ABSTRACT

MOTHAPO, NAPE VICTORIA. Nodulation and Rhizobia Diversity Associated with Distinct Hairy Vetch Genotypes. (Under the direction of Dr. Julie Grossman).

Presence of effective rhizobia strains in soils is essential for nodulation and enhanced biological nitrogen fixation (BNF) of the cover crop hairy vetch (*Vicia villosa* Roth, HV). This study evaluated nodulation effectiveness, BNF efficiency and genetic diversity of resident *Rhizobium leguminosarum* biovar *viciae* (Rlv) from 6 paired fields, three with HV cultivation history and three with no history. Ten distinct HV genotypes were used to trap rhizobia from each soil over a 6-wk period in a growth chamber. Nodulation efficacy was equated to nodule number and mass, and BNF efficiency to shoot nitrogen. Significant effects of both HV cultivation history and genotype on all nitrogen fixation parameters were found, except for lack of significant effect of genotype on plant biomass. Nodule numbers were significantly higher in all fields with HV history, and nodule mass in fields with HV history in two of the three sites. On average there were 60% more nodules and 70% greater nodule mass, in fields with history compared to fields without history. As with nodulation, plants inoculated with soil dilutions from HV history fields had significantly higher plant biomass and plant tissue N than those inoculated from fields without history, except one site where no difference in N was found between fields. These fields were in close proximity, suggesting that rhizobia population mixing may have occurred. Plant biomass and tissue N were linearly correlated to nodule mass (r^2 = 0.80 and 0.50, respectively), while correlation to nodule number was low (r^2 = 0.50 and 0.31 for biomass and N

respectively), indicating nodule mass to be a better indicator of symbiotic efficiency than number. A total of 519 Rlv strains were isolated from the root nodules of the HV genotypes. Repetitive element polymerase chain reaction (rep-PCR) with BOX-A1R primer showed that diversity was most impacted by site, within a site however, history of HV cultivation was the major driver of diversity. Cluster analysis of BOX-PCR banding patterns resulted in 36 genetic groups of Rlv at a similarity level of 70%, with 15 of the isolates from fields with HV history and 2 from fields without history not belonging to any of the clusters. The biggest cluster comprised 96 strains, 86 of which were from the Cedar Grove site. This particular site was contained many strains grouping at >90% similarity level, indicating a high level of similarity among strains and suggesting low rhizobia diversity. Except in one site, large clusters comprised at least 65% of strains from fields without history, suggesting lower diversity in fields with such histories. Although different strain profiles were sometimes obtained from distinct HV genotypes, genotypes appeared to have little or no effect on diversity of Rlv isolated. These results suggest that although rhizobia compatible with hairy vetch occur naturally in soils, past cultivation of HV enhances diversity of effective Rlv populations capable of high nodulation and effective N fixation. Information gained from this study emphasizes the important of legume cover crops in agricultural systems.

Nodulation and Rhizobia Diversity Associated with Distinct Hairy Vetch Genotypes

by
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DEDICATION

To my parents, Legodi and Raesetja Mothapo.

BIOGRAPHY

Nape Mothapo was born and raised in Limpopo Province, South Africa. She completed her Bachelor of Science degree in Agriculture majoring in Soil Science at the University of Limpopo. Her need to know what is meant by a developed country, coupled with desire for learning outside her country of birth, invoked Nape's application for Fulbright Scholarship. Upon receiving the scholarship, she embarked on a mission towards a master degree at North Carolina State University (NCSU). Following her M.S. completion, Nape will continue in a PhD program at NCSU characterizing nitrous oxide-producing fungi in different agricultural systems. Nape's long-term goal, alongside agricultural research, is to work as a policy advisor for agriculture in South Africa.

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Nodulation and rhizobia diversity of legume cover crops in agricultural systems, a review.

1. Introduction

Global population growth and food security, as well as increasing environmental and economic concerns associated with conventional farming, have necessitated development and adoption of alternative food production practices. Sustainable soil fertility management is a key to food production without compromising environmental stability (Brussaard et al. 2007; Lal 2009). Historically, cover crops have played an important role in soil fertility, particularly through addition of biologically fixed nitrogen (N) to the soil, enhancement of soil organic matter and prevention of nutrient leaching (Lyon and Wilson 1928). A cover crop is a non-harvested plant grown to improve soil fertility by reducing soil erosion and nutrient leaching (USDA 2009). Cover crops are often called 'green manures' when they are used specifically for nutrient and organic matter contribution, in which case they are plowed under and incorporated into the soils before maturity. The improvement of crop growth through legume production has been understood for many decades (Lyon and Wilson 1928) and although some studies have shown limited adoption of cover crops in certain areas (Mallory, Posner and Baldock 1998), there has been increased use in recent years due to their agroecological benefits (Blackshaw 2008; Fageria et al. 2005; Peoples et al. 2009 and Snapp et al. 2005).

Benefits derived from cover crop use depend on the needs of the grower, plant species, and the environment in which they are produced. Many soil properties including soil mineral N and pH, and management practices such as tillage and fertilization, influence growth and biomass production of legume cover crops, making it

critical that careful attention be paid to species selection within any agroecological system. Cereal cover crops such as barley (*Hordeum vulgare*), oat (*Avena sativa*) and rye (*Secale sereale*) are particularly important in reduction of nitrate leaching (Blackshaw 2008; Macdonald et al. 2005; Wang et al. 2005). Other benefits of cover crop use include weed control, reduction in soil loss, organic matter contribution (Lenzi et al. 2009; Sainju et al. 2002), and soil moisture conservation (Wang et al. 2005; Dabney et al. 2001). Weed suppression and erosion reduction during non-cash crop periods of the year is a common desired outcome of cover crop production, and both legume and cereal cover crops have been shown to have high rates of weed control (Brandsaeter and Netland 1999; Creamer and Baldwin 2000; Reddy 2001). Rye in particular has been shown to produce large amounts of biomass and have high weed suppression abilities (Blackshaw 2008).

Legume cover crops, including clover (*Trifolium* spp.), hairy vetch (*Vicia villosa*) and pea (*Pisum sativum*) fix atmospheric N through the process of biological nitrogen fixation (BNF). Such crops can be a significant source of N in organic agriculture and low-input farming systems, important because organic certification prohibits use of synthetic chemicals such N fertilizers derived from Harbor-Bosch (Bellow, 2005). This literature review summarizes the importance of symbiotic BNF between legume and rhizobia in farming systems and describes how nodulation and rhizobia diversity are modified by prevailing environmental and imposed agronomic conditions in agroecosystems. It will focus on the symbiotic association between hairy vetch (*Vicia villosa)* and its rhizobia partner, *Rhizobium leguminosarum* biovar *viciae* (Rlv).

2. Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is a natural process through which several species of bacteria (Galloway et al. 2004) convert atmospheric nitrogen into plant available nitrogen, usually ammonium (NH_4). Total biologically fixed N from all ecosystems is estimated at 122 Tg N yr^{-1} (Burris, 1980), with cultivated agricultural systems contributing about 25%, or 32 Tg N yr^{-1}, of the total BNF (Galloway et al. 2004). Nitrogen is an essential and often limiting plant nutrient in crop production. Nitrogen fixation resulting from mutual symbiosis of rhizobia and cultivated legume plants is therefore critical to food security as it directly affects agricultural production. Although the global contribution of non-harvested legumes such as cover crops is unknown, soybean, the major crop legume globally, has been estimated to fix 16.4 and 5.7 Tg N yr^{-1} globally and in the United States respectively (Herridge et al. 2008). Amount of N fixed varies widely within and between different legume cover crops, depending on species and environment (e.g. soil pH) and management (e.g. inoculation). Legume cover crops have been reported to fix up to 450 kg ha^{-1} yr^{-1} (Unkovich and Pate 2000).

There is a large body of literature documenting the usefulness of hairy vetch as a cover crop for improving and sustaining soil nitrogen (N) in agricultural systems (Anugroho et al. 2009; Kuo and Sainju 1998; Power et al. 1991; Utomo et al. 1990). Native to Europe or Western Asia (Undersander et al. 1990), the species has been domesticated in various geographic areas. Recently, varieties with specific plant characteristics have been developed, such as the Madison variety, developed in

Nebraska to have greater cold tolerance; and Purple Bounty, developed to be early flowering and high in biomass production (Maul et al. 2011; USDA 2009). Total biomass N in hairy vetch ranges from 100 kg ha^{-1} to 240 kg ha^{-1}, with N derived through fixation of up to 150 kg ha^{-1} (USDA 2009; Wagger 1989).

3. Nodulation

The legume-rhizobia symbiosis is highly specific (CleyetMarel et al. 1996); (Denarie et al. 1992) and depends on complex signaling processes between the host plant and rhizobia partner. Symbiotic N fixation between legumes and rhizobia takes place in plant-derived root organs called nodules, and competent nodulation is critical for efficient BNF. Molecular dialog or signal exchange between the legume and rhizobium (Denarie et al. 1993) is a complex process that involves both the legume symbiotic (*sym*) genes and the rhizobia nodulation (*nod*) genes. In the beginning of the signaling process, legumes exude flavonoid compounds into the rhizosphere, which then trigger soil dwelling rhizobia to release highly specific reverse signal molecules, *nod factors*, only comprehended by specific legume species (Cooper 2004) to initiate nodule formation. Rhizobia strains have a defined group of legumes species, or host range, with which they can nodulate, and in parallel, legumes select for specific rhizobia partner species (Denarie et al. 1992). For example, *Rhizobium leguminosarum* biovar *viciae* (Rlv) nodulates plant species belonging only to tribe *viciae*, which includes the genera *Vicia*, *Pisum*, *Lathyrus*, and *Lens*. Soybean is primarily nodulated by *Bradyrhizobium* spp., however some *Rhizobium* species also nodulate soybean hosts (Yamato et al. 1997). Specificity can be variable among legumes and rhizobia, with

some legume species such as *Phaseolus vulgaris* known to associate with a wide range of rhizobia (Andrade 2002). Nodulation, defined here as the number of nodules formed and the total nodule mass contained on a plant, is most related to soil rhizobial population size, with high nodulation occurring where compatible rhizobial population is high (Patrick and Lowther 1995). While nodulation success is vital to BNF efficiency, excessive nodule formation without an associated increase in N fixed can be detrimental to plant growth as this process is energy driven and uses a large percentage of the host plant's photosynthetic production to fuel the fixation of N. Hence, most legume hosts have a mechanism to control the number of nodules and zone of nodule development called autoregulation of nodulation (Oka-Kira and Kawaguchi 2006). Legume hosts that lack this regulatory system are characterized by excessive nodule numbers and are said to be hypernodulating mutants (Ferguson et al. 2010; van Brussel et al. 2002).

