, and use of inoculant was collected from site. Influence of edaphic factors (soil texture, soil salinity and drainage) and climate factors such as average annual temperature and mean annual precipitation were used as major factors to correlate genetic diversity of *B. japonicum* isolates to a given site location.

The proposed research relies on the assumption that due to the process of natural selection, the previously used commercial inoculants in these fields have resulted in the evolution of better adapted, more competitive and more infective strains among the indigenous populations of *Bradyrhizobium japonicum* present in the soil. Previous studies have shown that rhizobium evolves quickly in the soil usually by horizontal gene transfer among the inoculant strains and indigenous rhizobial populations (Sullivan *et al.*, 1995). Soybean

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cultivars that are well adapted to the edaphic and climatic conditions of this region were used as primary source to isolate rhizobia for this study. The assessment of genetic diversity of rhizobial populations and screening and selection of better, highly effective and well adapted strains are required for a given geographical region for developing superior inoculants and thus, would help to develop better management strategies leading to better yield and crop production in western Canada. This information will also help our industrial partner, *Philom Bios Inc.* in selecting best adapted strains infecting soybean in western Canada and also in US. *Philom Bios Inc.* is a leading inoculant producing company across North America and produces high quality products for the benefits of farmers in Canada and US. It has also been suggested that rep-PCR genomic fingerprinting could be used as tool for protection of intellectual property as fingerprints are unique for each individual strains (Rademaker & deBruijn, 1998). Hence, in order to improve the beneficial effect of soybean inoculation, it is important to determine the characteristics of rhizobial field populations.

In a companion study by Ms. Vanessa Kavanagh, *Bradyrhizobium japonicum* isolates were tested and screened for infectiveness and effectiveness on soybean plants under laboratory and greenhouse conditions to identify better and efficient strains isolated from naturalized populations. The ultimate goal of this research project along with the other companion study is to provide an assessment on the genetic diversity of naturalized population of *Bradyrhizobium japonicum* populations and to identify and test effectiveness and infectiveness of these rhizobial strains on soybean plants that could be potentially developed into commercial strains. This study will offer relevant and important information

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and expertise to our industrial partner that is needed to develop commercial soybean inoculant best suited for the soils of western Canada.

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Literature Review

For study and research on assessment of genetic diversity of rhizobial population, it is important to review several important topics that includes physiological and biochemical process required for nitrogen fixation, taxonomic characteristics and ecology of *Rhizobiaceae* and strategies and tools used in this field by various researcher to study and assess the genetic diversity of rhizobia (MartinezRomero & CaballeroMellado, 1996; Oyaizu *et al.*, 1993). Hence, this literature review will include the overview of legume- rhizobia symbiosis, taxonomy of rhizobia, ecology and genetic diversity of rhizobia and, molecular tools and strategies used to identify bacteria.

2.1. Symbiosis

2.1 (a). Overview of the legume-rhizobia symbiosis

Symbiotic nitrogen fixation is carried out in specialized organs called nodules, whose formation is induced on leguminous host plants by bacteria belonging to the family *Rhizobiaceae* (Hirsch *et al.*, 1992). In symbiotic nitrogen fixation, the host plant is directly benefited by the presence of microsymbiont (bacteria) which fix atmospheric nitrogen that can be used by the host plant (Vanrhijn & Vanderleyden, 1995). In symbiotic nitrogen fixation, formation of nodules depends on the availability of compatible rhizobia (Vanrhijn & Vanderleyden, 1995). Nodule development is a multistep, complex process, in which two partners continuously

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interacts among each other to facilitate the exchange of different signals and metabolites. Successful establishment of rhizobia induces several physiological changes on the plant organs for the nodule development (Vanrhijn & Vanderleyden, 1995). Detailed physiology and biochemistry of nodule development is explained in the next section.

2.2. Physiology and molecular biology of nodule development in soybean plants

Nodule development and successful establishment of soybean rhizobia into the compatible host plant is a complex process that can be divided in to three major steps as follows:

2.2 (a). Recognition and attachment of rhizobia

The symbiotic association between compatible rhizobia and host plant requires the recognition of both partners and subsequent attachment of bacteria to the roots of host plant (Haaker, 1988). Compatible plant-bacteria interaction starts with an exchange of molecular signals which regulates the expression of genes essential for infection and subsequent steps for nodule formation (Prell & Poole, 2006). Plants releases various compounds like isoflavonoids (Abd-Alla, 2001), flavonoids (Shaw *et al.*, 2006), isoflavones (Subramanian *et al.*, 2006) which are the secondary metabolities produces by phenylpropanoid metabolic pathway in higher plants (Prell & Poole, 2006). These compounds are identified by the compatible rhizobia and *nod*

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genes are expressed inside the bacteria (Gage, 2004). Rhizobia also releases Nod factors of low-molecular weight and thus, both plant and rhizobia identify their compatible partner (Abd-Alla, 2001; Giller & Cadisch, 1995). Nod factors are lipochitooligosaccharides (LCOs) that consists of an acylated chitin oligomeric backbone with various functional group substitutions at the terminal or non-terminal residues (Abd-Alla, 2001; Shaw *et al.*, 2006). The exact chemical structure of the Nod factor that is recognised by the plant varies between bacterial species and is the basis for host-symbiont specificity. Nod factors are recognized by a specific class of receptor kinases that have LysM domains in their extracellular domains (Shaw *et al.*, 2006). Once rhizobium recognizes its symbiotic partner, it attaches to the root surface of the host plant. Attachment of rhizobia to the roots of host plants apparently involves two major steps (Shaw *et al.*, 2006; Vanrhijn & Vanderleyden, 1995). First, single rhizobia adhere to plant receptor via a protein called rhicadhesin. Rhicadhesin is a calcium binding protein and is commonly found among the Rhizobiaceae. Subsequently, other rhizobia adhere to the trichoblast-bound bacterial cells (Gage, 2004; deBruijn & Downie, 1991). Lectins of plant origin have been frequently implicated to be involved in the recognition and attachment process, because they have specific binding sites for certain poly- or oligosaccarides found at the surface of bacteria (Gage, 2004). However, some studies have shown that lectins originating from the rhizobia may also be involved in the attachment process (Gage, 2004).

2.2 (b). Root infection

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The recognition and attachment of rhizobia to the roots of host plant is eventually followed by root infection of host plant due to the expression of nod genes in the bacteria that initiates the infection process (Debruijn & Downie, 1991). Rhizobial Nod factors influences the host root growth by causing an increase in the amount of root hairs and a subsequent root hair deformation and curling (Vanrhijn & Vanderleyden, 1995). Root hair curling is facilitated by the changes in the growth direction of the root hair cell at the site of rhizobial attachment. This process eventually led to entrapping of the bacteria following by subsequent ingestion into the root (Vanrhijn & Vanderleyden, 1995; Gage, 2004).

Subsequent to the root hair curling, bacteria penetrate the rhizodermis and the development of root infection thread occurs. Endocytosis is regarded as the initial step of infection thread formation (Hirsch *et al.*, 1992). Current evidence suggests that lipopolysaccrides may also be involved in the development of infection threads (Gage, 2004). An infection thread continues to develop by further penetrating into the cortex of the root. This penetration is facilitated by the continuous degrading of radial cell walls of the root. Eventually, the infection thread spread further into the root cortex (Hirsch, 1992; Hirsch *et al.*, 1992). The structure of infection threads is not identical in all legumes (Hirsch *et al.,* 1992; Gage, 2004). Infection thread consists of newly synthesized cell wall material surrounding the rhizobia (Gage, 2004). The centre of the tube is a glycoprotein containing some bacterial products and some host glycoproteins. The whole structure is surrounded by a membrane contiguous with root hair plasmalemma. The type of thread formation appears to be

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influenced by the surface polysaccharides of rhizobia (Debruijn & Downie, 1991; Shaw *et al.*, 2006).

2.2 (c). Root nodule formation

Simultaneous to the formation of infection threads, cortical cells of the root divides and finally lead to the formation of root nodule. In the nodules, the bacteria which have penetrated the root cortex in the infection thread are eventually released into the host cell (Oke & Long, 1999). Rhizobia differentiate into 'bacteroids' by loosing their cell wall and become substantially enlarged and gets enclosed in the peribacteroid membrane and thus kept separated from the cytoplasm of plant cell (Oke & Long, 1999). Synthesis of enzyme nitrogenase starts and leghaemoglobin is produced in the host cells and nodule acquires pink colour. Leghaemoglobin synthesis may be induced by the production of bacterial transacting factors giving rise to symbiotically induced plant genes involved in the production of leghaemoglobin (Prell & Poole, 2006). The nitrogen fixed by the bacteria is transported directly to the plant. Plant provides bacteria with a carbon supply for their survival and energy required for the nitrogen fixation by the bacteria (Hirsch, 1992; Debruijn & Downie, 1991). Nitrogenase synthesis follows in pea and soybean subsequent to the release of bacteria from the infection thread. Other plants do not release the bacteria but still form nitrogenase (Amarger, 2001; Prell & Poole, 2006).

2.3. Current Rhizobium taxonomy

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Rhizobia are Gram-negative, rod shaped and non- sporulating bacteria (Jordan, 1982). Phylogenetically, they belong to the alpha subdivision of *Proteobacteria* (Jordan, 1984). Rhizobia are diverse and their classification has undergone great changes during recent years (Young, 1996). This is an outcome of ongoing research on unexplored legumes in various parts of the world (Oyaizu *et al.*, 1993; Prakash & Annapurna, 2006; Ormeno-Orrillo *et al.*, 2006; Musiyiwa *et al.*, 2005; MartinezRomero & CaballeroMellado, 1996; Seguin *et al.*, 2001; Prevost & Bromfield, 2003). Recent advances in the molecular techniques led to the significant progress in characterization and identification of bacterial species. So far approximately only 23% of the total number of legume species has been characterized for their microsymbionts (~16500 and 19500). Rhizobial populations from tropical areas are poorly documented and previous and ongoing studies around the globe suggests that there is significantly high diversity in USA (Tlusty *et al.*, 2005); Canada (Vessey & Chemining'wa, 2006; Prevost & Bromfield, 2003), China (Chen *et al.*, 2005); India (Pandey *et al.*, 2004; Prakash & Annapurna, 2006), Brazil (Boddey & Hungria, 1997), Sudan (Nick *et al.*, 1999); Morocco and some other African countries (Musiyiwa *et al.*, 2005).

During early 1980's, all symbiotic nitrogen-fixing bacteria from leguminous plants were classified in the single genus *Rhizobium,* including six species *R. leguminosarum, R. meliloti, R. trifolii, R. phaseoli, R. lupine* and *R. japonicum based on* cross-inoculation groups (Young, 1996). This classification was later changed into two genera that included *Rhizobium* genus and the new genus called

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Bradyrhizobium, created for the slow growing ones (Young, 1996; Young & Haukka, 1996). Type species of this genus are classified as *Bradyrhizobium japonicum* (Kirchner 1896) Jordan 1982, comb. nov. (Jordon, 1982). Ever since, isolation of rhizobia from an increasing number of plant species around the world and use of modern molecular techniques for characterization of rhizobia has led to the description of additional new genera and species (Oyaizu *et al.*, 1993).Rhizobial species are currently classified in the following genera: *Allorhizobium* (emended genus *Rhizobium*), *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium* (Kirchner 1896) and *Methylobacterium* (Young, 1996; Young *et al.*, 2001). In the following section, information about the *Bradyrhizobium* genus is provided in detail.

2.3 (a). *Bradyrhizobium* branch

As described by Jordan (1982), the *Bradyrhizobium* genus includes all the so-called "slow growing" rhizobia. For a long period of time, it was only comprised of one type species, *Bradyrhizobium japonicum* (Kirchner 1896) Jordan 1982, comb. nov. (Jordon, 1982) and included all soybean nodulating strains. Kuykendall *et al.* (1992) created a new species, *Bradyrhizobium elkanii* which differ from the species *B. japonicum* by many features. Other very slow growing strains isolated from the nodules of *Glycine max* and *Glycine soya* in China (generation time between 16 and 24 hours), were given the proposed name *Bradyrhizobium liaoningense* (Chen *et al.*, 2005; Xu *et al.*, 1995). Groups of *Bradyrhizobium* sp. have been identified, and characterized by various phenotypic and genotypic methods (Lloret & Martinez-

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Romero, 2005; Young, 1996;Young *et al.*, 2001) The modern polyphasic taxomomy revealed by analysis of 16S rRNA gene coding sequence that *Bradyrhizobium* genus is phylogenetically closer to non-symbiotic bacteria, like *Rhodopseudomonas palustris* and *Blastobacter denitrificans*, than to *Rhizobium* and *Agrobacterium* (Barrera *et al.*, 1997; Lloret & Martinez-Romero, 2005; Yanagi & Yamasato, 1993) . Thus, the taxonomy of the *Bradyrhizobium* genus remains confused and taxonomic conclusions suggests that the taxonomy of the *Bradyrhizobium* genus is still at a beginning stage and increased research could result in further changes in taxonomy of rhizobia.

2.4. Rhizobial Ecology

Previous studies have shown that several abiotic and biotic factors could have direct influence on legume-rhizobial symbioses and could have an effect on soil rhizobial populations (Zahran, 2001). Several abiotic factors like osmotic stress, soil temperature, soil pH, soil texture, carbon and nitrogen content of soil have been suggested to influence genetic diversity of rhizobia (Zahran, 1999). Biotic factors like competition among rhizobial populations and competition with other soil microorganism, previous crop grown in the fields etc. could also have a direct influence on rhizobial population. Details of abiotic and biotic factors that usually influence rhizobial populations are explained in the following section:

2.4 (a). Abiotic factors

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Several abiotic factors are known to affect the growth and survival of rhizobial population. Soil pH, soil temperature, osmotic stress, soil texture, soil nitrate concentration and, presence or absence of carbon source are some of the important abiotic factors that are known to influence maintenance and survival of rhizobia in the soil (Brockwell *et al.*, 1995).

2.4 (i). Soil pH

Soil pH significantly influences the rhizobial population in the soil. Both high and lower pH affects nodulation by causing reduction in the colonization of soil and legume rhizosphere. Acidic conditions negatively affect soil populations of rhizobia and are also known to affect the soil nutrient distribution (Graham, 1992). Studies have shown that there are usually higher concentrations of aluminum and manganese in highly acidic soils which are generally toxic for both the legume and rhizobia (Bordeleau & Prevost, 1994). Thus, soil acidity could have negative effect on the survival and effectiveness of inoculant strains (Zahran, 1999). The majority of rhizobial strains cannot grow at pH lower than 4.5 (Zahran, 1999). In general, the most acidic conditions in which *B. japonicum* could survive is pH 4.0 (Wood & Cooper, 1988). *Rhizobium leguminosarum* biovars *trifolii* and *viciae* usually cannot survive below pH 4.7 and *S. meliloti* cannot survive below pH 5.0.(Wood & Cooper, 1988).The fast-growing strains of rhizobia have generally been considered less tolerant to acid pH than have slowly growing strains of *Bradyrhizobium* (Graham, 1992; Graham & Vance, 2000). Higher pH (pH>8.0) leads to increase in soil salinity and thus reduces nitrogen fixation (Bordeleau & Prevost, 1994).

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2.4 (ii). Temperature

Temperature is often a limiting factor in successful legume-rhizobium symbiosis. Several years of studies have shown that temperature affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule (Zahran, 2001). Rhizobia cannot survive under extremely high soil temperatures and high soil temperatures have been suggested to delay nodulation (Graham, 1992). Most rhizobia grow best in the range of 28°C to 31°C. However; 28°C is indeed the optimum temperature for culturing rhizobia (Graham, 1992). Many rhizobia are unable to grow at 37°C. However; *S. meliloti* grows well at 35°C (Zahran, 1999). Rhizobia from sub-artic regions are adapted to their environment and could grow and nodulate at comparatively lower temperatures than rhizobia from temperate regions (Ekjander & Fahraeus, 1971). Strains of *R. leguminosarum* bv. *viciae* isolated from artic legume *Lathyrus* species were found to be adapted to grow at low temperature (5°C) (Drouin *et al.*, 1996). In short season production areas, low soil temperature is suggested to be a major limiting factor affecting soybean growth in the field (Zhang *et al.*, 2003). Temperature could also affect ability of inoculant strains to nodulate and fix nitrogen and the persistence of the inoculant strains survival in the soil (Bordeleau & Prevost, 1994). It has been shown that ability of rhizobial strains to withstand high temperatures appears to be favored by dry rather than wet soil conditions (Graham, 1992).

2.4 (iii). Other abiotic factors that affects rhizobial diversity

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Abiotic factors like osmotic stress, soil texture, soil nitrate conditions and, presence or absence of carbon sources in the soil affects rhizobial growth and population. Growth of rhizobia is inhibited under extreme moisture conditions. Under high osmotic stress due to drought or salinity, there is reduction in infection and nodulation of legumes (Bussè & Bottomley, 1989). Also, the nitrogen-fixing activity in nodules is found to be significantly reduced (Streeter, 2003). Studies by Hunt *et al.* (1981) shows that low water content in soil could affect the success of soybean inoculation in soils with high indigenous population of *B. japonicum* (previously known as *R. japonicum*).

Previous studies have also shown that rhizobia have better survival in fine textured soils than in coarse textured soils because coarse textured soils are usually prone to nutrient deficiencies, water deficits, and acidification (Graham, 1992). Heijnen & Vanveen (1991) suggested that the chance of rhizobial survival is greater in soils with higher clay content as compared to the soils with lower clay content due to the presence of stable soil aggregates which help in preventing desiccation and nutrient loss.

Studies have also shown that the process of nodulation may be encouraged by relatively low levels of available nitrate or ammonia (Lucinski *et al.*, 2002; Supanjani *et al.*, 2006). A higher concentration of inorganic nitrogen in the soil leads to lower nodulation on legumes (Eaglesham, 1989). It has been established for many

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years that soil NO_3^- inhibits root infection, nodule development (Imsande, 1986), and nitrogenase activity (Purcell & Sinclair, 1990; Vessey & Waterer, 1992).

Rhizobial biomass is usually higher in more fertile soils and increases in proportion to soil organic carbon (Jenkinson & Ladd, 1981). Bradyrhizobia and other rhizobia can survive on a wide range of carbon sources that includes sugars (monosaccharide, disaccharides, trisaccharides, hexoses and pentoses); sugar alcohols (glycerol, mannitol, dulcitol); and aromatic compounds rising from root exudation and decomposition of plant residues (Stowers, 1985). *Bradyrhizobium* unlike other rhizobial species cannot utilize citrate (Stowers, 1985). Within the host root nodule, succinate is the most utilized carbon and energy source for the rhizobia which supports nitrogen fixation by bacteroids (Finan *et al.*, 1983).