Soil properties have been shown to affect legume nodulation either by impacting rhizobia population sizes and diversity, or interfering directly with the process of nodule formation. Soil acidity has been reported by several researchers as one of the main factors influencing rhizobial population size (Chemining'wa and Vessey 2006; Evans 2005; Lapinskas 2007), with extremely low population sizes of 10 cells g^{-1} soil or less being observed in cropped unlimed acidic soils (Coventry and Hirth 1992). Others have reported positive effect of liming on rhizobial cells (Roesner et al. 2005), with liming being shown to improve both size of soil rhizobial populations and nodulation (Andrade et al. 2002b; Staley 2002). Nodulation can also be restricted at very low soil moisture

levels (Leung and Bottomley 1994; Mahmood and Athar 2008), as moisture is necessary for overall plant germination and growth, and rhizobia viability, and also indirectly affects other soil properties such as salinity and soil temperature. The vetch-nodulating rhizobia species Rlv, in particular, when subjected to heat and moisture stress has been found to conserve its overall population size, but lose symbiotic efficiency with faba bean (Abdalla and Wahab 1995).

Agricultural management practices have also been shown to affect nodulation. One common management tool practiced by growers is inoculation of legume seeds with compatible rhizobia strains at planting with the goal of increasing nodulation success. In hairy vetch, inoculation with Rlv has been shown to improve productivity (Chemining'wa and Vessey 2006; Toro 1996). However, resident soil rhizobia, including indigenous rhizobia and those naturalized through past inoculation, have also been shown to compete for nodule occupancy with introduced rhizobial strains, impacting inoculation success (Thies et al. 1991; Toro 1996; Denton et al. 2002). Symbiotic efficiency of *Medicago sativa* and *Trifolium* legume species with resident rhizobia have been measured to range from -6 to 82% and from 10 to 130%, of BNF with commercial inoculants, respectively (Ballard and Charman 2000), suggesting that resident rhizobia can be equally, or more efficient at BNF than inoculant strains.

Previous legume planting has also been shown to improve nodulation, with fields previously grown to pea having significantly higher nodulation than fields without pea cultivation history (Chemining'wa and Vessey 2006). Tillage is a common practice in agricultural systems, serving to improve soil for seeds germination and

emergence, and also as a weed control measure. No-till systems have been shown to improve size and effectiveness of rhizobial populations (Andrade et al. 2003; Ferreira et al. 2000; Kaschuk et al. 2006; Snapp et al. 2005), conceivably by increased organic matter content and reduced soil and water loss. Synthetic agricultural inputs applied to soil, such as herbicides, Haber-Bosch N fertilizers, or pesticides can also affect rhizobial populations and legume nodulation (Caballero-Mellado and Martinez-Romero 1999; Depret et al. 2004), with negative effects such as reduction in rhizobial cells and nodulation being more common (Abdalla and Omar 1993; Bunemann et al. 2006; Niewiadomska 2004).

4. Rhizobia diversity

Many of the soil properties and agricultural management practices that affect nodulation also affect the genetic diversity of rhizobia found in a given site. For example, soil texture, and specifically high clay soils, has been observed to reduce rhizobia diversity in soybean cropping (de Fatima Loureiro et al. 2007). As with rhizobia population size, soil pH is also a driving factor in determining rhizobia diversity, where low diversity of *Rhizobium leguminosarum* has been reported in acid soils compared to limed, soils (Andrade 2002; Lapinskas 2007). Studies have shown that soil pH can select for tolerant species or strains of rhizobia (Andrade et al. 2002a; Bala et al. 2001; Bala and Giller 2007), which suggests reduced population of acid-intolerant strains in low pH soils. Like nodulation, high rhizobia diversity has also been reported in no-till systems compared to conventional tillage systems (de Fatima Loureiro et al. 2007). Monoculture of a non-legume grain crop has been shown to

affect rhizobia diversity, where long-term maize monoculture reduced diversity of resident Rlv populations present in the soil, but high Rlv diversity was reported in long-term wheat monoculture (Depret et al. 2004). Further, Ferreira et al. (2000) reported that maize/wheat rotation reduced *Bradyrhizobia* diversity, while rotation with soybean increased diversity, demonstrating that the effects of monoculture are crop species-dependent and also that past cultivation of legume hosts can serve to increase diversity of compatible rhizobia.

5. Hairy vetch-rhizobia interaction

An understanding of hairy vetch interaction with compatible soil rhizobia is critical to improve its N contribution to agricultural systems. As previously indicated, hairy vetch is nodulated specifically by *Rhizobium leguminosarum* biovar *viceae* (Rlv), the same rhizobium that nodulates pea (*Pisum sativum*), faba bean (*Vicia faba*) and common vetch (*Vicia sativa*). Like many cultivated legumes, HV is commonly inoculated with Rlv during planting with the aim of increasing efficiency of nodulation, BNF, and biomass produced. While some studies on pea inoculation have reported lack of inoculation response (Ballard et al. 2004), others have reported higher symbiotic efficiency in inoculated systems (Chemining'wa and Vessey 2006). Improvement of BNF has focused largely on development of competitive high N fixing rhizobial strains, with little attention on the symbiotic potential of resident soil populations. Microbial genetics have a vital role in cell functioning, and to some extent control the amount of N a particular strain of rhizobium can fix in symbiosis with its host. These concerns make our understanding of nodulation and rhizobia diversity critical for improvement of

cover crop BNF in organic systems. This study sought to determine the effect of hairy vetch cultivation history on nodulation and diversity of Rlv across distinct hairy vetch genotypes. We hypothesized that: 1) nodulation and Rlv diversity would be higher in fields with HV cultivation history compared to fields without history and ii) nodulation would vary across hairy vetch genotypes with genetically diverse rhizobial populations associating with distinct genotypes.

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Chapter 2:

Cropping history affects nodulation and symbiotic efficiency of distinct hairy vetch genotypes with resident soil rhizobia

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Abstract

Presence of compatible rhizobia strains is essential for nodulation and BNF of hairy vetch (*Vicia villosa*, HV). We evaluated how past HV cultivation affects nodulation and nitrogen fixation across host genotypes. Five groups of HV genotypes were inoculated with soil dilutions from six paired fields, three with 10-yr HV cultivation history (HV+) and three with no history (HV-), and used to trap rhizobia. Nodulation efficacy was equated to nodule number and mass, and nitrogen fixation efficiency to plant nitrogen. Both HV cultivation history and genotype affected all nitrogen fixation parameters; with the exception of genotype effect on plant biomass. Plants inoculated with HV+ soil dilutions averaged 60% greater nodule number ($P<0.005$) and 70% ($P<0.005$) greater nodule mass. Such plants also had greater ($P<0.005$) plant biomass and plant tissue N than those inoculated with soil dilutions from HV- fields, except one site where no difference in N was found perhaps as a result of rhizobia population mixing between the fields. Plant biomass and tissue N were strongly correlated to nodule mass ($r^2 = 0.80$ and 0.50, respectively), while correlations to nodule number were low ($r^2 = 0.50$ and 0.31, respectively), indicating nodule mass to be a better symbiotic efficiency indicator than number. Although hairy vetch rhizobia occur naturally in soils, past cultivation of HV appears to enhance populations of effective rhizobia capable of high nodulation and effective N fixation.

1. Introduction

Hairy vetch (*Vicia villosa* Roth; HV) is widely used in agroecosystems as a cover crop, with benefits including erosion control, weed and pest suppression, and soil fertility improvement (Campiglia et al. 2010, Lu et al. 2000; Teasdale and AbdulBaki 1997). As a legume, a wide body of literature documents its specific usefulness to improve soil nitrogen (N) status (Anugroho et al. 2009; Kuo and Sainju 1998; Power et al. 1991; Utomo et al. 1990). Native to Europe or Western Asia (Undersander et al. 1990), the species has been domesticated in various geographic areas, including the United States where it is commonly used in organic agriculture as a nitrogen source. Varieties with specific plant characteristics have been developed for various regions, such as the Madison variety developed in Nebraska to have greater cold tolerance, and Purple Bounty developed to be early flowering and high in biomass production (Maul et al. 2011; USDA 2009).

Legume-rhizobia interactions are known to be a powerful determinant of BNF efficiency in legume species. Many cultivated legume cover crops are commonly inoculated with compatible rhizobia at planting with the aim of increasing efficiency of nodulation, BNF and yield. Inoculation is often recommended in environments where compatible rhizobia are absent, soil rhizobial population density has been reduced, or where the soil rhizobia are shown to be less effective and efficient (Chemining'wa and Vessey 2006). In some cases, resident soil rhizobia, including native rhizobia and those naturalized through past inoculation, have been shown to compete for nodule occupancy with introduced rhizobial strains, impacting inoculation success (Thies et al. 1991; Toro

1996; Denton et al. 2002). In contrast, resident rhizobia symbiotic efficiency, defined here as the quantity of N fixed by rhizobia occupying root nodules and transferred to the host plant, can vary greatly, and in some cases can have equal or greater efficiency than inoculant strains. Symbiotic efficiency of *Medicago sativa* and *Trifolium* species with resident rhizobia has been measured to range from -6 to 82% and from 10 to 130%, respectively, of that resulting from recommended commercial inoculants (Ballard and Charman 2000; Drew and Ballard 2010).

Total biomass N contributed by HV ranges from 100 kg ha^{-1} to 240 kg ha^{-1} (USDA 2009; Wagger 1989) depending in part on the efficiency of the rhizobia symbiosis (Toro 1996). An understanding of how hairy vetch interacts with resident soil rhizobia is critical to improve the contribution of hairy vetch nitrogen to agricultural systems. Hairy vetch is nodulated specifically by *Rhizobium leguminosarum* biovar *viceae* (Rlv), the same rhizobia species nodulating pea (*Pisum sativum*), faba bean (*Vicia faba*) and common vetch (*Vicia sativa*). While some studies show Rlv inoculation to improve HV productivity (Chemining'wa and Vessey 2006; Toro 1996), others have reported lack of Rlv inoculation response (Ballard et al. 2004). From these contrasting observations emerge questions about the effect of cropping practices; particularly past history and use of host legume, on the infective ability and symbiotic efficiency of resident Rlv populations.

Many soil properties including pH, texture and temperature, as well as agricultural practices such as inoculation, contribute to variation in BNF across the landscape (Drew and Ballard 2010; Toro 1996), with legume genotype and interaction

with resident rhizobia being a critical consideration. Previous studies have shown large variation in BNF efficiency both between (Wagger 1989; Kitou et al. 2010) and within (Drew and Ballard 2010; Unkovich et al. 1997) cover crop species. Efficiency of BNF has been shown to be higher in legume genotypes that are compatible with a wide range of rhizobial strains (Drew and Ballard 2010).

Compatible resident rhizobia can exist in fields that have never been cultivated to a given host legume due to the presence of native legume species, transfer from adjacent fields or as part of the native microbiological soil community. Rhizobia are saprophytic and in the absence of a host species utilize a wide range of organic soil compounds as energy sources, allowing inoculant rhizobia to survive in soils long after a host legume is absent. Introducing a legume host to an environment lacking past or recent cultivation history presents an alternative survival strategy for already existing rhizobial populations, and has been shown to modify population size and symbiotic efficiency of existing resident populations (Andrade et al. 2002b; Chemining'wa and Vessey 2006). We understand little about the compatibility of resident hairy vetch rhizobia with introduced hosts, or how past HV cultivation affects nodulation and N_2 fixation efficiency. The objectives of the study were to: i) determine the effect of hairy vetch cultivation history on nodulation and BNF efficiency of resident soil rhizobia, and ii) determine the nodulation and BNF efficiency of distinct hairy vetch genotypes with resident soil rhizobia. Our guiding hypothesis was that fields with history of HV cultivation will have higher nodulation and BNF efficiency than fields without cultivation history.

2. Materials and Methods

2.1. Site selection and soil sampling

Soil samples were originally taken from five farms with history of hairy vetch cultivation, with selection criteria determined as a farm that had at least five seasons of HV since 1990. Originally selected farm sites included locations of Asheville (2 farms), Graham, Cedar Grove and Ivanhoe, all located in North Carolina. The Asheville and Cedar Grove fields were inoculated with Rlv each season hairy vetch was planted, Graham was never inoculated, and Ivanhoe had not been inoculated since 2004 (Table 2-1). From each farm, one field never planted to hairy vetch nor observed to have wild hairy vetch plants (Personal communication with farmers), was also sampled. Sampling was carried out in February 2010, during which time all fields with hairy vetch history were currently planted to hairy vetch, in combination with pea and rye in Graham, rye in Cedar Grove, and monoculture in Ivanhoe. Three of the five total farms (Graham, Cedar Grove and Ivanhoe) were eventually selected for further analysis based on frequency of HV and similar pH range between fields of the same farm.

Using a 2.5 cm diameter soil probe, 40 soil cores were randomly collected from each field to a depth of 15 cm, and thoroughly mixed in a composite sample. Samples were then divided in two; one for use as a microbial inoculant and the other for total inorganic N determination and standard nutrient analysis. The microbial inoculant sub-sample was kept cool during transport to NCSU campus and thereafter stored at 4 ^{o}C until processing. All sampling materials were sterilized with 75% ethanol prior to sampling and during sampling and handling, taking precautions to avoid cross-

contamination of soils of different fields and sites. Soils for inorganic N determination were dried at 45°C for 2 days, ground and sieved to pass a 2mm screen. For inorganic soil nitrogen (NH_4^+ and NO_3^-) analysis, samples were extracted using 1M KCl and shaken for 1 hour. The samples were allowed to settle for 20 minutes before filtration through a #42 Whatman filter paper. Extracts were frozen until analysis for NH_4^+ and NO_3^- on a QuikChem 2000 flow injection autoanalyzer (Lachat Instruments, Loveland CO). From each dried sample, an additional subsample was taken and sent to the North Carolina Department of Agriculture and Consumer Services, Agronomic Division for chemical analysis of soil pH, cation exchange capacity, base cations, base saturation, phosphorus, manganese, zinc, copper and humic matter (Table 2-2).

2.2. Hairy vetch genotypes

Nodulation assessment of resident soil Rlv was evaluated using five groups of distinct hairy vetch genotypes, each group comprising two closely related genotypes (Maul et al. 2010). Genotypes were previously collected from Afghanistan (two populations), Greece, Iran (two populations), Turkey (two populations), USDA *Purple Bounty* and *Purple Prosperity* early flowering varieties, (USA-MD 1 and USA-MD 2, respectively), and the *Madison* variety from Nebraska, USA-NE (Table 2-3). Seeds for Afghanistan, Greece, Iran and Turkey genotypes were obtained from National Plant Germplasm System (Washington State University, Pullman, WA), and seeds for USA genotypes were obtained from USDA-ARS Sustainable Agriculture Systems Lab (Beltsville, MD).

2.3. Experimental design and plant germination

The ten HV genotypes were used to trap soil rhizobia over a period of six weeks in a growth chamber. Two coupled magenta units (PlantMedia, Dublin, OH) were used (Tlusty et al. 2004); the bottom unit contained N-free nutrient solution (Broughton and Dilworth 1971), the top unit contained equal volumes of sand and vermiculite thoroughly mixed, and was drilled at the bottom and a cotton wick inserted to source water and nutrients from the bottom unit. Assembled magenta units were sterilized by autoclaving at 121^{o}C for 15 minutes.

Hairy vetch seeds were surface sterilized with 3% sodium hypochlorite, rinsed five times in sterile deionized water, placed on a sterilized germination paper in Petri dishes and left to germinate at room temperature for six days. Genotypes Turkey 1, Turkey 2, Iran 1 and Greece were scarified by soaking seeds in 80% H_2SO_4 for 30 minutes, then rinsing 5 times with deionized water prior to sterilization to improve germination. Germination rates for all genotypes were recorded. Two hairy vetch seedlings were planted per magenta unit. Each seedling was inoculated with 500 µl of a 5^{-1} soil dilution prepared by mixing 20 g of the reserved soil with 80 ml of 0.85% (w/v) NaCl solution (Bala 2001). A treatment was defined as a combination of each HV genotypes with soil inoculant from each of the six fields; with four replications in a completely randomized design. Due to growth chamber space constraints, replications one and two were established first in May/June 2011 (run 1) and replications three and four second in September/October 2011 (run 2). The growth chamber was set at 9 hr days with 22^{o}C day temperature, and 18^{o}C night temperature. After 7 days, plants were

thinned to one plant per unit, and sterile N-free nutrient solution was supplied as needed.

2.4. Assessment of symbiotic efficiency

Nodulation effectiveness, equated to nodule numbers and nodule mass, as well as symbiotic efficiency of resident rhizobia in each soil, were determined through evaluation of shoot biomass production and shoot tissue nitrogen. After 6 weeks of growth plants were harvested, and shoots dried at 65^{o}C for 24 to 48 hours then weighed. Due to low sample mass, shoots were manually ground using mortar and pestle, and samples sent to the Environmental and Analytical Testing Services Lab in the Department of Soil Science at N.C. State University for analysis of tissue nitrogen to be used as an estimate of N_2 fixation. Plant roots were harvested to assess nodulation, and nodule number per plant and total nodule mass per plant recorded. Nodules were then dried using a desiccant (Drierite Desiccant-Anhydrous, W.A. Hammond Drierite Co., Xenia, OH), nodules weighed and total nodule mass per plant recorded.

2.5. Data analysis

Statistical analyses were performed using SAS ver. 9.2 Statistical Software (SAS, Cary, NC). A combined analysis was performed for all replications. Number of nodules, nodule mass, plant biomass and biomass N were analyzed using the mixed models procedure (PROC MIXED), with run and all run interactions as random effects, and site as a fixed effect. All parameters were square root transformed for analysis, and reported least squared means back transformed for data presentation. Mean separations were performed using Tukey's honestly significant difference (HSD) with $\alpha = 0.05$.

3. Results

3.1. Chemical soil properties

Soil chemical characteristics varied more between sites than within sites (Table 2-2). Soil pH (H_2O) was in the range of 6.6 to 5.4 in Cedar Grove HV+ field and Ivanhoe HV- field, and across all sites, HV- fields had lower pH values than HV+ fields. Soil pH was nearly similar between paired fields within a site, except in Cedar Grove, where pH difference between the paired fields was 0.9. Soil phosphorus (P) was highest at Ivanhoe and almost 10 times lower in Graham. Similarly, the Graham and Cedar Grove copper levels were a little over a tenth those in Ivanhoe HV- field. Notably, manganese levels were greatest in the Graham HV+ field, 26% lower in the HV- field, and over 65% lower in all other fields.