2.4 (b) Biotic factors

Competition of rhizobial strains with other soil microorganisms and with indigenous population of rhizobia becomes particularly important for the successful establishment of legume-rhizobium symbiosis. Predation by protozoa and bacteriocins produced by other bacteria could reduce rhizobial population in the soil (Heijnen & Vanveen, 1991).

Competition of introduced strains or inoculant strains with the indigenous population of rhizobia is also an important factor determining the successful establishment of introduced strains in soil and for inoculation of the host plant. The

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presence of large populations of indigenous, well adapted and less efficient strains have infection advantages over inoculant strains due to their high number in the soil (Brockwell & Bottomley, 1995; Chemining'wa & Vessey, 2006).

Numerous evidences are found in the literature which suggests influence of different crops on rhizobial populations (Paffeti 1996; 1998; Triplett *et al.*, 1993). *Bradyrhizobium japonicum* populations are found to increase in the presence of soybean crops (Weaver *et al.*, 1972). Plant cultivar is also suggested to influence rhizobial diversity (Zhang *et al.,* 1999). Kucey & Hynes (1989) found that there was a 10 fold increase in *R. leguminosarum* bv. *viciae* in pea fields than in bean or cereal fields.

2.5. Genetic Diversity

During the last few years, the assessment of diversity within rhizobial natural populations in various regions of the world has received increased attention (Ando & Yokoyama, 1999; Boddey & Hungria, 1997; Judd *et al.*, 1993; Madrzak *et al.*, 1995; Prevost & Bromfield, 2003). Studies have shown that rhizobium population evolves quickly in the soil and many indigenous competitive strains have been identified by the researchers in many parts of the world (Hungria *et al.*, 1998; Hynes & Oconnell, 1990; Ormeno-Orrillo *et al.*, 2006; Sikora & Redzepovic, 2003). The possible prediction of this quick evolution of new strains is the lateral transfer of genes among the long-term resident population of rhizobia present in the soil

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(Sullivan *et al.*, 1995). The other source could be the commercial inoculant (s) used previously in the fields that could have resulted in the evolution of more competitive strains among the rhizobial population present in the soil (Amarger, 2001; Santos *et al.*, 1999). Thus, it becomes important to assess the diversity of indigenous rhizobial natural populations as it offers great advantage and opportunities for the selection of better, well adapted, and effective strains that could be potentially used for the development of superior commercial inoculant(s).

It has been suggested that the edaphic and climatic factor play an important role in influencing the genetic diversity of rhizobia (Amarger, 2001). Factors like temperature (Zahran, 1999; Zhang et al., 2003); moisture (Bordeleau & Prevost, 1994; Zahran, 1999); soil type (Garbeva *et al.*, 2004a; Garbeva *et al.*, 2004b) and, soil pH (Bordeleau and Prevost, 1994; Graham, 1992) have been suggested to play an important role in determining and influencing the microbial population in the soil. Other factors like plant genotype, plant cultivar and crop rotation has been suggested to play a significantly affects the rhizobial populations in the soil (Weaver *et al.*, 1972; Zhang *et al.*, 1999).

In a study on rhizobial symboints isolated from different legume species, Laguerre *et al.* (1997) found that genetic diversity of rhizobial isolates was independent of geographic origin and host plant affinity. Zhang *et al.* (1999) found a strong influence of plant cultivar on the genetic diversity of the bradyrhizobial strains isolated from the root nodules of two peanut cultivars from four different sites of in Sichuan, China. They also showed that there was a possibility of grouping of

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Bradyrhizobium isolates according to their geographical origin. Urtz & Elkan (1996) examined the symbiotic gene diversity in 33 *Bradyrhizobium japonicum* isolates using RFLP analysis of *nif* genes and *nod* genes. Most isolates were found to very stable for these markers and showed low genetic variability. In a study on *Sinorhizobium meliloti* population genetic diversity isolated from root nodules of several *Medicago sativa* varieties, Paffetti *et al.* (1996; 1998) found that significant genetic difference among strains were revealed by RAPD profiles suggesting that plant genotype is an important and influential factor in determining the genetic diversity of *S. meliloti* population in the field. Chiarini *et al.* (1998) did not find any significant influence of different maize cultivars on microbial population on bacterial community structures.

Genetic structure of *Rhizobium leguminosarum* bv. *phaseoli* populations associated with wild and cultivated beans were studied by Souza *et al.* (1992) over several spatial scales ranging from individual host plants to throughout the western hemisphere. They suggested that limited migration between *Rhizobium leguminosarum* bv. *phaseoli* populations contribute to substantial genetic variability.

2.6. Inoculation

Inoculation of legume plants with rhizobium inoculants has been practiced for centuries (Brockwell & Bottomley, 1 995; Peoples *et al.*, 1995). Inoculants are commercially available in powder, liquid and granular formulations. Peat-based

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inoculants are the most common form of inoculants and are applied to the seed surface prior to planting (Brockwell & Bottomley, 1995; Thrall *et al.*, 2005). Inoculants are generally applied directly to the seed surface to ensure greater chances of symbiotic association of commercial rhizobial strain to the crop plant (Brockwell & Bottomley, 1995). Studies have shown that commercial inoculants work best in the fields where they were being applied for the first time or where the field has no cropping history of compatible host crop.

Fields having no history of legume cultivation could still contain significant populations of compatible rhizobia due to invasion of rhizobia from different location or sites (Beringer & Bale, 1988). Dispersal via seeds, airborne dust arising from harvesting and human activities are some possible causes and source for the introduction of rhizobia into area where there is no history of cultivations of legumes (Beringer & Bale, 1988).

2.6 (a). Strategies for identifying competitive strains of rhizobia

In order to achieve maximum benefit from inoculation of legume crop, highly infective and effective strains of rhizobia must be sampled from the soils for developing them into commercial inoculants. It is also important for the inoculant companies to distinguish strains of rhizobia. Currently, genetic fingerprinting techniques, including rep-PCR genomic fingerprinting are effective tools for strain identification. The most common method of strain selection for legume involves re-isolation of rhizobial strains from fields where they have been introduced previously, usually as commercial inoculants (Santos *et al.*, 1999). Screening of collected strains of rhizobia for effectiveness and infectiveness

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under controlled environmental and field conditions is also important before they could be potentially developed into commercial inoculants by inoculant companies.

2.6. (b). An ideal inoculant:

From a commercial prospective, in order to acquire maximum benefits from inoculation, an ideal inoculant must have maximum efficiency in the field. The inoculant strains should stay stable and should remain persistent and effective under the edaphic conditions in a given agricultural settings (Catroux *et al.,* 2001, Smith, 1992). Compatibility of commercial inoculant strains with agricultural practices and with use of pesticides and other chemicals used by the farmers is also important (Brockwell & Bottomley, 1995, Smith, 1992). The inoculant must reach the standard of an appropriate inoculation rate and therefore it must contain the standard number of viable rhizobial cells of the appropriate species or strain per unit weight of inoculant. rhizobia (Smith , 1992). Longer shelf-life of inoculants is one of the most essential requirements for the inoculant companies for the success of their products (Catroux *et al.,* 2001). Most of the inoculants produced by the inoculant companies have an average shelf-life of 1-1.5 year. Inoculants must be free of contamination as it could have detrimental effects on the shelf-life of rhizobia (Date & Rougghley, 1997). Oleson et al. (1996) have shown in their study that in a large percentage of inoculants made with non-sterile carrier had more than twice as many contaminants as rhizobia and could also carry harmful microorganisms including human, animal, plant or rhizobial pathogens. Most of the inoculant companies in Europe, USA and Canada produce contamination free products with shelf-life longer than one year (Catroux *et al.,* 2001). In Canada, rhizobial inoculants are regulated and evaluated in

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a formal testing program by Agriculture Canada and Canadian Food inspection Agency before being approved for commercial use by the industry (Olsen *et al.*, 1994). However, it has been suggested that in order to optimize agronomic benefits from inoculation, high quality standards for the manufacture of inoculants should be maintained (Olsen *et al.*, 1994). An important future requirement for companies to increase their market is to increase shelf-life of their product, provide highest number of viable strains per seed and most importantly by raising high quality standards for the formulation of inoculant products that would beneficial for farmers and overall agricultural productivity of legumes (Catroux *et al.,* 2001).

2.7. Genomic Fingerprinting

Discovery of Polymerase Chain Reaction (PCR) technology in the year 1980 has revolutionized the whole concept of research in molecular biology. Various modification of PCR techniques have been designed in recent years according to research needs (Grundmann *et al.*, 1995). Rate of microbial evolution in the soil is very high and thus, identification of bacterial species is a great challenge. PCR-based genomic fingerprinting techniques namely PCR-RFLP, RAPD, and rep-PCR genomic fingerprinting techniques have made identification and classification of bacteria much easier (deBruijn, 1992; Judd *et al.*, 1993; Schneider & deBruijn, 1996). The utility of any genomic fingerprinting method depends on high differentiating power, low cost and reproducibility (Barry *et al.*, 1991, Bingen *et al.*, 1993; Grundmann *et al.*, 1995). The technique employed must be able to clearly differentiate unrelated strains, should be affordable, easy to perform and

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be reproducible (Grundmann *et al.*, 1995). Most of the currently used molecular techniques for typing rely on electrophoretic separation of DNA fragments of different molecular lengths. The electrophoretic result is represented by a pattern of bands on a gel. Since these patterns may be extremely complex, the ease with which the patterns are interpreted and related is an important factor in evaluating the utility of a particular typing method (Appuhamy *et al.*, 1997; Grundmann *et al.*, 1995).

Rep-PCR genomic fingerprinting is very efficient and robust technique and is very useful for determining phylogenetic relationships among microbial isolates and for assigning strains into specific groups and to identify the prokaryotic organism at species, sub-species and strain level (Laguerre *et al.*, 1994; Opgenorth *et al.*, 1996; deBruijn, 1992). Information about these genomic fingerprinting techniques is provided in the following section.

2.7 (a). Restriction Fragment Length Polymorphisms (RFLPs)

Restriction Fragment Length Polymorphism is widely used by molecular biologist for bacterial identification purposes. (Appuhamy *et al.*, 1997; Cockerill *et al.*, 1995). PCR-based RFLPs (PCR-RFLP) is an important research tool used for identification of rhizobial isolates (Haukka *et al.*, 1996; Laguerre *et al.*, 1994). 16S rRNA sequences are highly conserved among proteobacteria. However, the intergenic spacer (IGS) sequences between16S rRNA and 23S rRNA are highly variable and can be used for identification purposes and has been applied successfully in several studies to discriminate strains within various rhizobial species including *R. leguminosarum bv. viciae* (Vessey & Chemining'wa, 2006), *B. japonicum* (Vinuesa *et al.*, 1998, Wolde-Meskel *et al.*, 2005),

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and *S. meliloti* (Zribi *et al.*, 2004). This method is commonly used to differentiate between species of root nodule bacteria (Gault *et al.*, 1994; Hung *et al.*, 2005; Laguerre *et al.*, 1994). The RFLP analysis of amplified symbiotic genes (*nod* and *nif* genes) regions has been demonstrated to effectively differentiate *Rhizobium* isolates at species and sub-species level (Laguerre *et al.*, 1994; 1996; Louvrier *et al.*, 1996)

2.7 (b). RAPD

The random amplified polymorphic DNA (RAPD) assay are based on the use of short random sequence primers, 9 to 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the genome of the organism (Caetano-Anolles *et al.*, 1991). In comparison to other typing techniques, studies have shown that the RAPD assay is more discriminating than RFLP analysis. The major problem associated with RAPD assays is lack of reproducibility and standardization (Caetano-Anolles *et al.*, 1991; Lehmann *et al.*, 1992). Since the primers are not directed against any particular genetic locus, many of the priming events are the result of imperfect hybridization between the primer and the target site. Thus, the amplification process is extremely sensitive to slight changes in the annealing temperature which can lead to variability in the banding patterns (Caetano-Anolles *et al.*, 1991; Lehmann *et al.*, 1992).

The RAPD technique has been successfully employed for differentiating and characterization of strains of *B. japonicum* (Vanrossum *et al.*, 1995; Sikora *et al.*,

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1997), *R. fredii* (Young, 1998), *R. leguminosarum* bv. *viciae* (Ballard *et al.*, 2004), *S. meliloti* (Bradic *et al.*, 2003) and other rhizobia (Pinto *et al.*, 2004).

2.7 (c). Rep-PCR

Rep-PCR genomic fingerprinting makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, including the 35-40 bp repetitive extragenic palindromic (REP) sequence and the 124-127 bp entrobacterial repetitive intergenic consensus (ERIC) sequence present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (deBruijn, 1992; Lupski & Weinstock, 1992). These sequences appear to be located in distinct, intergenic positions around the genome. Pairs of specifically designed primers from these sequences and PCR can be used for selective amplification of distinct genomic regions located between REP or ERIC elements. The amplified fragments can be resolved on a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint (deBruijn, 1992; Laguerre *et al.*, 1994).

Rep-PCR genomic fingerprinting is a very efficient and robust technique. This technique is very useful in determining phylogenetic relationships between microbial isolates and for assigning strains into specific groups. Rep-PCR genomic fingerprinting techniques can identify the prokaryotic organism at species, sub-species and strain level (deBruijn, 1992; Laguerre *et al.*, 1996; Vinuesa, 1998). The utility of rep-PCR fingerprinting in characterization of rhizobial population has been well recognized (Santamaria *et al.*, 1997; Vanrossum *et al.*, 1995). Recently,

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genomic fingerprinting of soil bacteria in mainland of Brazil suggested greater diversity among the *B. japonicum* isolates (Boddey, 1997; Santos *et al.*, 1999). In agriculture, rep-PCR techniques are used to identify and characterize bacterial population in soil because of their use in biological nitrogen fixation. Various strains of symbiotic bacteria like *Rhizobium* sp. and *Bradyrhizobium* sp. are used as commercial inoculants for various crop plants by the farmers to optimize the overall crop yield. Plant biologists all over the world are trying to identify more efficient and competitive strains of symbiotic bacteria to decrease the cost and use of chemical fertilizers and to improve the overall crop production in the world (Brockwell & Bottomley, 1995; Zhang *et al.*, 2003; Vinuesa *et al.*, 2003). Rep-PCR genomic fingerprinting protocols have been successfully applied in medical, agricultural, industrial and environmental studies of microbial diversity (Judd *et al.*, 1993; Schneider & deBruijn, 1996; Versalovic *et al.* 1991; Vinuesa *et al.,* 2003). In addition to studying diversity, rep-PCR genomic fingerprinting has become a valuable tool for the identification and classification of bacteria. Rep-PCR genomic fingerprinting techniques are also used in molecular epidemiological studies of human and plant pathogens. These techniques have also been used for the characterization and identification of rhizobial species (Bradic *et al.*, 2003; Judd *et al.*, 1993; Schneider & deBruijn, 1996; Sikora & Redzepovic, 2003; Zhang *et al.*, 2003).

2.8. Summary

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Legume-rhizobium symbiosis is important for the maintenance of sustainable agriculture systems. Studies have shown that rhizobial populations evolve quickly in the soil and that can limit the success of the inoculant strains in the field. Many indigenous competitive strains have been identified by the researchers in many parts of world (Hungria *et al.*, 1998; Hynes & O'connell, 1990; Ormeno-Orrillo *et al.*, 2006; Sikora & Redzepovic, 2003). It has been acknowledged that ability of inoculant strains to compete with very diverse indigenous rhizobial strains is an important requirement for agronomically useful rhizobium-soybean associations (Ballard *et al.*, 2004; Brockwell & Bottomley, 1995; Moawad *et al.*, 2004; Vessey & Chemining'wa, 2006). A review of the topic of genetic diversity suggests that a better understanding of legume-rhizobia symbiosis and development and use of advanced techniques and molecular tools are required for characterizing rhizobial field population. The high rate of evolution of rhizobia in the soil and competition of indigenous strains with the inoculants is a major problem for the success establishment of inoculant strain in the soil and rhizosphere (Thies *et al.*, 1991; Triplett & Sadowsky, 1992). Assessment of the genetic diversity of the local indigenous populations of rhizobia and selective screening of competitive and efficient strains on crop plants is an effective process for the selection of better, well adapted and competitive strains that could be potentially used for production of superior inoculant(s). Therefore, in order to improve the beneficial effect of soybean inoculation, it is important to determine the characteristics of rhizobial field population that would help in improving legume-rhizobia symbiosis and thus, would help in overall improvement of legume crop production and yield on global scale.

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Materials and Methods

3.1. Strategies for sample collection

For this study, samples were collected from 29 sites in southern Manitoba and from North Dakota, South Dakota, and Minnesota in the US during the 2004 and 2005 growing seasons (Figure 1). Samples were randomly collected from all sites. Samples were collected from sites by using following strategies:

a) From soybean plants within soybean fields which had not been inoculated in the current year;

b) By the establishment of "trap plots" (1 m^2 microplots of uninoculated soybean) in the fields where inoculated soybean crop had previously been grown, but was not growing in the current year; and

c) From 'volunteer' soybean plants growing in the fields of other crops (e.g. corn.). A 'volunteer' is a plant that grows from mature seed shed by the crop in the previous year(s).

Selection of sites and collection of nodules were done by the members of *Philom Bios Inc.* and information about the previous crop grown, year since last soybean grown, inoculant(s) used and genotype of the soybean crop were recorded where the information was accessible. The edaphic factors like soil texture, drainage, and salinity and climatic factors like mean monthly and annual precipitation and average monthly and annual temperature for both US and Canadian sites were also considered for the analysis of genetic diversity. Table 1 contains information on the sites used for sampling in this study and, total number of sample collected from each site across Canada and the US.

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Figure 2. Site locations from where isolates were collected. GPS locations of each site were used to create map using ArcGIS software.