3.2. Effect of HV cultivation history on nodulation and symbiotic efficiency

There was a significant effect of hairy vetch cultivation history on all parameters tested, including number of nodules formed, nodule mass, plant biomass and plant tissue N. Across all sites, HV+ fields had significantly higher nodule number and mass (Figure 2-1a and 2-1b) than HV- fields. The Ivanhoe HV- field had the poorest nodulation, with nodulation reduction of more than 80% compared to Ivanhoe HV+ field. Averaged across all genotypes, HV- fields from Graham and Cedar Grove had 32% and 35% reduction in nodule numbers, respectively, compared to the HV+ fields at those locations. A positive effect of past hairy vetch cultivation on nodule mass was observed in all the three sites. Nodule mass was reduced by 23% in the HV- field in

Graham, with greater reductions of 58% and 73% in Cedar Grove and Ivanhoe, respectively.

There was a strong site-by-field history interaction effect on plant biomass ($p = 0.0003$) and shoot N ($p < 0.0001$). Plants inoculated with soil dilutions from the HV+ field from Ivanhoe had nearly 70% more biomass than those inoculated with dilutions from the HV- field. Overall, within a location, genotypes inoculated with soil with HV history had at least 20% greater shoot biomass than genotypes that were inoculated with soils with no HV history. In general, plants inoculated with soil dilutions from HV+ fields had greater mean N content than those inoculated with soil dilutions from HV- fields, with significant differences observed in Cedar Grove and Ivanhoe, but not in Graham (Figure 2-4). As with nodulation, N content from genotypes inoculated with -HV from Graham had over 25% more N content than –HV from Cedar Grove and Ivanhoe. As shown in Figure 2-2, shoot biomass was correlated with nodule mass and nodule numbers ($r^2 = 0.80$ and 0.50, respectively).

3.3. Effect of HV genotype on nodulation and symbiotic efficiency

Hairy vetch genotype had a significant effect on nodule numbers and nodule mass (Table 2-4). Group 1 genotypes, Turkey 1 and Turkey 2, had the lowest mean nodule numbers, 32 and 49 nodules per plant respectively. The highest mean nodule numbers were obtained from the Iran 2 genotype, with over 60% more nodules than the least-nodulated Turkey 1. Moreover, the difference in nodule mass between Iran 2, with the highest mean nodule counts, and Turkey 1, with the lowest mean nodule counts, was over 70%. The number of nodules found on Group 3 genotypes (including

Afghanistan 1 and Iran 2 genotypes) was more than three times the number found on Group 1.

We also observed differences in germination rates among genotypes, with a range from 16.7% in the Turkey 2 genotype to 95.5% germination in the USA-MD 1 genotype. The highest rates were observed in groups 3 and 5 with a mean of 89.6% (Table 2-4). The hairy vetch genotypes used in the study were from geographically diverse origins and were chosen based on their distinct biochemical characteristics (Table 2-3). A companion study provides further details on the plant biology characteristics of these genotypes (Maul et al. 2011). Poor nodulation, and in some case no nodulation at all, was observed for all genotypes with poor germination, suggesting that the significant effect of HV genotype on nodulation may have been related to germination rate differences between the genotypes. Although scarification increased germination rates by as much as 50%, there was no recorded increase in nodulation.

There was no significant genotype effect on biomass production ($p = 0.1299$), however, shoot biomass between cultivars varied from a mean of 390 mg plant $^{-1}$ in Turkey 1 to 1129 mg plant^{-1} in Iran 2; with values as low as 20 mg plant $^{-1}$ (Turkey 1) and as high as 2271 mg plant $^{-1}$ (Iran 2) recorded. Shoot nitrogen content varied significantly between genotypes (Fig 2-3, $p < 0.0001$). As with nodulation and biomass, highest N content, 5.38 % was obtained in Iran 2 and lowest N content, 4.01 %, in Turkey 1. Overall, Group 3 genotypes showed the highest symbiotic efficiency, fixing nearly 20% more N than group 1 genotypes. There was a close association between shoot biomass and % shoot N (Figure 2-3), with a significant correlation coefficient of

0.50. The Turkey 2 genotype had high N content, nearly 90% of the maximum, surprising since this genotype was one of the least nodulated and had relatively low shoot biomass.

4. Discussion

This study showed that fields where hairy vetch had been cultivated at least five times in the past 20 years contained resident populations of rhizobia able to successfully nodulate and fix nitrogen with multiple hairy vetch genotypes without additional inoculation. Hairy vetch plants inoculated with soil from fields with a history of hairy vetch cultivation were very well nodulated, while very poor, and in some cases negligible, nodulation was observed in plants inoculated with soil dilutions from fields having no history of HV cultivation. The results are consistent with previous work (Chemining'wa and Vessey 2006) showing optimal nodulation of pea in fields with histories of pea cropping, and very poor nodulation in fields never planted to pea. The reduced nodulation ability of these resident pea rhizobia was explained by a loss of symbiotic effectiveness over time without host legume (Chemining'wa and Vessey 2006). Rhizobia with high saprophytic efficiency, defined by their efficient use of available soil carbon resources for growth in the absence of a host plant, have been found to have a lower symbiotic efficiency when the host is subsequently introduced into the environment (Duodu et al. 2005). Rhizobia population size is known to affect the efficiency of nodulation (Patrick and Lowther 1995), with larger populations enhancing nodule development. Together these results suggest that despite infective soil resident rhizobia being present in all fields tested in this study, evidenced by nodule

formation on all genotypes, fields where hairy vetch had never been planted contained populations of rhizobia with possibly high saprophytic efficiency, yet low nodulation efficiency and/or population size.

The Graham HV+ field was unique in that it had never been inoculated with Rlv so that no intentional introduction of rhizobia occurred. This suggests that the significantly higher nodulation observed in the positive HV history field in Graham as compared to the Graham field never planted to hairy vetch can be attributed to previous hairy vetch cultivation. However, the lack of difference in total plant N between vetches inoculated with soil with and without vetch planting history suggests that while previous HV cultivation may have increased population size of vetch-nodulating rhizobia present in the field, the symbiotic efficiency of resident Rlv populations, indicated by total biomass N, appears not to have been affected by previous cultivation of hairy vetch. It is also possible that this is a result of rhizobia population mixing between the two fields as they were in proximity to each other. Across all sites our results provide evidence that in North Carolina farmers using hairy vetch regularly as a winter cover crop with or without inoculation, can increase the population size and nodulation ability of resident rhizobia, as well as in many cases improve N-fixation. It is still not known whether this nodulation and N fixation efficiency of resident strains would match or exceed that of inoculant strains. The role of inoculation in BNF improvement is less understood, and in some cases past inoculation may be a factor in further increasing high-fixing rhizobia populations in agricultural soils.

The Cedar Grove HV+ field had been inoculated with Rlv each season HV was planted, however nodulation was not significantly higher than the other two fields with HV history. Likewise, (Ballard et al. 2004) showed that inoculation of pea did not significantly increase nodulation in over 20 of 30 tested soils. This lack of significant differences between recently-inoculated and never-inoculated fields suggests that inoculation benefits may be minimal in systems that use legume hosts regularly. Although this study did not assess the effect of inoculation on nodulation effectiveness, questions remain regarding the need for inoculation in fields with history of HV, and further research on nodule occupancy and soil-resident strain efficiency in BNF is warranted.

All hairy vetch genotypes assessed in this study nodulated when inoculated with soil from the six field-sites, indicating that tested soils contain at least minimal populations of effective Rlv needed for effective nodulation across genetically distinct populations of hairy vetch. The differences in nodulation observed between HV- fields of different sites, with some clearly having less effective populations of resident rhizobia than others (for example, Ivanhoe), suggest impacts of site-specific factors on resident rhizobia populations. In addition to low pH in the Ivanhoe field without history, this field also had the highest copper content. Rhizobia, particularly Rlv, have been shown to be affected by soil copper levels, with high levels resulting in significant reductions in nodule numbers (Laguerre et al. 2006). Within all three sites, HV- fields had slightly lower pH values than the HV+ fields. Since pH is known to be a driving factor in rhizobia survivability in the field (Andrade 2002; Andrade et al. 2002a;

Lapinskas 2007; Hungria and Vargas 2000), this environmental factor may have impacted rhizobia survivability in soils where hairy vetch had never been cultivated, although the pH levels observed here are unlikely to induce conditions that challenge rhizobia survivability. Wolff et al. 1995 reported significant reduction in nodule numbers of *Phaseolus vulgaris* in pH lower than 4.5, and several other studies have also shown significant effects of soil acidity on the rhizobial population size and nodulation (Chemining'wa and Vessey 2006; Evans 2005), supporting our finding of high nodulation in all sites with pH greater than 6.0. Evidence provided in this study support that pH was not a driving factor in nodulation success in the assessed fields. No significant difference in nodulation was observed between fields with sharply contrasting pH values, such as field one in Graham, with a pH of 6.5, and field five in Ivanhoe, found to have a much lower pH of 5.7, both soils with history of vetch cultivation. Further, fields with similar pH values yet contrasting vetch histories, specifically fields five and six in Ivanhoe with pH values of 5.7 and 5.4, respectively, were found to have significantly different nodulation rates. In combination these data suggest HV history and not pH to be the primary driver in vetch nodulation by resident rhizobia in this study. Thus, agricultural systems that include legume hosts improve chances of optimal nodulation even if soil properties are not favorable.