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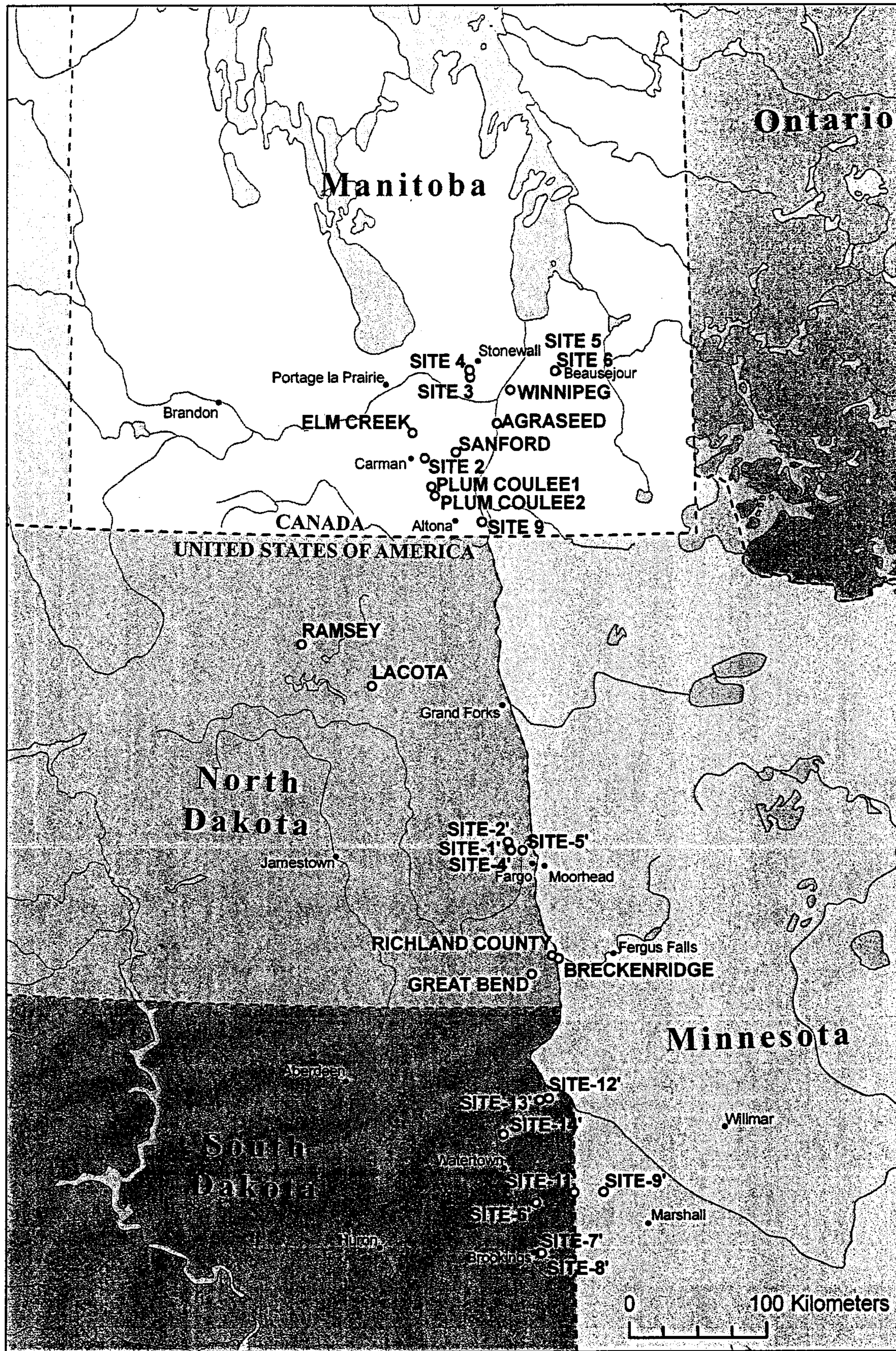

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Table 1. GPS locations and name of each site in Canada and US.

Site name	GPS	Geographical location	Country
Site 2	N49^{0}30' 39.4" / W97^{0}51' 54.7"	Manitoba	Canada
Site 3	N50^{0}2'5"/ W97^{0}24' 18.1"	Manitoba	Canada
Site 4	N50^{0}4'46.6"/ W97^{0}24' 49.7"	Manitoba	Canada
Site 5	N50^{0}10'48.8"/ W96^{0}38' 4.8"	Manitoba	Canada
Site 6	N50^{0}4'8.8"/ W96^{0}32' 38.2"	Manitoba	Canada
Site 9	N49^{0}5' 48.7" / W97^{0}17' 20.3"	Manitoba	Canada
Plum Coulee, #1	N49^{0}19' 32.4" / W97^{0}47' 52.8"	Manitoba	Canada
Plum Coulee, #2	N49^{0}15' 58.6" / W97^{0}45' 47.1"	Manitoba	Canada
Elm Creek	N 45° 09.774/ W 093° 24.682	Manitoba	Canada
Winnipeg	N49° 57.036' / W097° 00.068'	Manitoba	Canada
Sanford	N49° 32.998' / W097° 33.000'	Manitoba	Canada
Agra seeds	N49^{0}44'14"/ W97^{0}8' 11.8"	Manitoba	Canada
Breckenridge	N46^{0}15.257/ W96°34.421	Minnesota	US
Great Bend	N46^{0}09.219/ W96°49.794	North Dakota	US
Richland County	N46^{0}16.364/ W96°38.183	North Dakota	US
Ramsey	N48^{0}17.781/ W99°03.849	North Dakota	US

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Site name	GPS	Geographical location	Country
Lacota	$N48^{0}02$/ $W98^{o}22$	North Dakota	US
Site-1'	N46° 58.638' / W097° 01.826'	Fargo, North Dakota	US
Site-2'	N47° 01.0931' / W097° 02.952'	Fargo, North Dakota	US
Site-4'	N46° 57.848' / W097° 01.189'	Fargo, North Dakota	US
Site-5'	N46° 57.821' / W096° 54.419'	Fargo, North Dakota	US
Site-6'	N44° 39.261' / W096° 48.283'	Brandt, South Dakota	US
Site-7'	N44° 19.407' / W096° 46.051'	SDSU, South Dakota	US
Site-8'	N44° 19.293' / W096° 45.625'	Brookings, South Dakota	US
Site-9'	N44° 43.169' / W096° 11.251'	Yellow Medicine, Minnesota	US
Site-11'	N44° 43.132' / W096° 27.210'	Gary, South Dakota	US
Site-12'	N45° 21.095' / W096° 08.461'	Corona, South Dakota	US
Site-13'	N45° 20.174' / W096° 09.288'	Corona, South Dakota	US
Site-14'	N45° 06.846' / W097° 06.211'	Pioneer field trial South Dakota	US

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3.2. Nodule collection and rhizobial isolation

For nodule collection and rhizobial isolation, the soybean plant was used as a selection tool for the initial isolation of infective strains from the naturalized population of rhizobia in the field soil (Chemining'wa, 2002; Beattie *et al.*, 1998). Three cultivars of soybean (Gentlemen, Prudence and Apollo) were used for nodule collection. The nodules were collected from the soybean plants during early flowering stage (nodules are well developed by this stage of plant development). Healthy, robust nodules were removed from the plant roots in the field and stored in tubes in 20% glycerol and sent to lab for further analysis (Beattie *et al.*, 1998). In the lab, rhizobial lines were isolated from the nodules following the procedures of Chemining'wa (2002) as modified from Rice and Olsen (1993) by Ms. Vanessa Kavanagh. Bacterial extracts from crushed nodules were grown on yeast extract mannitol agar (YEMA) with the addition of Congo red dye and incubated at 28°C (Hahn, 1966).

In the companion study by Ms. Vanessa Kavanagh, *Bradyrhizobium japonicum* isolates were tested and screened for infectiveness and effectiveness on soybean plants under laboratory and greenhouse conditions to identify better and efficient strains isolated from naturalized populations.

3.3. rep-PCR Genomic fingerprinting

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Rep-PCR genomic fingerprinting of *B. japonicum* isolates requires several important steps that includes culturing of *B. japonicum,* DNA extraction, genomic fingerprinting using REP and ERIC primers, and analysis of band pattern and cluster analysis using fingerprinting analysis software, Gelcompar-II ® (Applied Maths, Austin, US).

3.3 (a) Culturing of *Bradyrhizobium japonicum* isolates

Bradyrhizobium japonicum isolates were grown in liquid Yeast Mannitol Broth (YMB) medium at 28°C on orbital shaker according to the protocol of Schneider & de Bruijn (1996).The *Bradyrhizobium japonicum* is slow growing bacteria and took approximately 10-12 days to reach an O.D value of 0.6. The cells were harvested from the cultures when the optical density was in the range for 0.6-0.9 O.D. value for successful DNA extraction during the later steps.

3.3 (b). DNA Isolation

Total genomic DNA was isolated from these strains for genomic fingerprinting according to the modified version of rep-PCR protocol of Schneider & de Bruijn (1996). Cultures of *B. japonicum* produce a lot of polysaccharides that can interfere with extraction process. Washing the cell pellet with an alkaline solution like NaCl helps in removing polysaccharides and improves the quality of PCR. The stepwise modified protocol used for DNA extraction was follows:

i) Cells were collected from liquid culture by centrifugation at 14,000 rpm for 15 min.

ii) Cell pellet was washed with 1 M NaCl at 14,000 rpm for 10min. This step was repeated thrice.

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iii) Cells were resuspended in 100 µl of TE buffer by vortexing.

iv). 3µl of lysozyme (10 g/ml TE) (final concentration of 200 µg/ml) was added to the solution and incubated for 60 minutes at 37°C until solution becomes clear (may add more lysozyme if needed).

v). 500 µl of GES was added to the solution and agitated gently. Solution was incubated at room temperature for 20 min. Solution was than incubated on ice for 5 min.

vi). 300 µl of 7.5 M ammonium acetate (pre-cooled) was added and solution was agitated gently. Solution was than incubated on ice for 30 min.

vii). 900 µl of Phenol/ Chloroform/ Iso-amyl-alcohol (25:24:1) was added and solution was vortexed and, centrifuged for 10 min at 14000 rpm at 4°C.

viii). Supernatant was transferred into new 2-ml microcentrifuge tube. Equal volume of chloroform/iso-amyl-alcohol (24:1) was added and solution was vortexed, and centrifuged for 10 min at 14000 rpm at 4°C.

ix). Supernatant was transferred into a new 1.5-ml micro-centrifuge tube, equal volume of isopropanol (pre-cooled at -20^{0}C) was added. Tube was inverted several times until DNA pellet became visible. Samples were kept at -80^{0}C for 30 min, melted at RT, and centrifuged for 20 min at 14000 rpm at 4°C.

x). Cell pellet was washed with 150 µl 70 % ethanol than centrifuged briefly. Ethanol was removed by using a micropipette. This step was repeated once.

xi). DNA pellet was air dried and re-dissolved in 100 µl TE, pH 8.

xii). 25 µl of RNase H (250 µg/ml) was added to the tube and mixed gently and incubated for 1 hour at 37°C and stored the DNA at -20^{0}C.

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xiii). Take 10 μl DNA solution, dilute into 500 μl. Determine the DNA concentration using a spectrophotometer at 260 nm/280 nm (1 OD_{260} = 50 g/ml) and adjust it to 50 ng/μl.

3.3 (c). PCR amplification

Oligonucleotide primers designed for REP and ERIC sequences were used for genomic fingerprinting. Primers were synthesized at National Research Centre-Plant Biotechnology Institute, Saskatoon, Canada. Sequences of these primers are as follows:

Primer	**Sequence**	**Reference**
ERIC 1R	5'-ATGTAAGCTCCTGGGGATTCAC-3'	Versalovic *et al.* 1991
ERIC 2	5'-AAGTAAGTGACTGGGGTGAGCG-3'	Versalovic *et al.* 1991
REP 1R	5'-IIIICGICGICATCIGGC-3'	Versalovic *et al.* 1991
REP 2I	5'-ICGICTTATCIGGCCTAC-3'	Versalovic *et al.* 1991

REP and ERIC fingerprinting was performed with primers REP 1R and REP 2I, and ERIC 1R and ERIC 2, respectively using the rep-PCR protocol of Schneider & de Bruijn (1996). The cycling programs for REP-PCR and ERIC-PCR fingerprinting differed only in the annealing temperature and time. PCR reactions were carried out in a 25μl PCR tube containing 5μl of 5X Gitschier Buffer, 0.2 μl of BSA (20 mg/ml), 2.5 μl DMSO (100%), , 1μl of each primer (0.3 μg/μl), 1.25 μl dNTPs mix (25 mM of each nucleotide mixed in the proportion of 1:1:1:1), 0.4 μl of 5U *Taq* DNA Polymerase (GoTaq® Flexi

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DNA Polymerase, Promega, Madison,WI) and 12.65 µl double distilled water (Schneider & de Bruijn, 1996). DNA amplification was performed in a thermal cyler (My Cycler ™ BioRad, BioRad Laboratories, Richmond, CA.). The reaction mixtures were incubated for 7 min at 95°C for initial denaturation, and then amplified for 35 cycles consisting of 1 min at 94°C, 1 min at 40°C, 8 min at 65°C for REP-PCR, and 1 min at 94°C, 1 min at 52°C and 8 min at 65°C for ERIC-PCR. PCR reaction was ended with an extension at 65°C for 8 min., and stored at 4°C.

3.3 (d). Gel electrophoresis

The amplification products from the PCR were separated by gel electrophoresis on 1.5 % agarose, 20 cm long gels. The run-time for all gels was 9.5 hours at 70 V, 25-30 mA at 4°C. Constant voltage was kept through out the run-time to avoid distortion in the gel lanes. Gels were usually run at 4°C to avoid distortion of gel lanes due to heat generated during electrophoresis. A 1kb molecular weight marker ladders were used in the first and last lane to estimate size of bands. Commercial inoculant strains 532C and USDA 110, commonly used in Canada and US were used as reference strains to compare isolate profile. 532C and USDA 110 Gel was stained in ethidium bromide solution 60 ng/ml in 0.5 x TAE (60 µl of a 10 mg/ml stock solution in 1 liter 0.5 x TAE), and destain for 30 min. in 0.5 x TAE. Gel was visualized under UV light using Chemi Genius Bioimaging System (Syngene, Frederick, US) and the gel image was saved as TIFF image.

3.3 (e) Data analysis: Analysis of band pattern and cluster analysis of rep-PCR fingerprints was performed using Gelcompar-II ® (Applied Maths, Austin, US). Gelcompar-II ® is specialized software for the analysis of genetic fingerprinting data.

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Individual gel images were loaded into the program and normalized with resolution and background subtraction depending on the background intensity of individual gels, as determined by the software. It is useful to use the same number of lanes and gelstrip thickness for analysis of different gels. A reference system can be set in the program using molecular marker. In this study we have used the 1 kb DNA ladder as molecular marker and bands ranging within the 13,000bp to 250 bp regions were analyzed for size and presence and absence of bands. All gels were normalized for the bands position in each gel relative to the specific bands in 1 kb reference ladder before scoring gel for presence and absence of bands to correct for any distortion in the gel lanes and for precise size estimation (Scheidner & deBrujin, 1996). Bands were coded in the binary form (Scheidner & deBrujin, 1996; Rademaker & deBruijn, 1998) and Jaccard's coefficient was calculated to construct a similarity matrix and the unweighted pair group method with an arithmetic mean algorithm (UPGMA) was used to perform hierarchical cluster analysis and to construct a dendrogram for REP fingerprints and ERIC fingerprints respectively (Scheidner & deBrujin, 1996). Multidimensional scaling (MDS) was also performed for analysis. Multidimensional scaling is also referred to as "perceptual mapping," is a procedure that "represents a set of individuals or genotypes (*n*) in a few dimensions (*m*) using a similarity/distance matrix between them such that the inter-individual proximities in the map nearly match the original similarities/distances" (Johnson & Wichern, 1992). Multi dimensional scaling represents the relationships among a set of genotypes, can be presented as a 2- or 3-dimensional representation that can be more easily interpreted. The pattern obtained from MDS can also be used to estimate the actual number of groups that may be obtained by cluster analysis.

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3.4. Shannon's diversity index

Shannon's diversity index was calculated for each site location using the formula

$$H = -\sum_{i=1}^{s} p_i \ln p_i$$

Where **H**= Shannon diversity index

p_i = The relative abundance of each strain, calculated as the proportion of individuals of a given strain to the total number of strains.

s = The number of strains. Also called species richness.

Shannon diversity has been used in previous studies for determining relative abundance and species richness of soil bacteria at strain level (Seguin *et al.,* 2001). The diversity index was calculated for each site considered in this study using the similarities identified among isolates at each site by REP and ERIC fingerprinting. Table 2 in chapter 4 includes the Shannon diversity index calculated for each site as identified by REP and ERIC fingerprinting.

3.5. Correlation of genetic diversity with edaphic and climatic factors

Factors like average monthly and annual temperature, mean monthly and annual precipitation, geographical location, soil type, inoculant use, last crop grown on the sampling site from the year of isolation and year since last soybean grown were considered in this study to correlate genetic diversity to site characteristics and to

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understand the influence of environmental and edaphic factors on genetic diversity of *B. japonicum* isolates (Table 2). Analysis of variance and regression analysis was performed using SYSTAT software Inc. (San Jose, California, USA). Geographical information systems (GIS) were used for obtaining soil information in USA and Canada. Information for Manitoba's soil data was obtained through Manitoba agriculture, food and rural initiatives website (http://geoapp2.gov.mb.ca/website/MAFRI/index3.html) and USA soil data was obtained through National Resources Conservation Service, United States Department of Agriculture website (http://websoilsurvey.nrcs.usda.gov/app/). Detailed soil surveys available on USDA website and Manitoba rural initiatives websites were used to collect soil data. Influence of soil texture, drainage, salinity was tested in relation to the diversity index of isolates from each site using non-parametric statistics (Kruskal-Wallis test). Table 2 contains information about year of nodule collection, crop history for the last two years (if available), and year since last soybean was grown, soil texture, soil drainage and if inoculant(s) was used in the field. In some cases information on crop history, soil data and use of inoculants at each site from where samples were collected for this study. In case where information was not available on crop history, soil data and, inoculant use is indicated 'unknown' in Table 2.

Climate data for Canada was obtained from Environment Canada website. (http://climate.weatheroffice.ec.gc.ca/climate_normals/stnselect_e.html) and from National Climate Data Centre site (http://lwf.ncdc.noaa.gov/oa/ncdc.html) for the US. Climate data on mean monthly and annual precipitation and monthly and annual temperature for both the US and Canadian sites was used for the analysis. Kruskal –

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Wallis one way analysis of variance was performed to test correlation between diversity index at each site location with mean annual and monthly precipitation and average monthly and annual temperature.

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Table 2. Site characteristics. Includes information year of nodule collection, crop history for the last two years (if available) and year since last soybean was grown, soil texture, soil drainage and if inoculant(s) was used in the field.