Rhizobia strains resident to soils in North Carolina (NC) were able to effectively nodulate hairy vetch genotypes from different centers of diversity, including Afghanistan, Greece, Iran and Turkey. Lei et al. (1987) suggest that when legume species from distant geographic locations are introduced to a new region they are likely

to have low nodulation. This is supported by further research demonstrating that rhizobia nodulating hosts with no history of co-evolution with that particular symbiont have poor nodulation (Howieson et al. 2005). Strains of *R. leguminosarum* have been shown to share a common phylogenetic origin, and some authors suggest them to have been spread trans-continentally with *Vicia sativa* seeds, including the continents of Africa, America and Asia (Alvarez-Martinez et al. 2009). Alvarez-Martinez et al. (2009) further showed that *Rhizobium leguminosarum* from *Vicia sativa* isolated from soils in Spain were phylogenetically related to *V. sativa* rhizobia strains isolated from Africa, America and Asia. This study provides evidence suggesting that NC resident Rlv strains are related to those found in the HV centers of diversity of Afghanistan, Greece, Iran and Turkey, and that through a process of co-evolution with HV, may have been introduced to NC along with populations of HV commonly cultivated in the United States.

Results showed variation in symbiotic capacity (the potential of a legume or rhizobia to nodulate with the partner and result in high N fixation rates) of distinct HV genotypes with resident rhizobia. Cultivar selection within the same legume species has been shown by others to affect nodulation and BNF in pea (Fettell et al. 1997; Abi-Ghanem et al. 2011), and subterranean clover, with some cultivars being compatible with a wide range of rhizobium strains resulting in high BNF efficiency (Drew and Ballard 2010). Due to great variation in nitrogen fixing ability of different rhizobial strains, and the difficulty in predicting the competitive ability of resident rhizobia, some have suggested selection of host genotypes as a means of improving the efficiency of

legume-rhizobia symbiosis (Ballard and Charman 2000; Drew and Ballard 2010; Ballard et al. 2002). Our study shows differences in nodulation and nitrogen fixation rate between distinct populations of hairy vetch. Such differences indicate that that these genotypes may possibly be able to be used as gene stocks in cover crop breeding programs, improving N_2-fixation ability while developing plants with traits that make hairy vetch more amenable to inclusion as a winter cover crop in farming system niches where specific plant biochemical characteristics are desired. Group 1 genotypes, as well as Iran 1 and Greece, expressed poor nodulation in all soils examined as well as lower germination rates. The possibility exists that the poor nodulation of these genotypes is related to their poor germination and perhaps root development rather than ineffectiveness of symbiosis.

As a surrogate for N_2-fixation, shoot N content results showed that resident rhizobia, particularly those from HV+ fields, are capable of efficient BNF. Although results showed a low correlation coefficient of nodule numbers to shoot biomass, nodule numbers can be important predictors of improved legume performance and yield. Sprent et al. 1988 in Viosin et al. 2010 reported nodules to play an important role in the ability of host to tolerate and adapt to environmental conditions, and nodule mass to influence the amount of N fixed. Thus, both nodule numbers and nodule mass are critical indicators of legume productivity, and mechanisms of how legumes regulate these parameters require further attention.

5. Conclusions

Hairy vetch is one of the most widely used winter annual cover crops in agricultural systems, especially among organic farms. Among other benefits, it can contribute significant amounts of N through a mutualistic relationship with its symbiotic partner, *R. leguminosarum* biovar *viciae*. This study determined the effect of hairy vetch cultivation history on symbiotic efficiency with resident rhizobia, and determined whether distinct HV genotypes vary in their symbiotic capacity when associating with resident rhizobia. Results suggest that infective strains of Rlv present in North Carolina soils are indeed able to nodulate a diverse array of hairy vetch genotypes. More importantly, decreased nodulation in fields without histories of HV as compared to fields where hairy vetch had been previously cultivated was observed, suggesting that Rlv population size in soils with no history is generally low and/or that populations have low symbiotic capability. Comparably higher nodulation was found in fields with HV history, suggesting that use and history of host legumes enhances the population size of resident rhizobia and their ability to competently nodulate the host. Variability in symbiotic efficiency of different genotypes provides evidence that BNF efficiency varies by plant biochemistry. This suggests that hairy vetch may be improved using plant genotypes of diverse origin to breed for cultivars with high symbiotic efficiency. Understanding the effects of agricultural practices on plant-microbe interaction will improve agricultural stability globally, and result in systems that utilize ecological processes more effectively for optimal food production.

Table 2-1: Characteristics of fields from which soils were collected for inoculation of the ten distinct HV genotypes.

Site	Soil Type	Average temperature from 1990 to 2010 (^{o}C)		Field history	Status during sampling	Last inoculation with Rlv	Other legume cover crops used
		Min	Max				
Graham	Appling Sandy Loam (Fine, kaolinitic, thermic Typic Kanhapludults)	8.4	21.2	+HV	Hairy vetch, pea and rye	Never been inoculated	Crimson clover, cowpea and pea
				-HV	Asparagus grass	---	---
Cedar Grove	Appling Sandy Loam (Fine, kaolinitic, thermic Typic Kanhapludults)	8.6	20.9	+HV	Hairy vetch and rye	Every season hairy vetch was planted	Crimson clover
				-HV	Grass	---	---
Ivanhoe	Chipley Sand (Thermic, coated Aquic Quartzipsamments)	10.8	23.4	+HV	Hairy vetch	Not since 2004	Crimson clover
				-HV	Weeds	---	---

---Field does not have history of legume cropping or growth, therefore, no inoculation.

Table 2-2: Soil physiochemical properties for the 6 fields used in the study to test efficiency of resident rhizobia in nodulation of hairy vetch.

Soil property	Graham		Cedar Grove		Ivanhoe	
	HV+	HV-	HV+	HV-	HV+	HV-
pH (H_2O)	6.5	6.1	6.6	5.7	5.7	5.4
P (mg dm^{-3})	67.00	103.30	328.20	77.70	497.30	658.00
K (meq $100cm^{-3}$)	0.34	0.27	0.22	0.22	0.82	0.85
Ca (meq $100cm^{-3}$)	4.30	3.18	10.05	2.84	7.63	4.32
Mg (meq $100cm^{-3}$)	1.04	1.12	2.03	1.40	1.92	1.38
Na (meq $100cm^{-3}$)	0.0	0.0	0.1	0.0	0.1	0.1
Mn (mg dm^{-3})	95.2	69.8	31.4	32.6	12.0	9.6
Zn (mg dm^{-3})	15.5	18.9	7.1	2.3	18.2	13.9
Cu (mg dm^{-3})	2.7	1.4	2.7	1.0	1.1	10.1
Base saturation (%)	89.0	79.0	94.0	79.0	85.0	64.0
Cation exchange capacity	6.4	5.8	13.1	5.7	13.3	10.1
Humic matter (g $100cm^{-3}$)	0.41	0.32	0.18	0.41	0.86	3.28
Inorganic N (%)	2.96	9.58	4.42	5.92	2.97	5.54

Table 2-3: Hairy vetch genotypes used to evaluate symbiotic efficiency of soil resident rhizobia from North Carolina.

Group	Country of Origin	PI number	Germination by National Plant Germplasm (%)	Germination in lab (%)**	Code
1	Turkey	167259	88	33	Turkey 1
	Turkey	206493	90	17	Turkey 2
2	Iran	229970	74	25	Iran 1
	USA-NE	Nebraska	nd	75	USA-NE
3	Afghanistan	317447	95	88	Afghanistan 1
	Iran	429408	99	92	Iran 2
4	Afghanistan	222217	95	79	Afghanistan 2
	Greece	289482	71	21	Greece
5	USA-MD	Bounty	nd	96	USA-MD 1
	USA-MD	Prosperity	nd	83	USA-MD 2

nd Not determined for a given hairy vetch genotype.
** A total of 50 hairy vetch seeds were used to determine germination rate, *i.e*. n=50.

Table 2-4: Nodulation and biomass production of the hairy vetch genotypes.

Group	Hairy vetch genotype	Nodule numbers (plant^{-1})	Nodule mass (mg plant^{-1})	Shoot biomass (mg plant^{-1})	Shoot N (%)	Sample size (n)
1	Turkey 1	32b	18.8c	390.3a	4.01b	12
	Turkey 1	49ab	29.3bc	483.3a	4.82ab	17
2	Iran 1	50ab	34.4abc	632.2a	4.33ab	24
	USA-NE	70ab	51.3ab	854.0a	5.11ab	24
3	Afghanistan 1	70ab	62.2a	876.3a	5.35a	24
	Iran 2	87a	70.2a	1129.5a	5.39a	24
4	Afghanistan 2	84a	62.2a	974.4a	5.21ab	24
	Greece	49ab	38.3abc	527.0a	4.50ab	14
5	USA-MD 1	58ab	43.4abc	790.7a	4.96ab	24
	USA-MD 2	78ab	50.6ab	929.1a	5.07ab	24

Within a column, different letters following least squared means indicate significant differences at $\alpha = 0.05$.
Within a column, same letters following least squared means indicate non-significant difference at $\alpha = 0.05$.