Site name	Year of isolation	Crop History (current crop/last yr)	Year since last soybean grown	Soil texture	Drainage	Inoculant used
Site-1'	2005	Corn/Soybean	Two	Silty Clay loam	poor	Yes
Site-2'	2005	Corn/Soybean	Two	Silty Clay loam	very poor	Yes
Site-4'	2005	Corn/Soybean	Two	Silty Clay loam	well	Yes
Site-5'	2005	Corn/Soybean	Two	Sioux loam	excessive	No
Site-6'	2005	Corn/Soybean	Two	Clay loam	well	No
Site-7'	2005	Corn/Sod (30 yrs)	Never	Silt Loam	well	No
Site-8'	2005	Corn/Soybean	Two	Silt Loam	well	No
Site-9'	2005	Corn/Soybean	Two	Loam	well	No
Site-11'	2005	Corn/Soybean	Two	Silty Clay loam	very poor	No
Site-12'	2005	Corn/Soybean	Two	Unclassified	Unclassified	No
Site-13'	2005	Corn/Soybean	Two	Unclassified	Unclassified	No
Site-14'	2005	Corn/Soybean	Two	Clay loam	well	No
Breckenridge	2004	unknown	Never	Silty Clay	poor	No
Great Bend,	2004	Soybean/Wheat	Current	Silt Loam	poor	No
Richland County	2004	Unknown/	Never	Silty Clay	poor	No
Ramsey	2004	Corn/Soybean	One	Loamy	well	Unknown
Lacota	2004	Corn/ unknown	One	Silty Clay loam	very poor	Unknown
Site 2	2004	Oats/Soybean	One	Loamy	imperfect	Yes
Site 3	2004	Canola/ unknown	One	Unclassified	Unclassified	Unknown
Site 4	2004	Barley/Soybean	One	Unclassified	Unclassified	Yes
Site 5	2004	Oat/Soybean	One	Marsh	Imperfect	Yes
Site 6	2004	Corn/Soybean	One	Unclassified	Unclassified	Yes
Site 9	2004	Corn/Soybean	One	Unclassified	Unclassified	Unknown
Plum Coulee1	2004	Wheat/Soybean	One	Coarse Loamy	imperfect	Unknown

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Site name	Year of isolation	Crop History (current crop/last yr)	Year since last soybean grown	Soil texture	Drainage	Inoculant used
Plum Coulee2	2004	Wheat/Soybean	One	Eroded slope	imperfect	Unknown
Elm Creek	2005	Unknown/Soybean	One	Sandy loam	Unclassified	Unknown
Winnipeg	2005	No soybean	Never	Clay	Imperfect	Unknown
Sanford	2005	unknown	Never	Unclassified	Unclassified	No
Agra seeds	2004	Clover/Soybean	One	Unclassified	Unclassified	Unknown

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Results

In this study, we have used hierarchical and nonhierarchical clustering methods for the analysis of the fingerprints obtained by REP and ERIC fingerprinting. Reproducible and specific banding patterns were obtained with both REP-PCR and ERIC-PCR fingerprinting (See examples, Figure 2a and Figure 2b; all gels generated are provided in Appendix A). One-hundred and nine isolates including control strains 532C and USDA110 were successfully analysed by ERIC fingerprinting. One-hundred and three isolates including control strains were successfully analysed by REP fingerprinting. The profiles for 8 isolates (13, 15, 22, 35, 37, 39, 50 and U73) were not obtained by REP fingerprinting despite of several repeated trials. Profile for two isolates, W 6A and U43 were not obtained by ERIC fingerprinting. The DNA concentration and other factors that could affect the successful PCR amplification were taken into consideration to overcome this problem but no success was achieved.

4.1. Rep-PCR genomic fingerprints

Amplification reactions with both primers generated a sufficient number of distinct polymorphic bands for reliable strain discrimination. In this study, we found that ERIC primers produce more discriminatory bands as compared to REP primers.

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Figure 2a. An example of an ERIC-PCR gel. ERIC-PCR fingerprints of 17 isolates collected from different sites in North Dakota, South Dakota and, Minnesota in US and from southern Manitoba in Canada. Lanes 1 and 22 consist of 1kb DNA ladder. Lanes 2 and 3 are the control strains, 532C and USDA110 respectively. Lanes 4-21 consists of rest of the field isolates of *Bradyrhizobium japonicum*. A weaker profile was generated for isolate in lane 19 and was excluded in cluster analysis.

Figure 2b. An example of a REP-PCR gel. REP-PCR fingerprints of 17 isolates collected from different sites in North Dakota, South Dakota and Minnesota in US and from southern Manitoba in Canada. Lanes 1 and 22 consist of 1kb DNA ladder. Lanes 2 and 3 are the control strains, 532C and USDA110 respectively. Lanes 4-21 consists of field isolates of *Bradyrhizobium japonicum*. Weaker profile was generated for isolate in lanes 4 and 5 but numbers of bands were clear and sufficient for cluster analysis. It should be noted that for isolates in lane 4 and 5 very few and weak band were detected in REP fingerprinting however sharp and clear band were generated by ERIC fingerprinting for the same isolates in Figure 1a.

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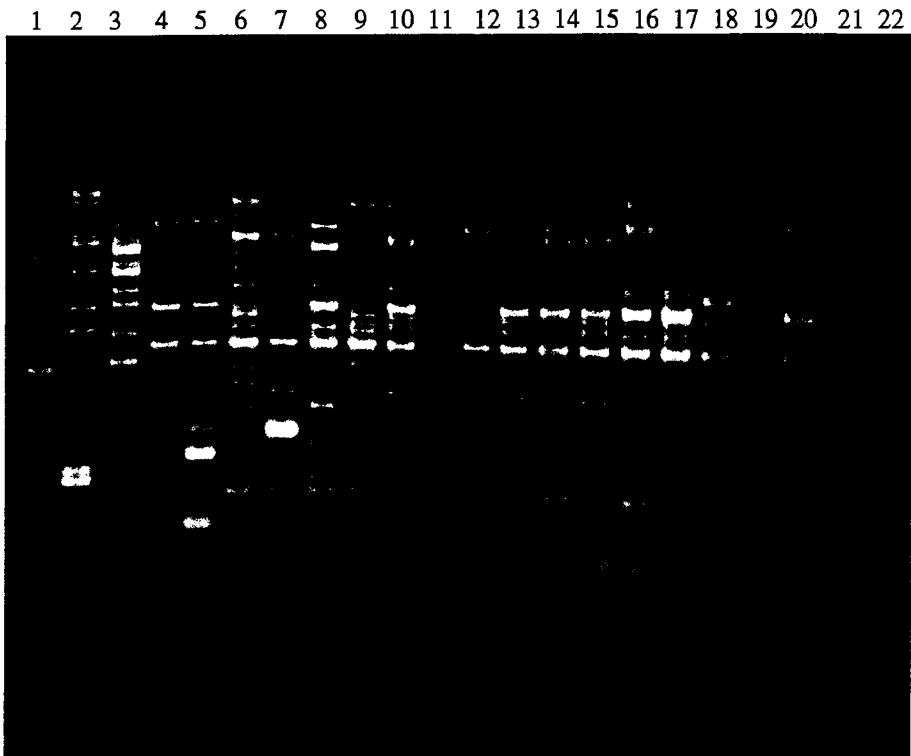

Figure 2a. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

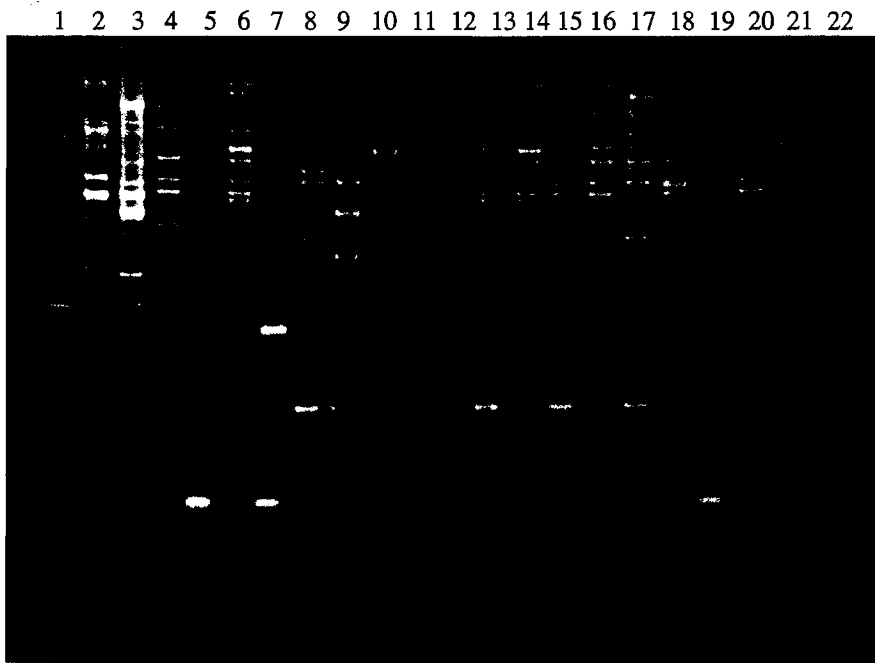

Figure 2b. REP fingerprints for *Bradyrhizobium japonicum* isolates

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4.2. Proximity analysis

Isolates collected from different site locations as shown in Table 2 were assigned to six different groups according to their relative closeness to each other (site closer to each other within a given radius were grouped together) (Table 3). Proximity analysis was performed to detect differences in the diversity index among the sites. No significance differences among the six groups were detected in the Kruskal-Wallis test.

Isolates were grouped based on their geographical origin (US and Canada) (Table 4). Grouping of isolates on the basis of their geographical origin (Canada or US) yield significant results ($p<0.05$). Significant differences were found between the diversity index of sites located in Canada and US. Results of analysis of variance are shown in section 4.2 (a).

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Table 3. Grouping of sites for US and Canadian isolates based on their relative closeness to each other. Information on geographical location and Country is also included.

Group	Site name	Geographical location	Country
Group 1	Site 3	Manitoba	Canada
	Site 4	Manitoba	Canada
	Site 5	Manitoba	Canada
	Site 6	Manitoba	Canada
Group 2	Site 2	Manitoba	Canada
	Site 9	Manitoba	Canada
	Plum Coulee, #1	Manitoba	Canada
	Plum Coulee, #2	Manitoba	Canada
	Elm Creek	Manitoba	Canada
	Winnipeg	Manitoba	Canada
	Sanford	Manitoba	Canada
	Agra seeds	Manitoba	Canada
Group 3	Breckenridge	Minnesota	US
	Great Bend	North Dakota	US
	Richland County	North Dakota	US
Group 4	Ramsey	North Dakota	US
	Lacota	North Dakota	US

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Group	Site name	Geographical location	Country
Group 5	Site-1'	Fargo, North Dakota	US
	Site-2'	Fargo, North Dakota	US
	Site-4'	Fargo, North Dakota	US
	Site-5'	Fargo, North Dakota	US
Group 6	Site-6'	Brandt, South Dakota	US
	Site-7'	SDSU, South Dakota	US
	Site-8'	Brookings, South Dakota	US
	Site-9'	Yellow Medicine, Minnesota	US
	Site-11'	Gary, South Dakota	US
	Site-12'	Corona, South Dakota	US
	Site-13'	Corona, South Dakota	US
	Site-14'	Pioneer field trial, South Dakota	US

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Table 4. Grouping of sites on the basis of their geographical location (Canada or US). Diversity index calculated by ERIC analysis and REP analysis for each site is also included.

Group	Site name	REP data analysis	ERIC data analysis	Country
		Shannon diversity index	Shannon diversity index	
Group 1	Site 3	0	0	Canada
	Site 4	0.693	0.693	Canada
	Site 5	1.386	1.386	Canada
	Site 6	0	0.693	Canada
	Site 2	1.386	1.386	Canada
	Site 9	1.609	1.332	Canada
	Plum Coulee, #1	1.098	0.693	Canada
	Plum Coulee, #2	1.039	1.039	Canada
	Elm Creek	0	0	Canada
	Winnipeg	0.693	0	Canada
	Sanford	1.098	1.098	Canada
	Agra seeds	0.693	0.693	Canada
Group 2	Breckenridge	1.098	1.098	US
	Great Bend	1.609	1.039	US
	Richland County	2.271	1.549	US
	Ramsey	2.271	1.468	US
	Lacota	1.386	1.092	US
	Site-1'	0.693	0.693	US
	Site-2'	1.039	0	US

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	Site name	REP data analysis	ERIC data analysis	Country
		Shannon diversity index	Shannon diversity index	
Group 2	Site-4'	0	0	US
	Site-5`	1.386	1.332	US
	Site-6'	0	0	US
	Site-7'	1.332	0.5	US
	Site-8'	0	0	US
	Site-9'	1.098	1.098	US
	Site-11'	0.693	0	US
	Site-12'	1.043	1.285	US
	Site-13'	1.098	1.039	US
	Site-14'	0	0	US

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4.2(a). Kruskal –Wallis one way analysis of variance (ERIC fingerprints)

Shannon diversity index for each site obtained from cluster analysis of ERIC fingerprints as shown in Table 3 was used in Kruskal –Wallis test. Isolates were tested for the difference in level of diversity for the two groups, US and Canada (Table 3).

Kruskal-Wallis One-Way Analysis of Variance for 29 sites.

Dependent variable is Shannon Diversity Index
Grouping variable is GROUP

Group	Sample size	Rank sum
Group1	17	304.500
Group2	12	130.500

Mann-Whitney U test statistic = 151.500
Probability is 0.026
Chi-square approximation =4.970 with 1 df

4.2(b). Kruskal –Wallis Analysis of variance (REP fingerprints)

Shannon diversity index for each site obtained from cluster analysis of REP fingerprints as shown in Table 3 was used in Kruskal –Wallis test. Isolates were tested for the difference in level of diversity for the two groups, US and Canada (Table 3).. Diversity index calculated from REP fingerprinting as shown in Table 4 was used in Kruskal –Wallis test.

Kruskal-Wallis One-Way Analysis of Variance for 29 sites

Dependent variable is Shannon Diversity Index

Grouping variable is GROUP

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Group	Sample size	Rank sum
Group1	17	297.500
Group2	12	137.500

Mann-Whitney U test statistic = 59.500
Probability is 0.057
Chi-square approximation = 3.635 with 1 df

Significant differences were found in the analysis of variance using non-parametric test (Kruskal –Wallis test) between the diversity index of sites located in Canada and US for both REP and ERIC fingerprinting. The results show that there is difference in the levels of diversity among isolates from two countries, US and Canada.

4.3. Cluster Analysis

Individual dendrograms for REP and ERIC fingerprints were constructed from the profiles of each primer set by UPGMA method using the similarity matrix calculated by Jaccard's coefficient. Isolates with similarity value of 80% or higher were considered as similar isolates. Previous studies have also considered isolates with 75 % (Leach *et al.,* 1992); 85 % (Van Berkum *et al.,* 1993) and; 95 % (Jarabo-Lorenzo *et al.,* 2003) similarity as same strain. Low correlation (r = 0.19, p= 1.0) was found between the two dendrogram using the Mantel's test (Mantel, 1967). However, inspite of low correlation between REP and ERIC dendrograms, many significant clusters and pattern were

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common between both methods. In this study, we have found that ERIC fingerprints are more discriminatory than REP fingerprints and detected larger genetic variability among the isolates considered in this study. In addition to these, multidimensional scaling (MDS) was also performed on both matrix separately to create a three dimensional plot. The pattern obtained from MDS is found to be similar to cluster analysis pattern obtained by each individual dendrogram.

4.4 Hierarchical cluster analysis of ERIC fingerprints by UPGMA method

In cluster analysis of dendrogram derived from ERIC fingerprints, 107 isolates and two reference strains (532C and USDA 110) grouped together into three major groups (arbitrarily defined) that could be further divided into nine clusters and fifteen loosely associated strains (Figure 3).

Group A contains total 17 isolates and control inoculant strain 532C. This group consists of two clusters (Cluster 1 and Cluster 2).

Cluster 1: Consists of five isolates (three from Canada and one from Ramsey in North Dakota) clustered together with the control strain (532C) at 41 % similarity value. Within this group, two isolates from site Winnipeg clustered tightly with high similarity value of 79% and were 62 % similar to the control strain, 532C. Isolate U11 was loosely associated with this cluster.

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Cluster 2: Contains 10 isolates which joined at 32.5 % similarity. Isolate EC 2B, EC 3A, EC 3B, EC 3C, EC 4C, EC 5A, EC 5B and, EC 5C from site Elm Creek, MB shared similar profile (83 % similar). Isolate EC 1C is found to less similar to rest of the isolates from Elm Creek and shared 42 % similarity with control inoculant strain, USDA110.

Group B is the largest group consisting of total 80 isolates and consist of six clusters and some loosely associated strains that are explained in section 3.2. (Cluster 3, cluster 4, cluster 5, cluster 6, cluster 7, cluster 8 and, cluster 9).

Cluster 3: This cluster consists of total twelve isolates. This cluster diverged into three sub-clusters (cluster3a, 3b, 3c) which were joined at 32 % similarity.

Cluster 3a: Isolates from different sites from North Dakota (Ramsey), South Dakota (site 8` in Brookings and site 7` in SDSU) and Minnesota (site 9` in Yellow medicine) were grouped together in this cluster at 42 % similarity along with two isolates from Elm Creek, MB. Isolate U91 and U93 from site 9` in Yellow Medicine, MN were closely related to each other with 92 % similarity. Isolate U73 and U85 from site 7` and site 8` respectively shared 70 % similarity.

Sub-cluster 3b: This sub-cluster consists of only three isolates (Isolate EC 2A and EC 2C from Elm Creek and U22 from Site 1` in Fargo) joined at 50% similarity.

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Sub-cluster 3c: Consists of only two isolates, isolate 65 and 68 from Richland County and Lacota in North Dakota respectively joined at 59 % similarity.

Cluster 4: In this cluster, two divergent sub clusters were identified which were joined at 42% similarity (sub cluster 4a and 4b).

Sub-cluster 4a: Within this cluster comprising of total fourteen isolates from various sites in US and site Plum Coulee #1 and Plum Coulee # 2 in Canada, cluster together at 44 % similarity. Isolate 32 from site Plum Coulee # 2 is similar to isolate 52 from Richland County in North Dakota (88 % similarity). Isolate 33 from site Plum Coulee # 2 was found similar to isolate 49 from site Breckenridge in Minnesota (89 % similarity).

Sub-cluster 4B: Contains total thirteen isolates joined at 50 % similarity. Twelve isolates in this cluster are from Canadian sites. Only one isolate (38) was from Great Bend, ND in this cluster. Isolates are from different site locations within Manitoba shows high level of diversity within this cluster. Isolate 17 and 19 from site 6 were similar (89.5 % similarity). Isolate 2 from site Agra-seeds, MB and isolate 24 and 31 from site 2 shared similarity value of 89.5 %. Isolate U51 from site 5` in Fargo, ND was loosely joined to cluster 4 at 36 % similarity.

Cluster 5: Cluster 5 consists of eight isolates and two divergent groups (sub clusters) joined at 38 % similarity.

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Figure 3. ERIC dendrogram of 107 isolates and two control strains 532C and USDA 110.

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Sub-cluster 5a: Consists of only two isolates (U142 from site 14` in South Dakota and isolate 15 from site 6 in Manitoba) joined at 43 similarity.

Sub-cluster 5b: This cluster has isolates from various site locations in South Dakota. One isolate (U135) from Site 13` in Corona, SD joined to isolate U123 and U62 at 58 % similarity. None of the isolates from South Dakota within this group shared similar profile and high genetic variability was found among isolates within this sub-cluster.

Group C: Contains total forty isolates and consists of four clusters (cluster 6, cluster 7, cluster 8 and, cluster 9).

Cluster 6: Consists of two sub clusters (Cluster 6a and Cluster 6b) containing six isolates and one loosely associated isolate (U141) joined at 36 % similarity value.
Sub-cluster 6a: Consist of three isolates (U74 from site 7` in SDSU, South Dakota and U23 from site2`and U44 from site 4` in Fargo, north Dakota.) joined at 50% similarity value.

Sub-cluster 6b: Contains three isolates, isolate U63 from South Dakota and EC 1B from Elm Creek in Manitoba and U14 from site 1`in Fargo, ND joined at 58 % similarity.

Cluster 7: This cluster mainly consists of two divergent groups joined together at 49 % similarity. Isolate 70 was found loosely associated with this cluster at 44 % similarity.

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Sub-cluster 7a: Consists of isolate 22 and isolate 35 from site 2 in Agra-seeds, MB and Great Bend, ND joined at 56.5% similarity.

Sub-cluster 7B: This cluster consists of total 12 isolates joined at 58 % similarity. Most isolates in this cluster shared very close or similar profiles. U83 and U84 from site 8` in Brookings, SD shared 93 % similarity. Isolate U111 from Site 11` in Gary, South Dakota shared similar profile (83 % similar). Isolate U141 from site 14` (Pioneer field trial) in South Dakota with 72 % similarity with these isolates. Isolate 41 and 61 from Great Bend and Richland County respectively were found similar (90 % similarity).

Cluster 8 : This cluster has total four isolates from US, isolate U121 and U131 from site 12` and site13` in Corona, SD and U52 and 70 from site 5` in Fargo and Richland County respectively. All isolated share 66 % similarity in this sub-cluster.

Cluster 9: Six isolates from site Richland County, ND clustered together at 46 % similarity. Only two isolates, 56 and 57 from Richland County, ND were similar to each other within this cluster (87.5 % similar).

4.4 (a). Loosely associated strains

Branching points consisting of only one isolate and two isolates branching at 36.2% similarity or less were considered as loosely associated strains in the cluster analysis of ERIC dendrogram. Isolate U11 was found loosely attached to cluster 1. Isolate EC 1A

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and W 5C were also loosely associated with cluster 2 at 32.5 % similarity. Isolate U55 and U65 were found loosely attached to cluster 2 at 32 % similarity. Isolate U51 was joined at 36 % similarity to cluster 4. Isolate 39 and 42 were joined to cluster 4 at 29 % similarity. Isolate U121 was loosely associated with cluster 7 at 42 % similarity. Isolate 10 from site 3 in Canada ; isolate U24 from site 2` in Fargo; isolate 64 from Richland County ND and; isolate 8 and 37 from Great Bend, ND were found loosely associated to the other isolates at 22 % similarity value. Isolate U22 and U112 grouped separately from rest of the isolates and were loosely joined at 30% in the analysis of ERIC fingerprints.

The overall pattern within ERIC fingerprint dendrogram shows high genetic variability amongst the isolates at individual sites and among different site locations within US and Canada. At the Elm Creek site in Manitoba, most isolates were found to be similar (8 out of 13 isolates are similar sharing similarity value of 84 %). Isolates from other site locations in Canada (Site 2, Agra-seeds, Plum Coulee #1 and, Plum Coulee # 2) revealed considerable diversity. Within some of the individual sub-clusters, Cluster 2 consists of isolates from Canadian sites only and mainly isolates are from site Elm Creek. This site revealed the least diverse population as compared to other sites. Cluster 5 consists of all isolates from South Dakota except one isolate (isolate 15) from site 6 in Canada. In sub-cluster 7b, all isolates were from Richland County, ND in the US. Some isolated pattern was observed within some sub-clusters where US and Canadian isolates clustered separately. Overall, isolates from site Great Bend, ND and Richland County, ND were found to be most diverse and isolates at site Elm Creek, MB has the least diversity compared to the other sites.

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4.5. Hierarchical cluster analysis of REP fingerprints by UPGMA method

In cluster analysis of dendrogram derived from REP fingerprints, 103 isolates and reference strains (532C and USDA110) grouped together into three groups (arbitrarily defined) that could be further divided into seven clusters and three loosely associated strains as shown in Figure 4.

Group A consists of a one cluster (Cluster 1) of total 8 isolates and two control inoculant strains (532 C and USDA110) joined together at 66 % similarity.

Cluster1: In this cluster, USDA110 was found similar to EC 1C at a 94.5% similarity value. Also isolates W6A and W6C from Winnipeg found to be similar at 100% similarity value. The isolates from this site share 84 % similarity with inoculant control strain 532C which is commonly used commercial inoculant strain in Canada and is used as one of the controls in our study. Isolate U11 from Fargo in North Dakota also share 82 % similarity with the control strain 532C.

Group B consists of total sixty three isolates and three loosely associated strains. This group contains 4 main clusters (cluster 2, cluster 3, cluster 4 and, cluster 5).

Cluster 2: Contains total seven isolates joined together at 74.8% similarity value. All isolates within this group are from different sites in North Dakota and South Dakota from

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US. The overall diversity within this group is low as five isolates shared similar profile (83 %). Two isolates, U65 and U55 shared similarity with these five isolates at 78 % and 75 % respectively.

Cluster 3: Consists of two divergent sub-clusters (Sub-cluster3A and Sub-cluster 3b) in which isolates from each clusters joined together at 79 % similarity. Most of the isolates in this cluster are from different site in US and Canada and have very similar profiles.

Sub-cluster 3a: All isolates within this sub-cluster clustered together with a high similarity values. The isolates within this sub-cluster are from different sites within US and Canada.

Sub-cluster 3b: Similar to sub-cluster 3a, isolates in this sub-cluster have very similar profile and share very high similarity. Nine isolates from different site locations within North Dakota and South Dakota clustered together along with one isolate from Canada (isolate 26 from Plum Coulee # 1) within this sub-cluster at 82 % similarity.

Cluster 4: Isolates within sub-cluster also share highly similar profiles and grouped together at 78 % similarity. This sub-cluster have isolate U61 from site 6`, isolate U143 from site 14` in South Dakota, eight isolates from various site locations (Fargo, Lacota, Richland, Great Bend) in North Dakota and one isolate (isolate 10) from Site 3 in Manitoba are found in this sub-cluster.

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Figure 4. REP dendrogram of 101 isolates including control 532C and USDA110.

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Cluster 5: Consists of only two isolates (isolate U51 from site 5` in Fargo, ND and isolate 8 from site 4 in Manitoba) sharing similar profile (84 %). These isolates were joined to other clusters in group B at 69 % similarity.

Group C: Contains twenty five isolates. Two main clusters were found in this group (cluster 6 and cluster7).

Cluster 6: Consists of total ten isolates joined together at 54 % similarity. Isolate 41 and 61 from site Great Bend and Richland County in North Dakota shared 82 % similarity. Isolate 4 and 28 from site 4, MB and Plum Coulee # 1, MB respectively shared 82 % similarity. Profiles of the most isolates within this cluster are considerably diverse.

Cluster 7: Consists of two divergent clusters joined together at 59 % similarity.

Sub-cluster 7a: Within this sub-cluster consisting of total nine isolates from Elm Creek in Manitoba clustered together at 73 % similarity value. Six isolates shared high similarity and have similar profiles (87 % similarity value). Isolate EC 1C, EC 2C, EC 5B were not exactly similar to the other isolates from site Elm Creek, MB within this group but share high similarity values with rest of the isolates and joined this sub-cluster at 77 %, 79 % and 73 % similarity respectively. Overall, the isolates from site Elm Creek were found to be least diverse compared to other sites considered in this study.

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Sub-cluster 7b: consists of total 7 isolates joined together at 73 % similarity value. Two isolates from Site 9` in Yellow Medicine, MN and two isolates from Site 8`from Brookings, SD were highly similar (85.5%). Isolate EC 2A and EC 5C from Elm Creek, MB and U22 from Fargo, North Dakota also found to share high similarity with rest of the isolates (EC 2A joined at and EC 5C and U22 sharing 73 % similarity with rest of the isolates).

4.5 (a). Loosely associated strains

Branching points consisting of only one isolate at 60% similarity or less were considered as loosely associated strains in the cluster analysis of REP dendrogram. EC 1B and U21 were found loosely associated to clusters in group A and group B. Isolate U21 from site 2` in Fargo, ND was loosely associated to the clusters in group A and group B at 49.5 % similarity and isolate EC 1B was loosely associated to the main cluster at 63 % similarity.

The overall clustering pattern identified in the hierarchal cluster analysis of REP fingerprints of isolates revealed considerable diversity among different site locations across US and Canada. However, cluster analysis of REP fingerprints revealed low genetic variability among the isolates as compared to cluster analysis of ERIC fingerprints. Much higher genetic variability was detected among the different site locations in US and Canada by ERIC fingerprinting. Nonetheless, some similar pattern was observed in both dendrograms. It has been found in a previous study that ERIC fingerprints are more discriminatory as compared to the REP fingerprints (Cartelle *et al.,* 2004). In Cluster 1, isolates from Winnipeg were found 84 % similar to the control strain.

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This pattern was also found in the ERIC dendrogram where Isolate W6C shares 62 % similarity with the control 532C strain. This suggests that the isolates at this site (Winnipeg) could have originated from inoculant strain 532C. USDA110 shared 80 % similarity to the isolate EC 1A from site Elm Creek. This pattern was not observed in the ERIC dendrogram. USDA110 was 42 % similar to isolate EC 1A in ERIC dendrogram. In group B of REP dendrogram, most of the isolates from different sites within US and Canada shared very similar profile. Isolate 2 and 31 from sites Agra-seeds and Plum Coulee 2 in Manitoba, respectively, were found similar in REP dendrogram (100 % similarity) as compared to ERIC (89.5 % similarity). Isolates in cluster 4 were found to be highly diverse. In sub-cluster 7b consisting of nine isolates from site Elm Creek, six isolates were found similar and other three sharing very similar profile (87 % similar). This pattern was also evident in the cluster analysis of ERIC fingerprints where eight isolates had similar profile (84 % similar) in Cluster 2 of ERIC dendrogram. Overall in this cluster analysis, isolates from site Great Bend and Richland County from North Dakota state are highly diverse. Site Elm Creek from Manitoba revealed least diverse population in REP dendrogram among all the sites considered in study. Cluster analysis of ERIC fingerprints also revealed high diversity among the isolates at site Great Bend and Richland County in North Dakota and least diverse population of *B. japonicum* isolates at site Elm Creek in Manitoba.

4.6. Non-hierarchical cluster analysis of fingerprints using three dimensional MDS plots

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Multidimensional Scaling was performed on the REP matrix and ERIC matrix as shown in Figure 5a and Figure 5b respectively. In this non-hierarchical presentation of the relationships among the strains, similar pattern was observed as in the hierarchical cluster analysis explained above. Multidimensional scaling (MDS) is a set of data analysis techniques that display the structure of distance-like data (similarity matrix) as a geometrical picture. There are two important things to realize about an MDS map. The first is that the axes are, in themselves, meaningless and the second is that the orientation of the picture is arbitrary. The orientation and scale of these configurations is completely arbitrary, so no axis labels have been shown in Figure 5a and Figure 5b.

In the MDS plots for both matrixes (Figure 5a and 5b), Site Elm Creek, MB isolates were found to be more similar and grouped together as tight clusters. Isolated patterns were also observed for most of the isolates from North Dakota state in U.S. Isolates from site Great Bend, ND and Richland County, ND revealed highest diversity among the all isolates.

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Figure 5a. Three dimensional MDS plot created by using similarity matrix of ERIC fingerprints. Same groups were obtained as in the hierarchical cluster analysis of REP fingerprints in Figure 3. Site Elm Creek, MB isolates were found to be more similar and most isolates from this site grouped together as tight clusters. Isolates from site Great Bend, ND and Richland County, ND were found to be spreaded in three dimensional spaces without any specific pattern. Specific colors were assigned for the isolates from each state in US (blue for North Dakota, pink for South Dakota and dark green for Minnesota). Isolates from all sites in southern Manitoba except site Elm Creek are represented by light green colour. Isolates from site Elm Creek are shown in Yellow colour.

532C
USDA 110
North Dakota
South Dakota
Minnesota
Southern Maniotba (except site ElmCreek)
Elm Creek, MB

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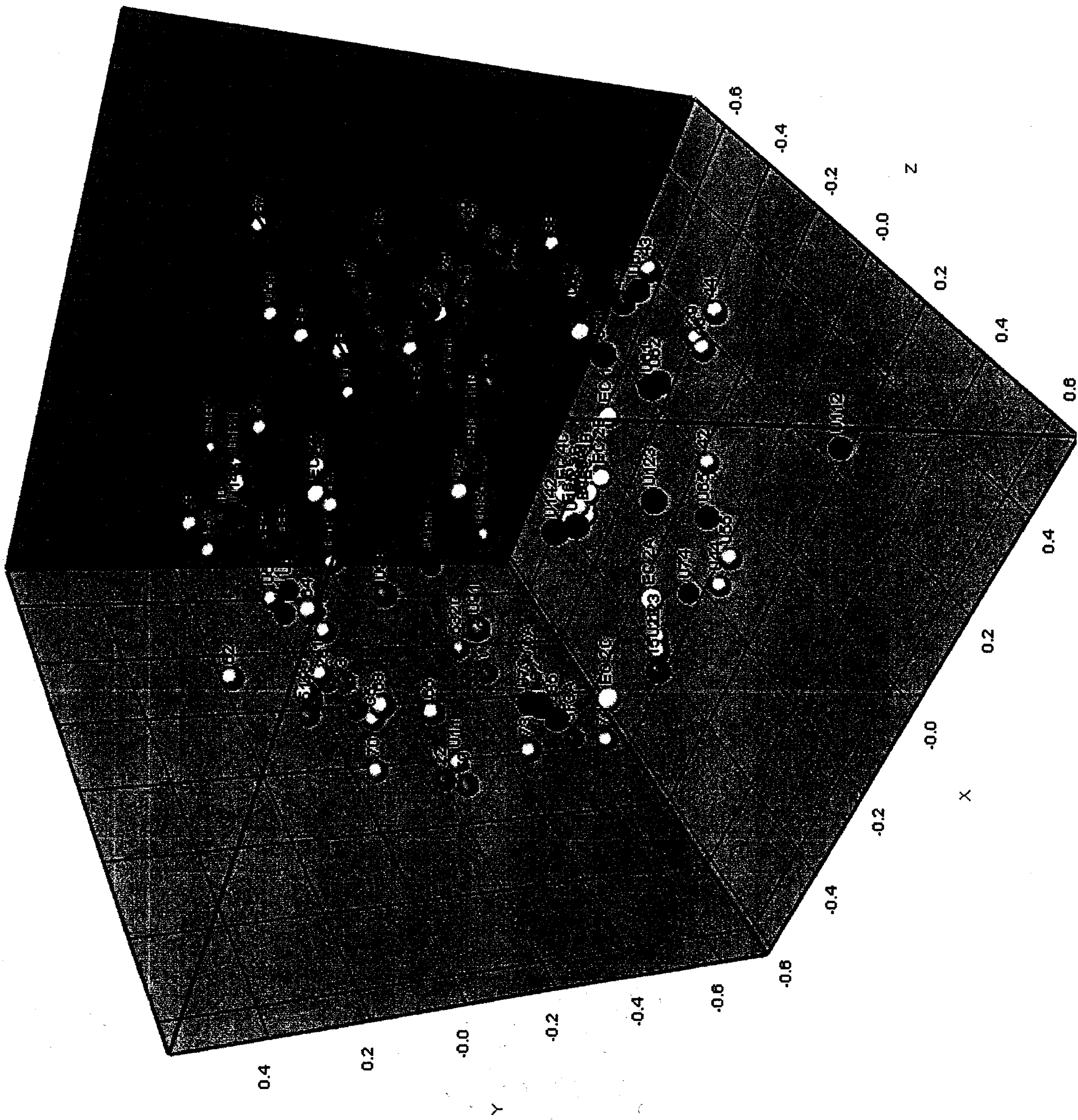