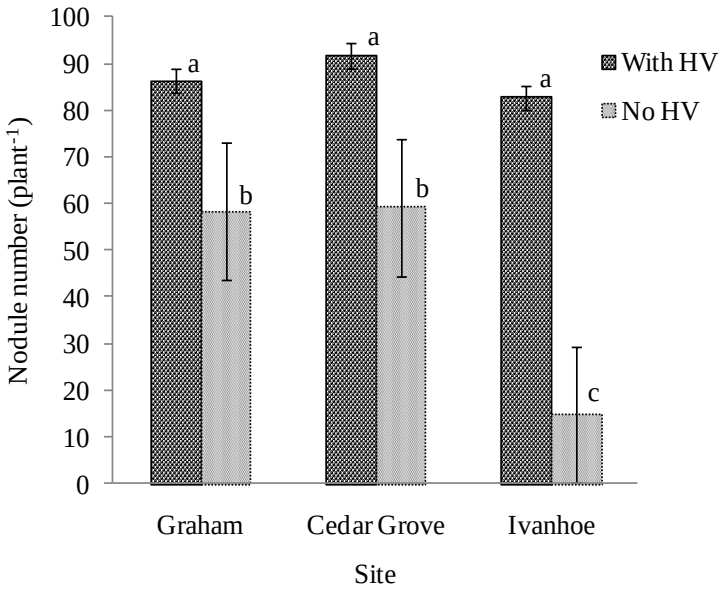

Figure 2-1a. Effect of hairy vetch cultivation history on nodule numbers.

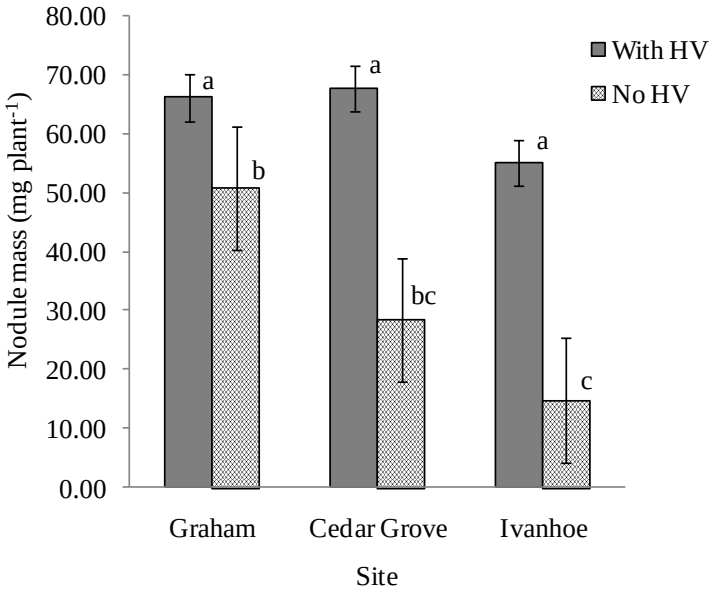

Figure 2-1b. Effect of hairy vetch cultivation history on nodule mass.

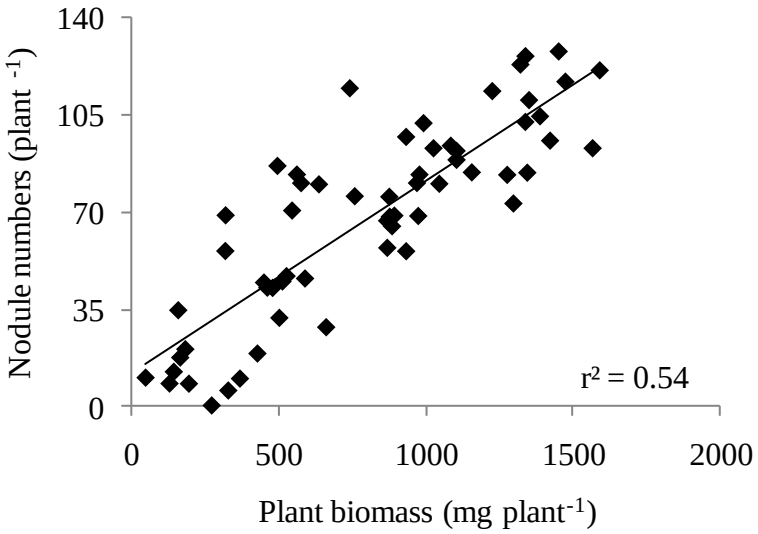

Figure 2-2a. Correlation of hairy vetch shoot biomass and nodule numbers.

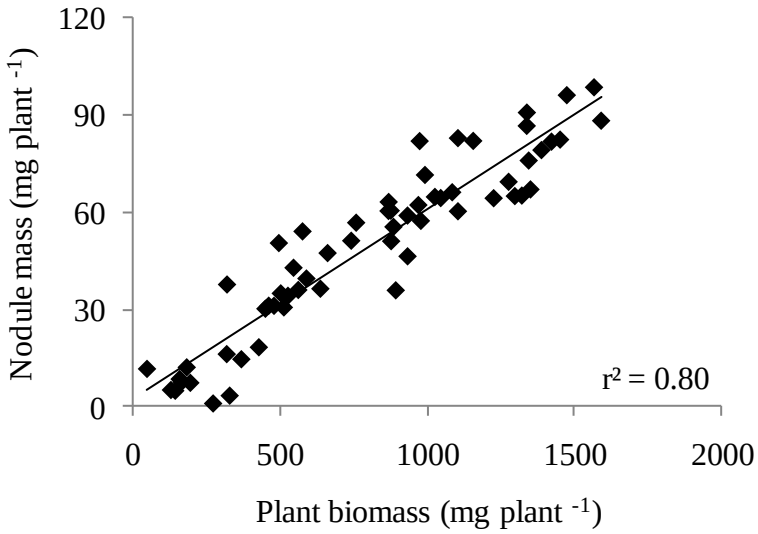

Figure 2-2b. Correlation of hairy vetch shoot biomass and nodule mass.

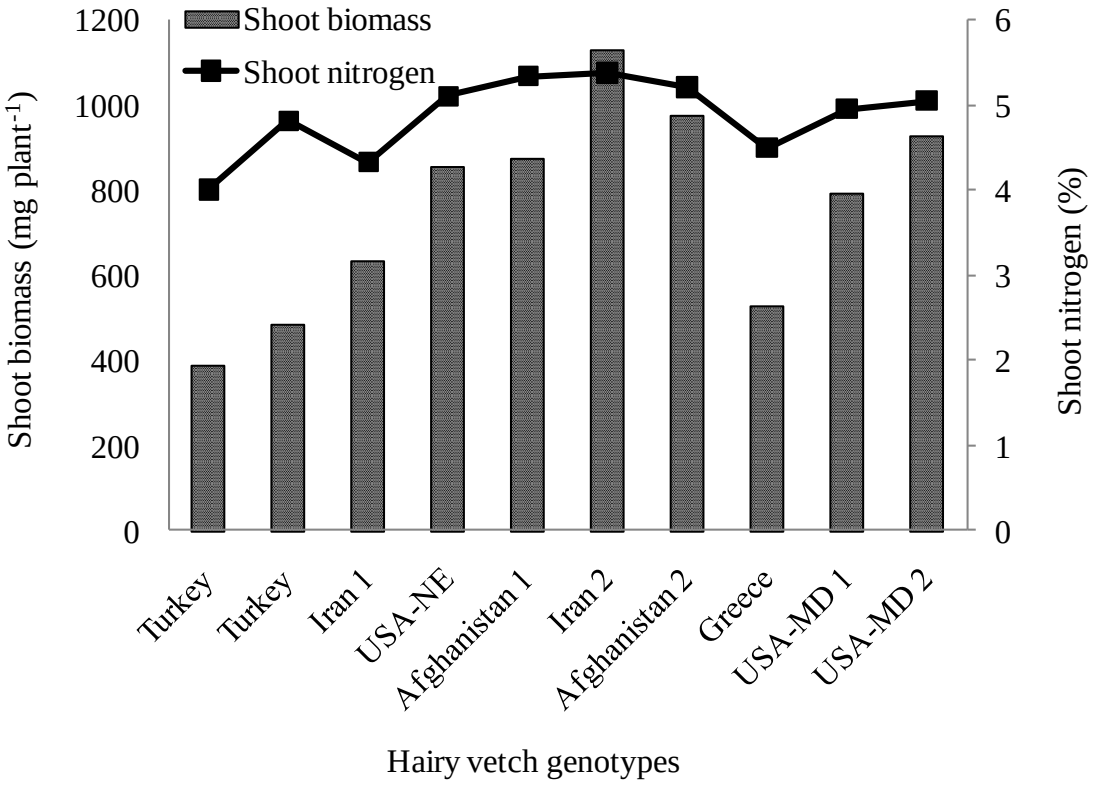

Figure 2-3. Shoot biomass and nitrogen content of the 10 hairy vetch genotypes.

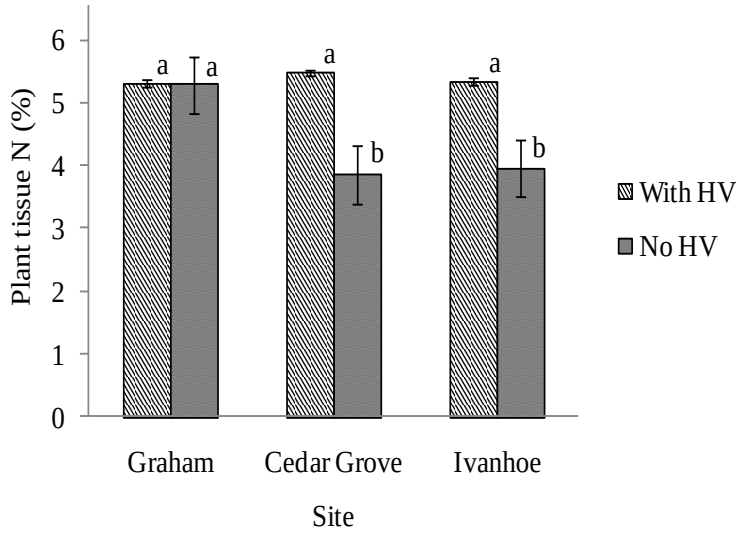

Figure 2-4. Hairy vetch shoot N content across the three sites.

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Chapter 3:

Genetic diversity of resident soil rhizobia isolated from nodules of distinct hairy vetch genotypes

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Abstract

Hairy vetch (*Vicia villosa* Roth) is widely grown as a legume cover crop throughout the U.S.A., with biological nitrogen fixation (BNF) through symbiosis with *Rhizobium leguminosarum* biovar *viciae* (Rlv) being one of the most sought after benefits of its cultivation. This study determined if HV cultivation history and genotype have an effect on the genetic diversity of resident Rlv. Soil samples were collected from within farmers' fields at Graham, Cedar Grove and Ivanhoe sites in North Carolina. Using ten distinct hairy vetch genotypes as trap hosts, a total of 519 Rlv strains were isolated from soil dilutions of six paired fields, three with and three without histories of HV cultivation. A total of 46 strains failed to PCR-amplify the *nifH* gene; however *nodC* PCR amplification of these *nifH*-negative strains resulted in amplification of 22 of the strains. Repetitive element polymerase chain reaction (rep-PCR) with BOX-A1R primer showed that diversity of rhizobia varied greatly within and between fields. Over 30 BOX banding patterns were obtained across the six fields. There was evidence of strain domination for both the fields at Cedar Grove. Cluster analysis of BOX-PCR banding patterns resulted in 36 genetic groups of Rlv at a similarity level of 70%, with 15 of the isolates from fields with HV history not belonging to any of the clusters. The biggest cluster comprised 96 strains, 86 of which were from the Cedar Grove. Rlv tended to group based on site from which they were isolated, and within a site by field history. Hairy vetch genotypes appeared to have little or no effect on diversity of Rlv isolated, particularly in fields with no HV cultivation history. Our results show that HV cultivation history changes and increases the genetic diversity of resident Rlv in soils.

Although different strain profiles were sometimes obtained from distinct HV genotypes, a consistent impact of HV genotypes on diversity is not pronounced.

1. Introduction

Hairy vetch (*Vicia villosa* Roth, HV) is a winter annual legume whose cultivation as a cover crop is common in the US, particularly in organic and low input farming systems. Through symbiosis with *Rhizobium leguminosarum* biovar *viciae* (Rlv), HV can contribute significant quantities of nitrogen (N) to farming systems (Power et al. 1991; Sainju et al. 2001), and inoculation of HV with effective Rlv strains to increase N fixation is a common practice. *R. leguminosarum* biovar *viciae* are capable of nodulating several legume species belonging to tribe viciae (Laguerre et al. 2003), including pea (*Pisum sativum*), faba bean (*Vicia faba*) and common vetch (*Vicia sativa*). In the absence of legume hosts these soil-borne saprophytic bacteria survive on decomposed organic compounds, and environmental variables and management practices have been shown to affect the Rlv population structure and diversity of Rlv existing in the field in the absence of a legume host (Andrade et al. 2002a; Bala et al. 2001; Depret et al. 2004; Kaschuk et al. 2006).

Although, several studies have reported that already-existing resident soil rhizobia negatively affect successful symbiotic relationships with legume hosts through competition with inoculant strains (Lima et al. 2009), other studies have shown resident rhizobia to form effective associations, both nodulating and fixing nitrogen with their legume hosts. Resident rhizobia can be defined as those existing in a given soil, including native rhizobia and those previously introduced and naturalized over time.

Ballard (2002) reported symbiotic efficiency, in which N is fixed and subsequently translocated to the host for improved growth and production, between resident clover rhizobia (*Rhizobium leguminosarum* biovar *trifolii*) and balansa clover (*Trifolium michelianum* Savi) to be as high as 128% of that of the recommended inoculant strain. Other studies have shown symbiotic efficiency between resident rhizobia and pasture medics to range between -6 and 72% of the inoculant strain (Ballard, 2000).

Understanding how resident rhizobial populations are affected by management and environmental changes is critical for improved N fixation in managed agroecosystems where legumes are used for N contribution. Soil pH has been shown to greatly affect rhizobia survival and diversity in soils (Andrade et al. 2002a; Bala et al. 2001; Frey and Blum 1994; Graham et al. 1994), and fields with low pH have low nodulation (Chemining'wa and Vessey 2006). Reduced diversity of *Rhizobium leguminosarum* biovar *phaseoli* has been reported in acid soils compared to limed soils (Andrade 2002; Lapinskas 2007). Soil management practices have also been shown to affect rhizobial populations, including reports of reduced diversity in long-term monocultures as compared to crop rotation with legume host (Depret et al. 2004), and high diversity of bean rhizobia in no-till fields compared to conventional till fields (Kaschuk et al. 2006). Past cultivation of a legume host in a field has been shown to be particularly important in impacting resident rhizobia population size in beans (Andrade et al. 2002a) and peas (Chemining'wa and Vessey 2006).

Hairy vetch cultivars adapted to specific environmental conditions have been developed in order to utilize them in particular agroecological management systems.

Examples include cold-tolerant Madison variety from Nebraska, and recent early-maturing USDA-ARS varieties Purple Bounty and Purple Prosperity, which reach peak biomass one to two weeks before standard varieties and allow for early/timely planting of cash crops (Maul et al. 2011). Previous reports assessing diversity among strains of Rlv in agricultural systems demonstrated higher diversity in no-till compared to conventional tillage systems (Andrade et al. 2003; Ferreira et al. 2000; Kaschuk et al. 2006) and higher diversity in arable lands compared to grassland (Palmer and Young 2000). However, little attention has been given to diversity of rhizobia associating with distinct HV genotypes. Numerous studies have shown that the presence of a legume host can affect population size and structure of compatible rhizobia in agroecosystems (Hynes and Oconnell 1990; Laguerre et al. 2003; Laguerre et al. 2007; Depret and Laguerre 2008; Mutch et al. 2003; Mutch and Young 2004). Some Rlv hosts, such as faba bean, appear to be highly specific and symbiotically associate with only a specific *nod type* of Rlv strain (Hynes and Oconnell 1990; Mutch and Young 2004; Laguerre et al. 2003; Laguerre et al. 2007). Even subtle differences in host genotype at the sub-species level can select for different populations of rhizobia in a soil (Depret and Laguerre 2008).

High rhizobial diversity is important to soil health and productivity (Ferreira et al. 2000; Kaschuk et al. 2006). Although many organic growers utilize hairy vetch on an annual basis in their fields, we do not yet understand how past planting of this species might impact the population structure and diversity of resident Rlv populations. In this study we evaluated genetic diversity of Rlv isolated from root nodules of ten distinct HV

genotypes inoculated with soil dilutions from fields with and without HV cultivation history. Our specific objectives were to: i) determine the effect of HV cultivation history on the genetic diversity of soil resident Rlv and ii) to determine genetic diversity of resident Rlv populations able to nodulate distinct HV genotypes.

2. Materials and Methods

2.1. Field sites and soil sampling

The field study was conducted in 2010 in North Carolina an agriculturally important area located in the Southeastern region of the U.S.A. with numerous certified organic farms. Soil samples were originally taken from five farms with histories of hairy vetch cultivation, including at least five seasons of HV since 1990, located in the cities of Asheville (2 farms), Graham, Cedar Grove and Ivanhoe (Table). On each farm a paired field never been previously planted to hairy vetch and never observed to have had wild hairy vetch varieties (Personal communication with farmers) was also sampled. Inoculation history on the fields with hairy vetch differed among farms, with Asheville and Cedar Grove fields being previously inoculated with each past hairy vetch planting, Graham having no history of inoculation and Ivanhoe not being inoculated since 2004.

All fields with vetch histories were planted to hairy vetch at the time of sampling, some in mixtures with grass species. Forty soil cores to a depth of 15 cm and diameter of 2.5 cm were randomly collected at each field, thoroughly mixed into a representative sample and then stored at field condition at 4^{o}C in the laboratory. All sampling materials were sterilized with 75% ethanol prior to sampling; during sampling and handling, precautions were taken to avoid cross-contamination of soils of different fields

and sites. From each representative sample, a subsample was taken, dried at 45^{o}C and sent to North Carolina Department of Agriculture and Consumer Services, Agronomic Division for chemical analysis. Three of the five farm soils were subsequently chosen to be included in this study in order to maximize number of past hairy vetch cultivations since 1990, and to standardize pH values between fields on the same farm.

2.3. Rhizobia isolation

Ten distinct hairy vetch genotypes (Table) were used to trap soil rhizobia over a period of six weeks in a growth chamber. A modified Leondard jar construction comprising of two coupled magenta units (PlantMedia, Dublin Ohio, (Tlusty et al. 2004) were used; the bottom unit containing N-free nutrient solution (Broughton and Dilworth 1971), the top unit containing equal volumes of thoroughly-mixed sand and vermiculite, and drilled at the bottom to hold a cotton wick inserted to source water and nutrients from the bottom unit. Assembled magenta units were sterilized by autoclaving at 121^{o}C for 15 minutes.

Five groups of distinct hairy vetch genotypes were used as Rlv hosts (Table), each group comprising two genotypes closely related in biochemical characteristics (Maul et al. 2010). Seven of the ten total genotypes were previously collected from Afghanistan, Greece, Iran, Turkey, and three were varieties recently developed for use as cover crops in U.S. based systems, including USDA releases *Purple Bounty* and *Purple Prosperity* early flowering varieties, (USA-MD 1 and USA-MD 2, respectively), and the *Madison* variety from Nebraska, USA-NE. Seeds for Afghanistan, Greece, Iran and Turkey genotypes were obtained from National Plant Germplasm System (Washington

State University, Pullman, WA), and seeds for US varieties were obtained from USDA-ARS Sustainable Agriculture Systems Lab (Beltsville, MD).