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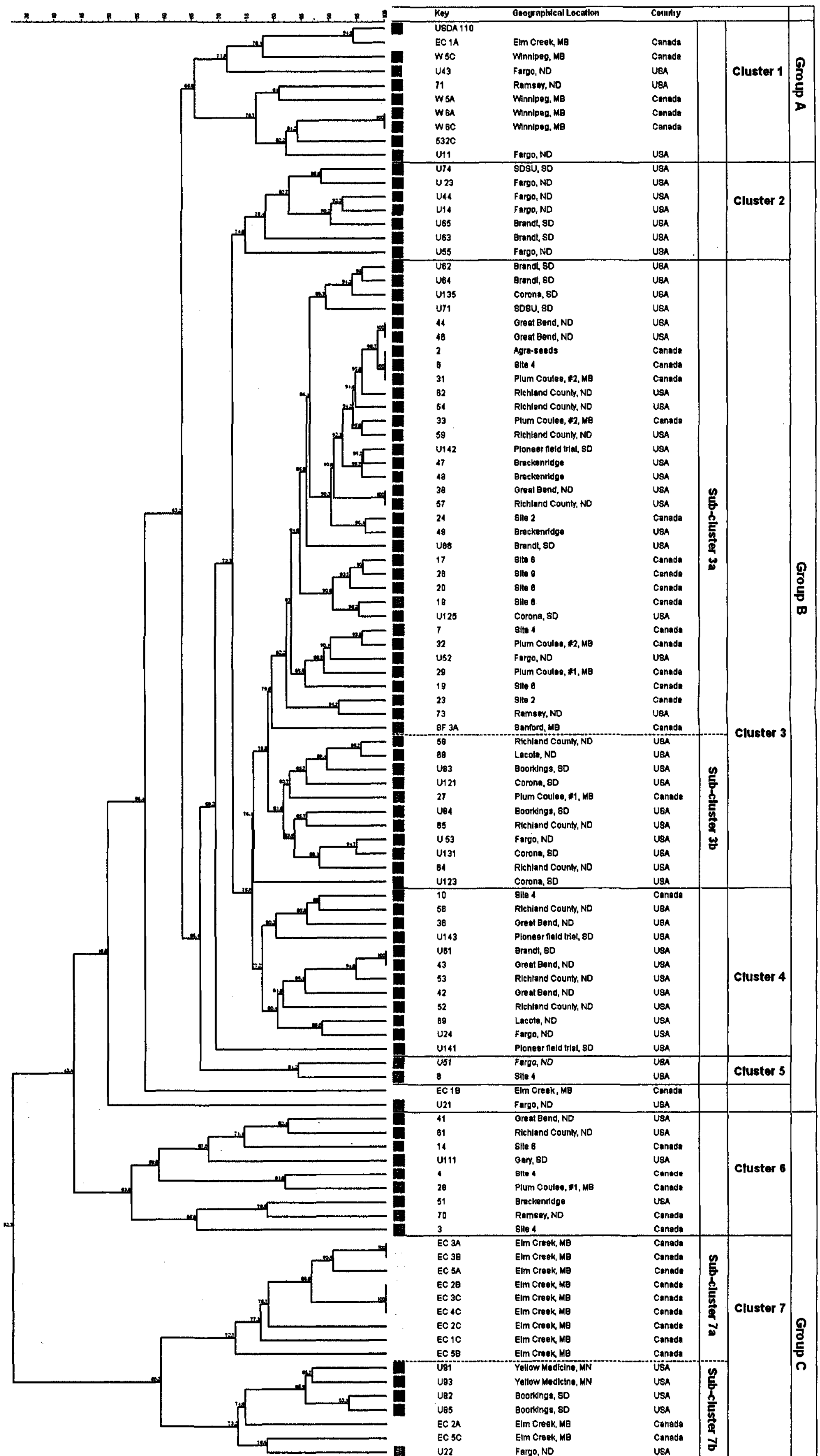

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Figure 5b. Three dimensional MDS plot created by using similarity matrix of REP fingerprints. Same groups were obtained as in the hierarchical cluster analysis of REP fingerprints in Figure 4. Nine isolates from Site Elm Creek, MB clustered together in tight group and found to spatially separated in three dimensional space from rest of the isolates. Isolated patterns were also observed for most of the isolates from North Dakota state in U.S.

532C
USDA 110
North Dakota
South Dakota
Minnesota
Southern Maniotba (except site ElmCreek)
Elm Creek, MB

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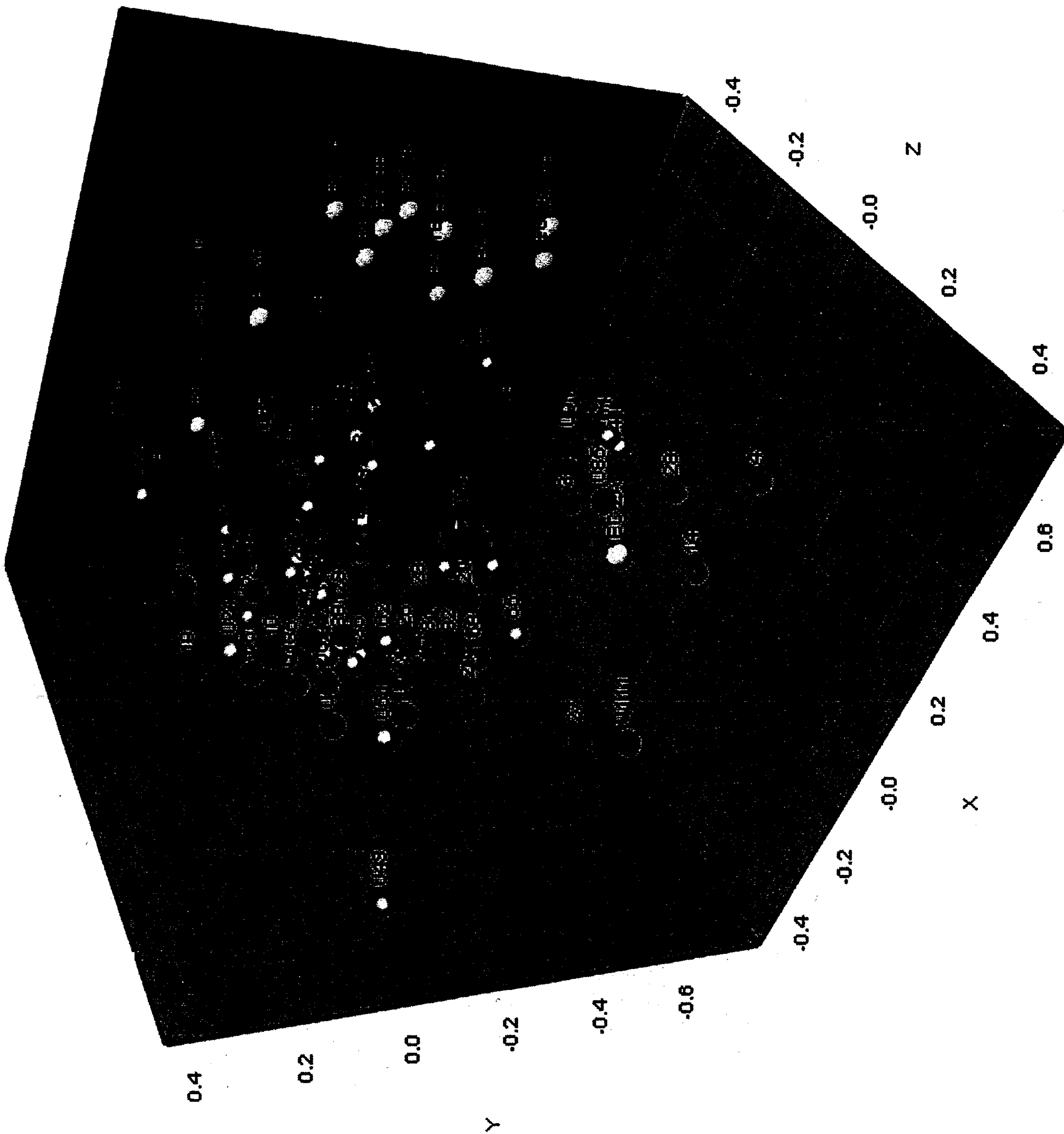

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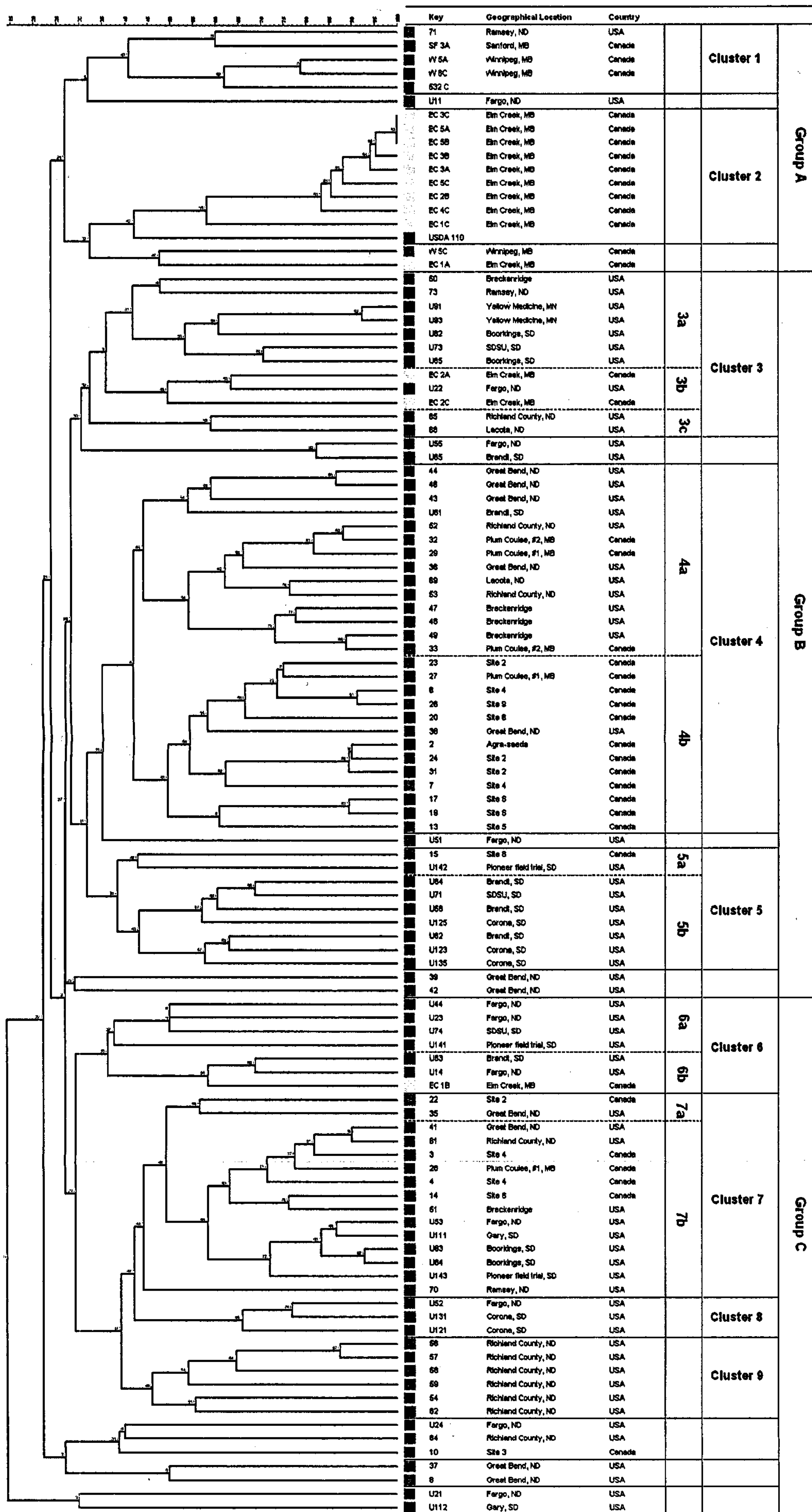

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Discussion

The aim of the present study was to provide an assessment of the genetic diversity of field populations of *Bradyrhizobium japonicum*, isolated from the soybean growing areas in western Canada and the northern US. In this study we have used PCR-based genomic fingerprinting techniques – namely, REP-PCR and ERIC-PCR (Versalovic *et al.*, 1991). DNA amplification with consensus sequences, such as REP and ERIC has proven to be valuable for the detection of genetic diversity at strain levels (deBruijn, 1992). The results of this study shows that the *B. japonicum* strains isolated from different field sites in southern Manitoba and from three states in the northern US (North Dakota, South Dakota, and Minnesota) are genetically diverse.

Cluster analysis of REP and ERIC fingerprints of *B. japonicum* isolates has revealed that high level of genetic diversity exists within individual site locations and across different sites in the US and Canada and these findings are in agreement with the results obtained in previous studies on genetic diversity of natural rhizobial populations in different parts of the world (Chen *et al.*, 2000; Lima *et al.* 2005; Loureiro *et al.*, 2006; Prakash & Annapurna, 2006). A previous study on soybean rhizobial population revealed high levels of genetic diversity among *B. japonicum* strains in Eastern Croatia (Sikora & Redzepovic, 2003). Hungria et al. (2006) genetically characterized 30 fast growing rhizobial strains using rep-PCR that were isolated from the nodules of different soybean genotypes in Brazil. In their study, eighteen reference strains and 22 isolates were used for cluster analysis. Eight strains produced either only two bands or no bands and were

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excluded from the cluster analysis. Seven strains clustered together at a similarity of 38% in one cluster. Another cluster included six strains clustered at 46% similarity. The remaining eight strains showed low relatedness to the reference strains. Overall, a high level of genetic diversity was revealed among the strains in their study. In an another study by the same group, the genetic diversity of 240 soybean rhizobia isolated from Brazilian Cerrados was examined using rep-PCR fingerprinting with BOX primers. A high level of genetic diversity was observed among the strains isolated from 12 different sites (Loureiro *et al.* 2006). Doignon-Bourcier et al. (2000) examined the genotypic diversity of 64 *Bradyrhizobium* strains isolated from nodules of 27 native leguminous plant species in Senegal (West Africa). Fifty-three reference strains of the different *Bradyrhizobium* species and described groups were included for comparison. Substantial diversity was discovered among the strains. In Paraguay, high levels of diversity were detected among the 78 isolates from nodules of field-grown soybean from 16 sites located in the two main producing states with most isolates representing unique strains (Chen *et al.*, 2000). Musiyiwa *et al.* (2005) studied the physiological diversity of indigenous rhizobia nodulating promiscuous varieties of soybean in Zimbabwean soil. There results indicate high genetic variability among the indigenous soybean rhizobia. Prakash & Annapurna (2006) studied the genetic diversity of 69 isolates from the nodules of four soybean varieties that were adapted to Indian soil. There results showed that high level of genetic diversity exists among the indigenous *B. japonicum* populations in Indian soils. Lima et al. (2005) studied the phenotypic diversity and symbiotic efficiency of *Bradyrhizobium* sp. strains from Amazonian soils. There findings shows that native populations comprise of highly diverse strains of *Bradyrhizobium* with variable symbiotic

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efficiency. Gao et al. (2001) studied genetic diversity of rhizobia isolated from *Astrgalus adsurgens* from eight northern provinces in China. They found high genetic diversity exists among the isolated rhizobia. High phenotypic diversity was found among the 56 rhizobia strains isolated from root of two chickpea cultivars growing in soils from different areas in Morocco (Maatallah *et al.,* 2002). Genetic diversity of forty five bradyrhizobial isolates that nodulate several *Lupinus* and *Ornithopus* species in different geographical locations was investigated by Jarabo-Lorenzo et al. (2003). There analysis showed an enormous diversity among the isolates. Madzak et al. (1995) investigated the diversity among the field populations of *B. japonicum* isolated in Poland. They found that that genetically diverse *B. japonicum* population could be isolated from some polish soils but at most of the sites where there was no previous cultivation of soybean, no soybean nodulating bacteria was found. Taurian et al. (2005) found that heterogeneity exists among the members of rhizobia nodulating *Arachis hypogaea* L. in central Argentina soils.

The results of our study are in agreement with previous studies on genetic diversity of rhizobia that shows that the rhizobial population evolves quickly in the soil. In western Canada, soybean is an introduced crop and its rhizobia have been introduced as inoculants (Beversdorf *et al.*, 1995). Substantial diversity amongst the nodule population has been generated quite rapidly within few decades. Diversification of rhizobia could results from mutation, conjugation and transduction events (Martinez *et al.*, 1990). The adaptation can be acquired by horizontal gene transfer among the populations of *B. japonicum* (Ochman & Moran, 2001). The most convincing example of lateral transfer of

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genetic information in the field is reported by Sullivan et al. (1995). In their study, horizontal gene transfer was reported for four non-symbiotic *Rhizobium loti* strains which had acquired a 'chromosomal island' from an inoculant strain confirming that horizontal gene transfer occurred from inoculant strain to the indigenous population of *R. loti*.

5.1. Influence of inoculants on genetic diversity of *Bradyrhizobium japonicum* populations in western Canada and the northern US.

Significant difference in the levels of diversity from two geographical regions (US and Canada) was revealed by proximity analysis (Kruskal-Wallis test, $p<0.05$). Also, some isolated patterns were observed in cluster analysis of ERIC and REP dendrogram where some US and Canadian isolates were grouped into separate clusters. Some previous studies were able to group isolates obtained from different sites on the basis of their geographical origin (Dalmastri *et al.,* 1999; Zhang *et al.*, 1999). The results of our study indicate that some of the existing population of *B. japonicum* could have developed from the inoculant strains used commonly in the region. Strain 532C is commonly used commercial inoculant strain in Canada. Strain 532C was originally isolated from soils in Ontario, Canada (Hume & Shelp, 1990). The profiles of some of the isolates from site Winnipeg in Manitoba were found to have relatively high similarity to 532C (REP profile of isolate W6C from site Winnipeg, MB is 82 % similar to 532C strain and ERIC profile is 62 % similar to 532C strain) indicating that the substantial diversity amongst the *B. japonicum* populations at this site might have originated from 532C or other inoculant strains commonly used in western Canada. However, the REP fingerprint of one isolate

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from site Elm Creek, MB in Canada was found to be more close to strain USDA 110 (82 % similar). USDA 110 is an inoculant strain that originated in US and is commonly used in American inoculants (Coutinho *et al.*, 1999). Nonetheless, the control strains 532C and USDA 110 did not show higher similarity to other isolates. Use of different inoculants in the soybean growing region of the northern US and western Canada might be an important factor that could possibly influence genetic diversity of *B. japonicum* populations in our study. These finding also suggests that currently used commercial strains (532C and USDA110) change quite rapidly after being introduced into the soil and indigenous populations of *B. japonicum* have significantly diverged from the inoculant strains. Sikora & Redzepovic, (2003) also found that soybean rhizobial isolates from eastern Croatia have significantly diverged from the inoculant strains that were previously used to inoculate the soybean crops. Batista et al. (2007) studied the variability in conserved and symbiotic genes of *B. japonicum* and *B. elkalani* inoculant strains seven years after they were introduced into a Brazilian Cerrados soil along with soybean host plants. They isolated 263 isolates from the nodules. Only 6.4% of the isolates showed high similarity to the inoculant strain CPAC 15 and none of the isolates were similar to CPAC 7 which were used as control strains in their study and are commonly used commercial inoculant strains in Brazilian Cerrados. They concluded that the high genetic variability is certainly related to the plasticity of the *Bradyrhizobium* genome and was accelerated by interaction with the host plant, adaptation to the environment and agricultural practices. Variability in their study appears to have resulted from a variety of events including strain dispersion, genomic recombination, and horizontal gene transfer.

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5.2. Influence of agriculture practices on genetic diversity of *Bradyrhizobium japonicum* populations in western Canada and the northern US.

In our study, we have found significant difference in the levels of diversity from two geographical regions (US and Canada) as revealed by the proximity analysis. This was verified by Kruskal-Wallis test where significant difference was found between the diversity index of isolates from the US and Canada ($p<0.05$). In a previous study on genetic diversity of Maize-root associated *Burkholderia cepacia*, Dalmastri et al. (1999) were able to group strains obtained from distinct soils on the basis of their origin. Zhang et al. (1999) were able to group bradyrhizobial isolates from different sites in China according to their geographical origin. However, some previous studies on genetic diversity of rhizobia were unable to correlate genetic diversity to geographical locations (Laguerre *et al.*, 1997). Wong et al. (1994) analysed diversity among isolates of *B. japonicum* and rhizobia, respectively and found that the strains isolated from distant places showed similar levels of diversity compared to rhizobia isolated from close places. Jarabo-Lorenzo et al. (2003) studied the genetic diversity of 45 bradyrhizobial isolates of *Lupinus* and *Ornithopus* species collected from diverse geographical origin and did not find correlation among genomic background and geographical location. In a study on genetic diversity of fast growing soybean rhizobia isolated from three different geographical regions (Papua New Guinea, China and Vietnam), levels of diversity of rhizobia was found unrelated to the geographical sites of isolation (Saldana *et al.*, 2003)

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Difference in the level of diversity of isolates from the US and Canada in our study point toward the influence of agricultural practices on genetic diversity of *B. japonicum* populations isolated from two geographical regions. Factors like crop rotation (Lupwayi *et al.,* 1998), application of nitrogen fertilizers (Hardarson *et al.,* 1984) and plant cultivar (Weaver *et al.,* 1972, Zhang *et al.*, 1999) have been shown to affect rhizobial diversity in the soil.

In the northern US, soybeans are most commonly grown in a crop rotation with corn and also with winter wheat (Padgitt *et al.*, 2000). At most of the sites considered in this study in western Canada, soybean is grown in crop rotation with different crops mostly with cereals including wheat, barley, oat (Erin Burton, *Philom Bios Inc.*, pers. comm.). Lupwayi et al. (1998) investigated the effects of tillage and crop rotation on the diversity and community structure of soil bacteria in the northern Alberta. Results of their study indicate that conservation tillage and legume-based crop rotations support diversity of soil microbial communities. The difference in crop rotation at the sites considered in this study in western Canada and the northern US could be a possible factor causing differences in diversity among the *B. japonicum* populations. Triplett et al. (1993) studied the effects of crop rotation on *B. japonicum* and *R. melolti* populations in Wisconsin soil. Their findings showed that populations of *B. japonicum* did not differ significantly unless the field had been planted continuously to corn and populations of *B. japonicum* are far more persistent during crop rotation than are *R. meliloti.*

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Nitrogen fertilizers are used at much higher rates in US than in Canada because of the subsidies provided on fertilizers to farmers (Figure 6). Application of nitrogen fertilizers affects nodulation (Scharf & Wiebold, 2003) and nitrogen fixation by rhizobia (Hardarson *et al.,* 1984). However, small doses of nitrogen have been found beneficial especially if the initiation of nodules is retarded (Mahon and Child, 1979). Gorissen et al. (1993) found negative correlation between the NH_4^+ concentration in the soil and root microbial numbers. They also observed that rhizosphere microbial population density of juvenile Douglas-fir significantly decreased upon application of ammonium sulphate to the soil. Hoflich *et al.* (2000) found that the leghemoglobin content of pea nodules, an indicator of nitrogen fixation activity was reduced by high nitrogen application in crop rotation. Different rates of application of nitrogen fertilizers witihin the field combined with other ecological factors might be an important factor in influencing the genetic diversity in the northern US and western Canada.

Plant cultivar is also suggested to influence rhizobial diversity. *B. japonicum* populations are found to increase in the presence of soybean crop (Weaver et al., 1972). Kucey & Hynes (1989) found that there was 10 folds increase in the *R. leguminosarum* bv. *viciae* in pea fields than in bean or cereals. Zhang et al. (1999) found strong influence of plant cultivar on the genetic diversity of bradyrhizobial strains isolated from the root nodules of two peanut (*Arachis hypoglea*) cultivars from four different sites in China. Chiarini *et al.* (1998) studied the influence of four maize cultivars on microbial population and did not found significant differences in bacterial community structures. Paffeti et al. (1996; 1998) suggested that plant genotype is an important and influential factor in determining the

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genetic diversity of *S. meliloti* populations in the field. The influence of cultivar on genetic diversity of soybean rhizobial populations has previously shown in other studies (Weaver *et al.*, 1972; Zhang *et al.*, 1999). Introduction of early maturing cultivars of soybean in western Canada within the last decade and their better adaptability to the environment in western Canada might be a possible cause leading to diverse populations of *B. japonicum* in the region.

5.3. Influence of environmental and edaphic factors on genetic diversity of *Bradyrhizobium japonicum* field populations.

In this study, there was no significant correlation found between the effects of climatic factors such as average annual and monthly temperature and mean annual and monthly precipitation on genetic diversity of *B. japonicum*. No significant correlation was found between the genetic diversity of *B. japonicum* isolates and ecological factors like previous crop grown in the field, year since last soybean grown and, use of inoculants. However, an interesting clustering pattern was revealed by the dendrogram and multidimensional scaling analyses showing that the diversity of *B. japonicum* at site Elm Creek in southern Manitoba is quite different from rest of the isolates considered in this study. This site revealed the least diverse population and low genetic variability was detected among most of the isolates by both ERIC and REP fingerprinting. In the cluster analysis of ERIC dendrogram, eight out of thirteen isolates had similar profiles (80% similarity or more).Cluster analysis of REP fingerprints also revealed similar results with six isolates from this site having similar profiles. The low genetic variability among the

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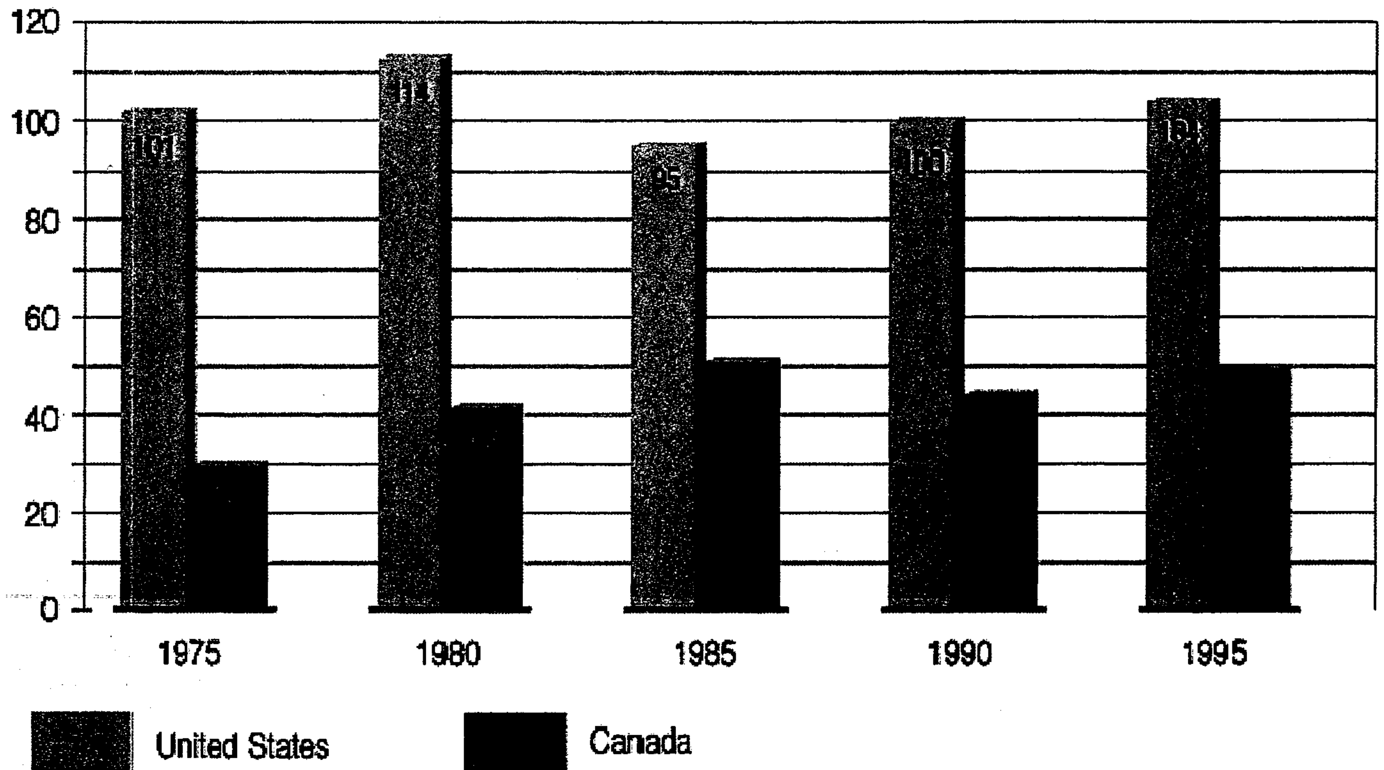
kg/hectare arable land/year



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isolates at this site suggests that the indigenous strains of *B. japonicum* are well adapted to the existing environmental conditions. This may be due to the unidirectional and predominating influence of variety of environmental forces which may have selected for a relatively narrow genetic spectrum among the existing population of *B. japonicum*. Elm Creek was the only site in our study that has sandy loam soil. The other environmental and ecological factors were mostly common to the other sites in western Canada. The low genetic variability and stability among the isolates might be due the influence of soil texture. Previous studies have shown that soil texture plays an important role in influencing the rhizosphere density (Hagen *et al.*, 1997). Chiarini et al. (1998) suggested that the percentage of sand in soil has a negative effect on the rhizosphere population density. Dalmastri et al. (1999) compared the effects of soil type, maize cultivar and root location on the microdiversity of root –associated *B. cepacia* populations using 180 bacterial isolates. In their study, they showed that the soil type has a major effect on the degree of genetic diversity of the maize-root associated *B. cepacia* populations. They confirmed the effect of soil type on genetic diversity of *B. cepacia* strains on the basis of clustering of the strains according to their origin. Their study also shows that the percentage of variation among populations was significantly higher among the maize cultivars planted in different soils. Latour et al. (1996) have shown that the phenotypic diversity of populations of flouroscent pseudomonads was largely influenced by soil texture and composition. Yohalem & Lorbeer (1994) studied the intraspecific metabolic diversity among 218 strains of *B. cepacia* and found the strong influence of soil type on metabolic diversity of *B. cepacia*. It has been suggested by Dalmastri et al. (1999) that " factors like soil type and metabolites produced by root system depending on the plant species might select not only

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some species among all those present in the entire rhizosphere, but, also within one species, some strains characterized by distinct haplotypes". High sand content in the soil such as that found at the Elm Creek site is more susceptible to desiccation stress and lower nutrient content (Bentham *et al.*, 1992; Uhlirova *et al.*, 2005). This may have led to the selection for a relatively narrow genetic spectrum among the existing population of *B. japonicum*. Further studies are necessary to determine the mechanisms by which soil texture may influence rhizosphere community and affects genetic diversity *B. japonicum*.

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Conclusions

Based on the cluster analysis and multidimensional scaling of REP and ERIC fingerprints, we conclude that there are high levels of genetic diversity among *B. japonicum* strains isolated from important soybean growing areas in western Canada and the northern US (North Dakota, South Dakota, and Minnesota). Most of the strains isolated from the US soils clustered together and a separate pattern was observed for most of the Canadian isolates. This spatial separation of diversity of two geographic regions suggests that the regulation of diversity is influenced by ecological and environmental factors. In our study, we have found that the soil texture could have an influence on the stability of isolates at site Elm Creek, MB in Canada. Desiccation stress and lower nutrient content conditions posed by the sandy loam soil texture might be an influencing factor at this site. This finding is in agreement with a previous study on maize- root associated *B. cepacia* (Dalmastri *et al.*, 1999). A less diverse site like Elm Creek in Manitoba may show better yield responses to a highly competitive inoculant because the introduced inoculant strain(s) would face less competition with indigenous populations. Continuous inoculation of the site for few years would assist in the successful establishment of effective commercial inoculant strains in the field. Future studies should compare influence of different soil texture on genetic diversity of soybean rhizobia field populations along with plant cultivars. This will help us in better understanding ecology of soybean rhizobia.

This study has provided the very first assessment on the genetic diversity of *B. japonicum* populations from the soils of southern Manitoba in Canada. The findings of this research

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would help in implementing and developing effective strategies that could lead to increase soybean production in western Canada and which could also significantly influence the crop production in other soybean growing regions of the world. This knowledge is also required for the development of effective strategies for delivery and maintenance of exogenous microorganisms in association with root systems. This study also demonstrated the successful application of rep-PCR fingerprinting as a tool for strain identification. Studies has shown the utility of rep-PCR genomic fingerprinting in identification and classification of bacteria, and for molecular epidemiological studies of human and plant pathogens (van Belkum *et al.,* 1994; Louws *et al.,* 1996, Schneider & deBrujn, 1996). Thus, rep-PCR genomic fingerprinting in combination with other molecular techniques could be successfully used by inoculant companies as a tool for the protection of intellectual properties. In future studies, genetic diversity of soybean rhizobia should be covered on broader scale. Sampling rhizobia across various soybean growing regions of the world and using more like Argentina, Brazil, India, China, and U.S etc could help researchers in identifying the impact of various ecological and edaphic factors that could possibly influence the genetic diversity of soybean rhizobia in general. Future studies should use more reference strains (commercial inoculant strains) in their study to examine the effects of inoculant use on rhizobial diversity. The finding of this study, along with the companion study by another colleague on testing the infectiveness and effectiveness of these isolates on soybean plants under field house conditions, have provided *Philom Bios Inc.* with four competitive strains that could be successfully developed into commercial inoculants strains. The assessment provided on genetic diversity of *B. japonicum*

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populations in western Canada in this study would help the farmers, agronomists and inoculant companies to design better strategy for inoculant application within the region.

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APPENDIX

Appendix A: Gel images

All gel images are included in this section. The lanes where there was no amplified product was found and where the bands were not clear or very weak were not analyzed for this study.

Figure I a and I b.

Gel sequence:

Lane	Isolate
1	1kb DNA ladder
2	532C
3	USDA 110
4	2
5	6
6	7
7	9
8	13
9	16
10	17
11	19
12	20
13	23
14	24
15	26
16	27
17	29
18	31
19	32
20	33
21	35
22	1kb DNA ladder

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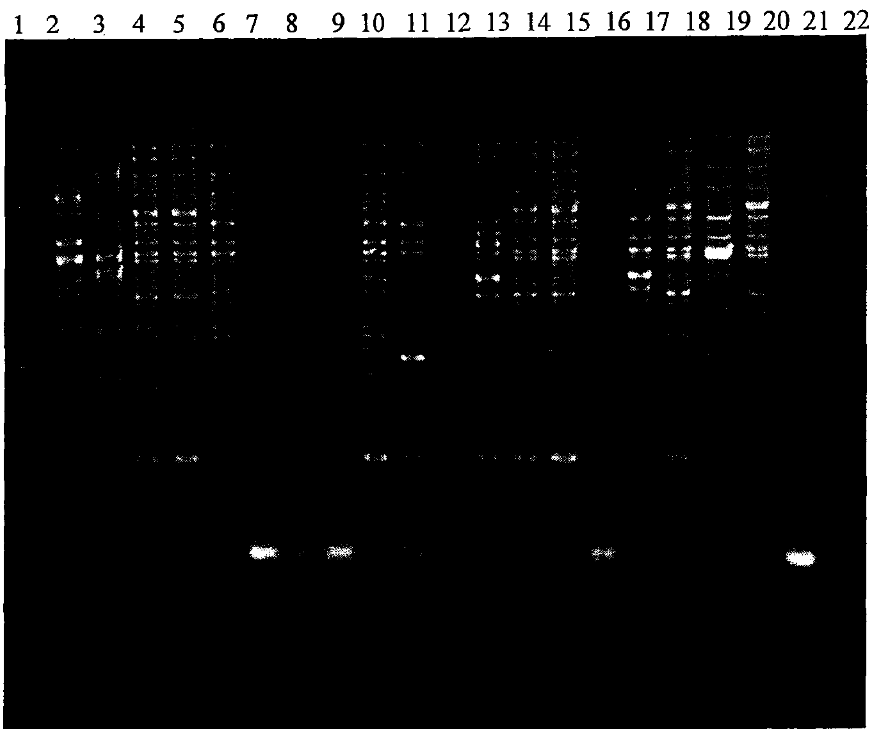

Figure I a. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

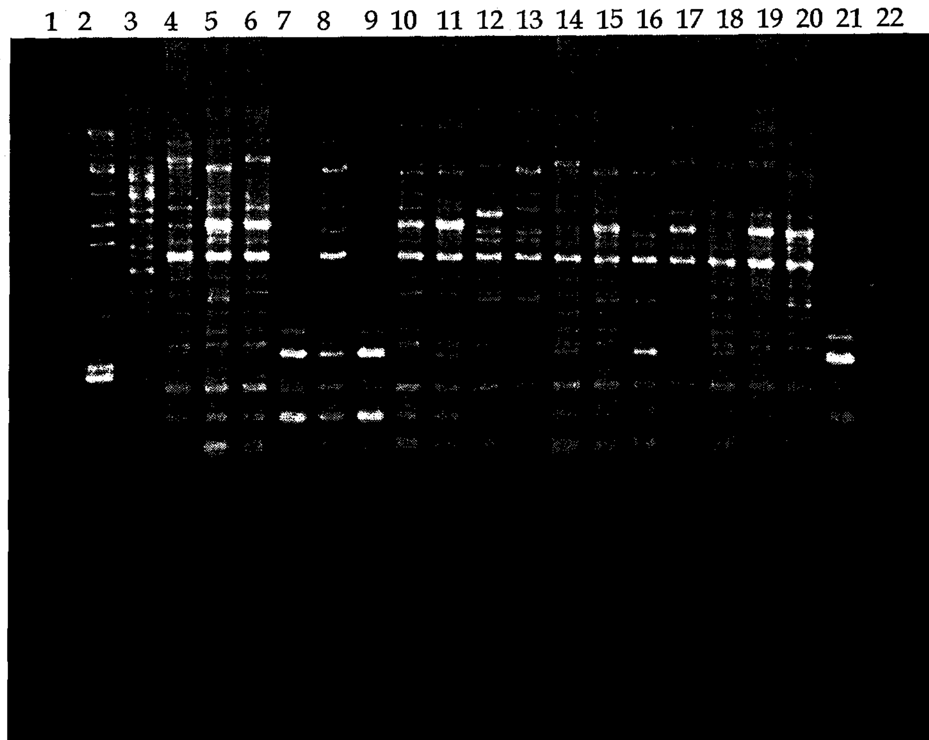

Figure I b. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

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Figure IIa and II b.

Gel sequence:

Lane	Isolate
1	1kb DNA ladder
2	532C
3	USDA 110
4	36
5	37
6	38
7	39
8	40
9	42
10	43
11	44
12	46
13	47
14	48
15	49
16	52
17	53
18	54
19	55
20	56
21	57
22	1kb DNA ladder

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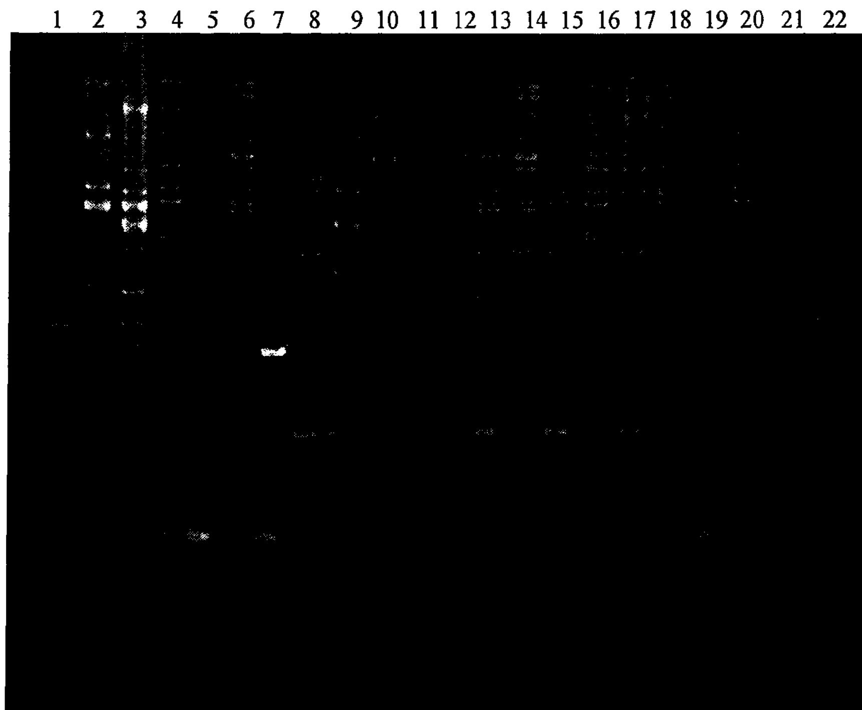

Figure Ia. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

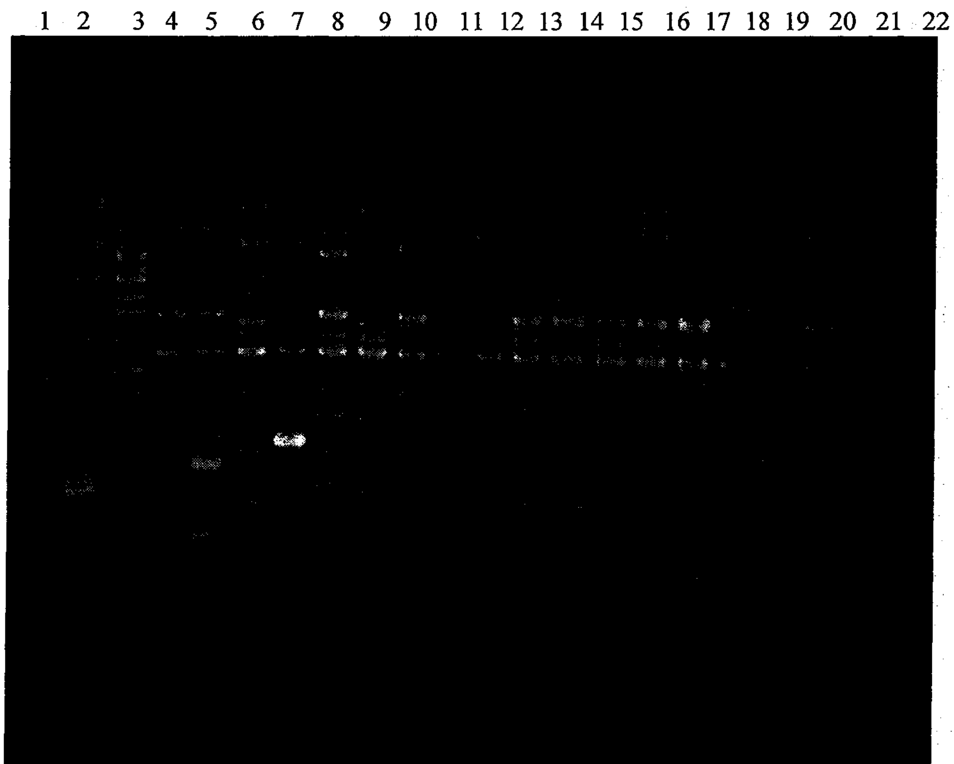

Figure II b. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

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Figure III a and III b.

Gel sequence:

Lane	Isolate
1	1kb DNA ladder
2	532C
3	USDA 110
4	58
5	59
6	60
7	62
8	63
9	64
10	65
11	66
12	68
13	71
14	72
15	73
16	SF 3A
17	EC 1A
18	W 5A
19	W 6A
20	W 6C
21	U23
22	1kb DNA ladder

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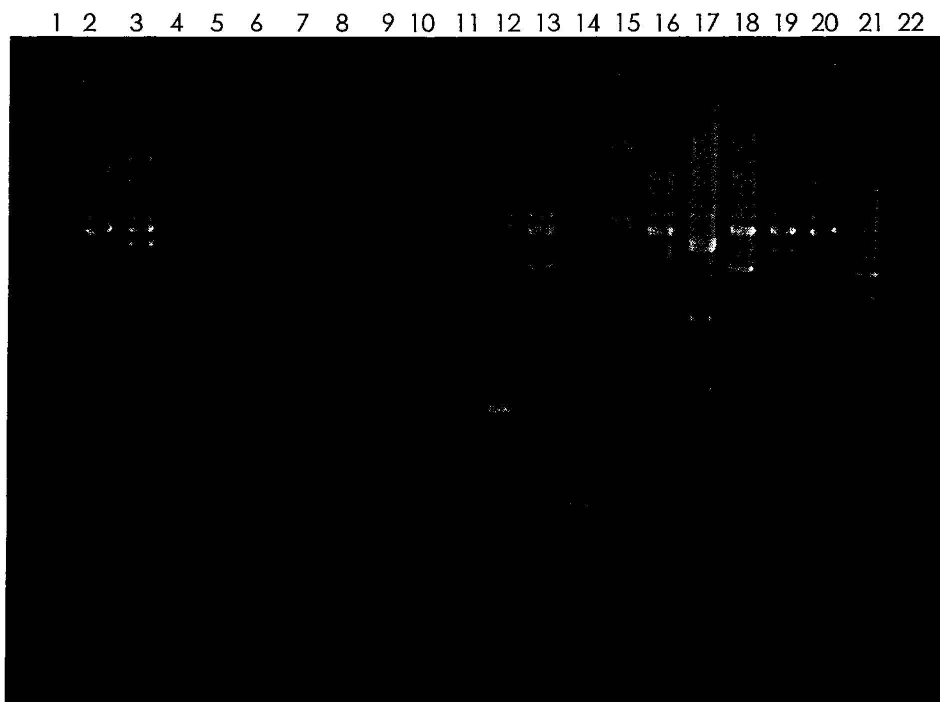

Figure IIIa. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

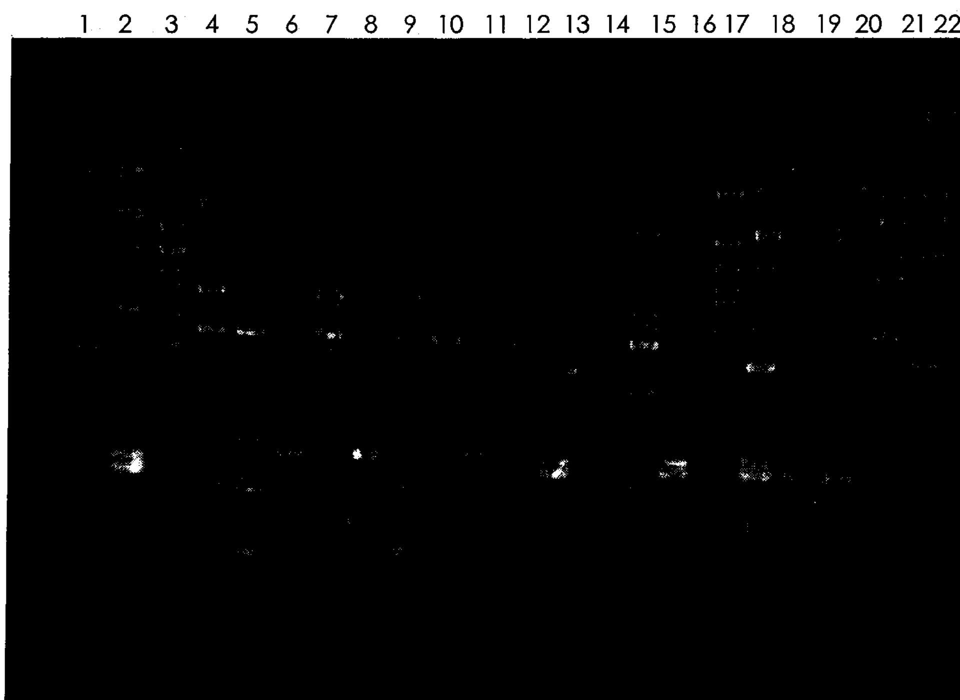

Figure IIIb. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

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Figure IV a and IV b.

Gel sequence:

Lane	Isolate
1	1kb DNA ladder
2	532C
3	USDA 110
4	U24
5	U43
6	U51
7	U55
8	U61
9	U62
10	U64
11	U65
12	U71
13	U92
14	U123
15	U124
16	U125
17	U135
18	U142
19	8
20	10
21	15
22	1kb DNA ladder

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Figure IVa. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Figure IVb. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

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Figure Va and Vb.

Gel sequence:

Lane	Isolate
1	1kb DNA ladder
2	532C
3	USDA 110
4	EC 1C
5	EC 2A
6	EC 2B
7	EC 2C
8	EC 3A
9	EC 3B
10	EC 3C
11	EC 4C
12	EC 5A
13	EC 5B
14	EC 5C
15	U22
16	U61
17	U73
18	U82
19	U85
20	U91
21	U93
22	1kb DNA ladder

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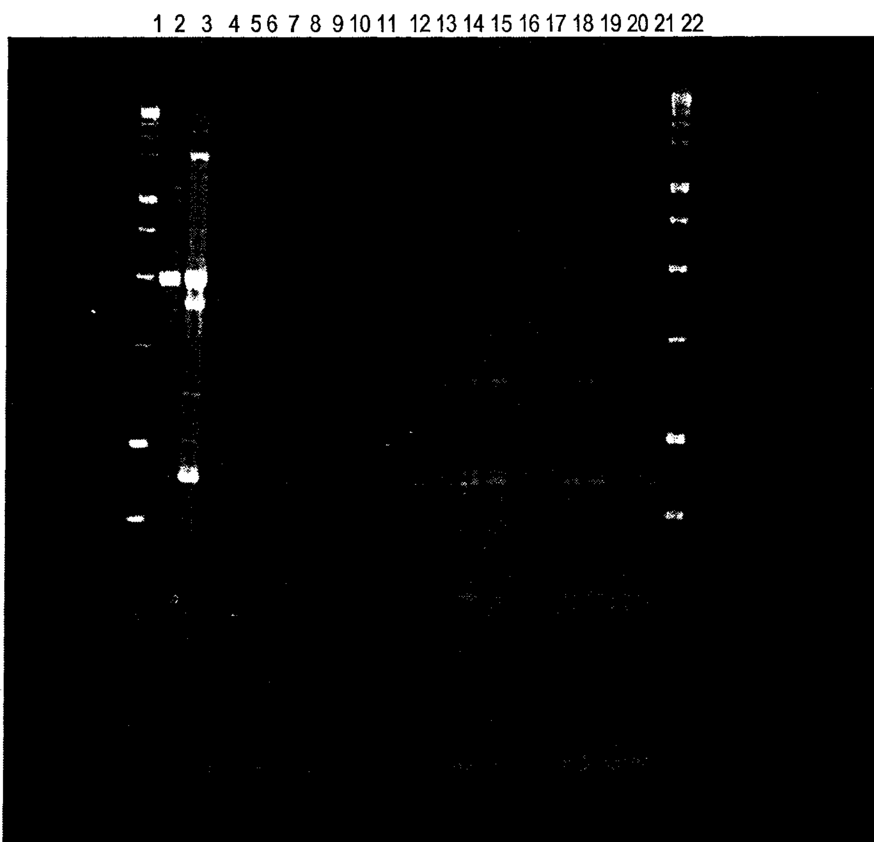

Figure Va. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

Figure Vb. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

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Figure VIa and VI b.

Gel sequence:

Lane	Isolate
1	1kb DNA ladder
2	532C
3	USDA 110
4	5
5	8
6	10
7	12
8	15
9	16
10	18
11	33
12	38
13	40
14	47
15	49
16	50
17	53
18	57
19	62
20	67
21	69
22	1kb DNA ladder

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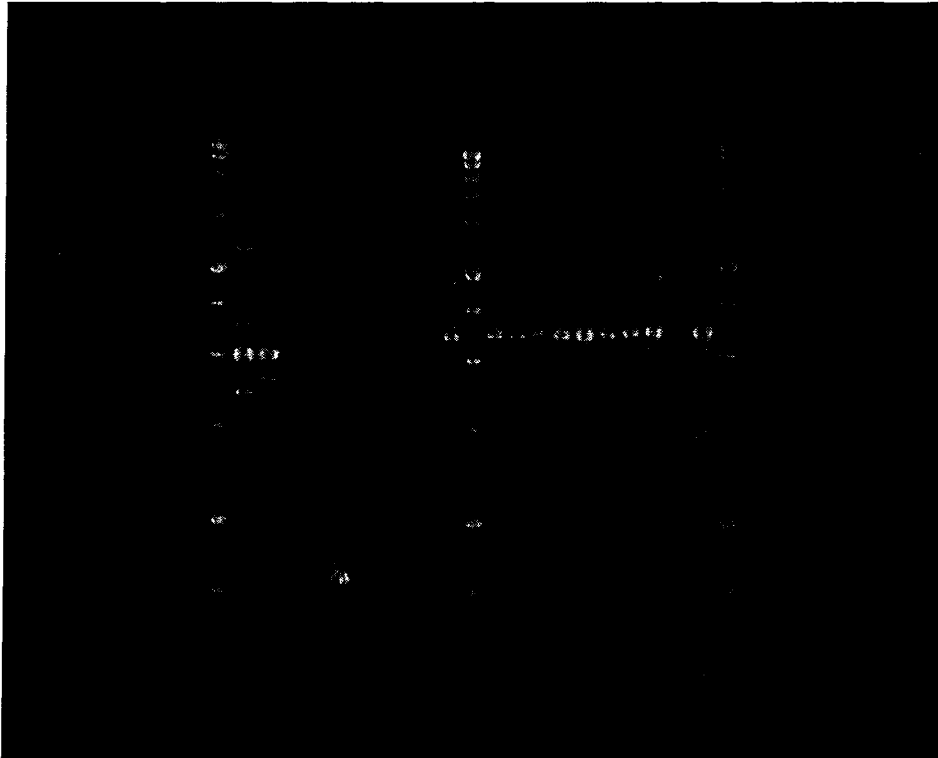

Figure VIa. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

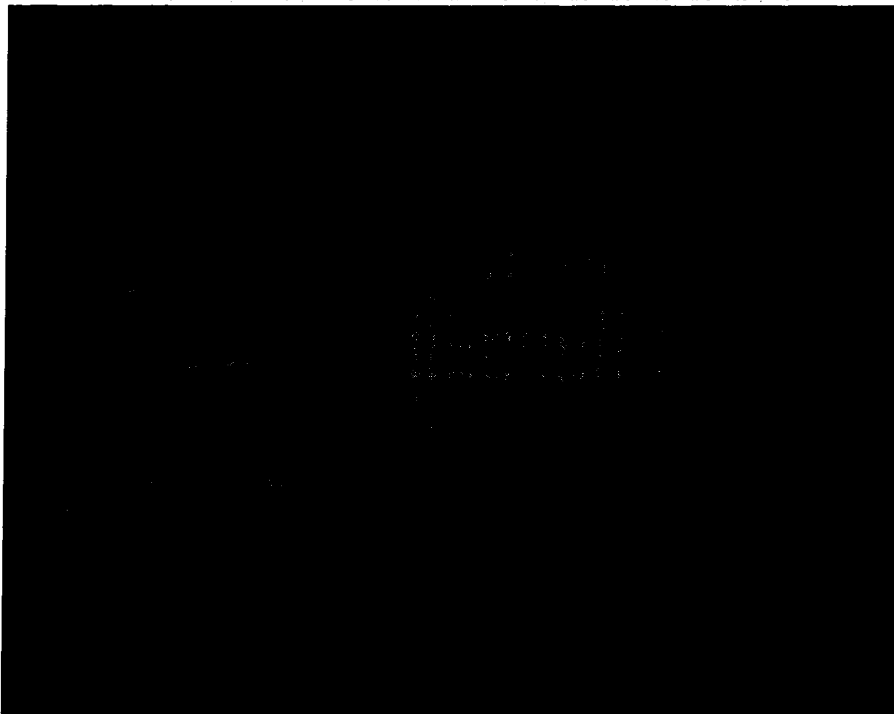

Figure VIb. ERIC fingerprints for *Bradyrhizobium japonicum* isolates

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Figure VIIa and VII b.

Gel sequence:

Lane	Isolate
1	1kb DNA ladder
2	532C
3	USDA 110
4	1
5	3
6	4
7	9
8	11
9	14
10	21
11	22
12	26
13	28
14	30
15	34
16	35
17	39
18	41
19	51
20	61
21	70
22	1kb DNA ladder

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