Hairy vetch seeds were surface sterilized with 3% sodium hypochlorite, rinsed five times in sterile deionized water, placed on a sterilized germination paper in Petri dishes and left to germinate at room temperature for six days. Seeds showing low initial germination rates were scarified by soaking in 80% H_2SO_4 for 30 minutes, then rinsing 5 times with deionized water. Two hairy vetch seedlings were planted per magenta unit. Each seedling was inoculated with 500 µl of a 5^{-1} soil dilution prepared by mixing 20 g of soil with 80 ml of 0.85% (w/v) NaCl solution (Bala et al. 2001). A treatment was defined as a combination of a HV genotype with soil inoculant from one of the six fields. The 60 combinations were evaluated in the growth chamber in a randomized complete block design with four replications. Due to growth chamber space constraints, replications one and two were established first in May/June 2011 (run 1) and replications three and four second in September/October 2011 (run 2). The growth chamber was set at 9 hr days with 22^{o}C day temperature, and 18^{o}C night temperature. After 7 days, plants were thinned to one plant per unit, and sterile N-free nutrient solution was supplied when needed.

Plant roots were harvested after six weeks of growth. Three nodules were randomly selected from each root system, surface sterilized with 3% sodium hypochlorite and rinsed 5 times in sterile deionized water. The surface sterilized nodules were crushed onto yeast mannitol agar media plates (YMA, Vincent 1970) containing 0.1% Congo red and incubated at 28^{o}C for 3 days. Cultures were repeatedly streaked on

YMA to ensure purity and obtain single colonies. A single typical colony was then transferred into tryptone yeast (TY, Vincent 1970) and shaken for five days at 180 rpm. Resulting colonies were centrifuged, supernatant discarded, and isolates placed in 300 µl sterile water and stored at -20^{o}C. Each nodule was assumed to contain a single strain, and each colony was assumed to represent a single strain (Vessey and Chemining'wa, 2006). Live cultures were maintained on YMA slants at 4^{o}C.

2.3. Confirmation of isolates: *nifH*-PCR

A total of 519 rhizobia were isolated from the hairy vetch genotypes across all treatment replications. Isolates were assumed to be *Rhizobium leguminosarum* biovar *viciae* (Rlv) due to the high specificity between hairy vetch and Rlv. To further support the inclusion of only Rlv in the study, *nifH* PCR was performed. Amplification of *nifH* was performed using primers *nifH*-1 and *nifH*-2 (Table 3-1) from New England Biolabs (Ipswich, MA). The PCR reaction contained 1 µl DNA template, 3.3 µl Taq polymerase, 0.2 µl *nifH*-1 and 0.2 µl *nifH*-2, made up to final volume of 10 µl with sterile water. Amplification was carried out in a Mastercycler^{ep} thermocycler (Eppendorf, Germany), using the following PCR cycles: initial denaturing at 94^{o}C for 5 min, 30 cycles of, denaturing 94^{o}C for 1 min, annealing at 60^{o}C for 1 min extension at 72^{o}C for 1 min, and final extension at 72^{o}C for 5min. *nifH*-PCR products were examined via electrophoresis using 1% agarose gel containing 5% ethidium bromide (EtBr), run at 100 V for 40 min. Gels were viewed under UV radiation.

2.4. *NodC*-PCR amplification

NodC-PCR amplification was performed on a total of 117 isolates, 71 of which were amplified by *nifH* and 46 were not. Primers *nodC*-forward and *nodC*-reverse (Table 3-1) from New England Biolabs were used. The PCR reaction contained 1 µl DNA template, 3.9 µl Taq polymerase, 0.2 µl *nodC*-F and 0.2 µl *nodC*-R, made up to final volume of 11 µl with sterile milliQ water. Amplification was carried out in a Mastercycler^{ep} thermocycler, using the following PCR cycles: initial denaturing at 94^{o}C for 3 min, 30 cycles of, denaturing 94^{o}C for 1 min, annealing at 55^{o}C for 1 min extension at 72^{o}C for 1 min, and final extension at 72^{o}C for 7min. *NodC*-PCR products were examined via electrophoresis using 1.5% agarose gel containing 5% ethidium bromide (EtBr), run at 100 V for 40 min. Gels were viewed under UV radiation.

2.5. PCR amplification with BOX A1R primer

Isolates confirmed to contain the *nifH* gene were amplified by repetitive element polymerase chain reaction (rep-PCR) using BOX-A1R primer to assess genetic diversity. The PCR reaction contained 1 µl template, 8.75 µl Taq polymerase, 0.5 BOX-A1R primer, made up to final volume of 25 µl using water. The following thermocycler settings were used: initial denaturing at 94^{o}C for 5 min, 30 cycles of, denaturing 94^{o}C for 1 min, annealing at 60^{o}C for 1 min extension at 72^{o}C for 1 min, and final extension at 72^{o}C for 5min. Products obtained using BOX-PCR were analyzed using horizontal gel electrophoresis in a 3% agarose gel containing 5% EtBr. A 10,000 bp molecular marker (Quick Load DNA Ladder III, Apex™ Bioresearch Products) was loaded alongside

BOX-PCR products to estimate the size of band patterns. Gel was run at 300 V for 5 min followed by 18 hours at 80 V and visualization under UV radiation.

2.6. Cluster analysis

Cluster analyses performed on the BOX-PCR patterns using the GelCompar II program version 6.1 (Applied Mathematics, Belgium) was used to construct a dendrogram of similarity for all isolates. One analysis across all sites was performed, followed by three separate analyses, one for each site. The unweighted pair-group method with arithmetic mean (UPGMA) algorithm and the Jaccard coefficient were used. Grouping of isolates into clusters was based on 70% level of similarity (Loureiro et al. 2007; Kaschuk et al. 2006; Grange and Hungria 2004; Giongo et al. 2007).

3. Results

3.1. *NifH*-PCR and *NodC*-PCR

A total of 519 isolates were obtained from root nodules of 10 distinct hairy vetch genotypes inoculated with soil dilutions from six fields (Table 3-2). The *nifH* gene of 473 Rlv isolates could be PCR amplified, and despite characteristics corresponding to rhizobia phenotypes, 46 of the isolates failed to amplify, and are henceforth referred to as *nifH*-negative strains (Table 3-3). Nearly 60% of the *nifH*-negative strains were from Ivanhoe, and across all sites, 50% were from fields with HV history (Table 3-2). Interestingly, 22 of the *nifH*-negative strains were able to be positively amplified using PCR for the *nodC* gene, supporting characterization as rhizobia. The fewest *nifH*-positive isolates were obtained from the Iran 1 and Turkey 1 hairy vetch genotypes,

whereas the most isolates were obtained from the USA-NE genotype. Averaged across groups, most *nifH*-positive isolates were obtained in Group 5 genotypes.

3.2. Cluster analysis of BOX-PCR patterns

Site was found to be the main driver of isolate diversity overall (data not shown), however within sites, history of vetch cultivation was shown to be a strong determinant of grouping patterns. Over all three sites, number of BOX-PCR bands obtained per strain ranged from 3 (strain 557, isolated from Iran 2 inoculated with soil from Graham field with HV history) to over 25 (strains isolated from genotypes inoculated with soil from Cedar Grove) (Figures 3-1a and 3-1b). Great diversity was observed across all sites, with a total of 36 clusters identified that contained isolates with greater than 70% similarity to each other. Fifteen isolates from fields with HV history, and two from fields without HV history were found to have distinct banding patterns differing from a majority of the collected strains, and did not belong to any one cluster, with their most closely related cluster similarity levels ranging from only 28.8 to 55.9%. Significant diversity was observed across the sites, with almost 30% of the clusters (11) being small, containing only a few strains that were at least 80% similar to each other. The largest cluster comprised 96 of the total strains, with 90% of these strains originating from the Cedar Grove site. Within this cluster, there were 21 subgroups (defined as strains that are >90% similar within a cluster) with 2 to 6 strains each that grouped at 100% similarity level. Further, 16 of these subgroups contained only strains from -HV field from Cedar Grove, indicating high similarity level among Cedar Grove isolates. In addition, over 60% of the strains with 100% similarity were isolated from distinct HV

genotypes, suggesting that hairy vetch genotypes have little effect on the banding patterns, thus genetic diversity of strains. Except a few cases with the USA-MD genotypes, it was only when inoculated with soils from different sites and different field histories that genotypes were nodulated by unique Rlv strains. The second largest cluster across all 519 strains comprised 77 strains, with 44 strains from Graham, 8 strains from Cedar Grove and 25 strains from Ivanhoe.

Separate cluster analyses performed by site (Figures 3-2, 3-3 and 3-4) showed that except Ivanhoe, fields with HV history had higher levels of diversity than fields without history, with more identified clusters related at 70% similarity or higher in the fields with history (Figures 3-2, 3-3 and 3-4). The greatest number of clusters was identified at Ivanhoe, with 20 clusters identified, followed by Cedar Grove with 18, and Graham with 16 (Table 3-2). A few strains from each site did not fit into any identified cluster, including 9 from Graham, 4 from Cedar Grove and another 9 from Ivanhoe. The Graham site in particular contained one very large cluster comprised of 51 of the total 161 strains at that site, of which a little over half (65%) were from the field with no HV history. This mixing of strains from both field types within a cluster demonstrates genetic similarity of rhizobia found in both fields at this site. Cedar Grove contained a large cluster of 91 isolates of the total 173 grouping at greater than 70% similarity, of which 85% came from the field with no HV history and suggesting great rhizobial similarity in fields where no HV has been planted. Within this large cluster, 33 subgroups were identified that clustered at 100% similarity, and 24 subgroups clustering at >92% similarity level, again indicating a high level of similarity and low genetic

diversity among resident rhizobia strains at this site. Contrary to Graham and Cedar Grove, data suggest that Ivanhoe soils contained a higher level of diversity in the no HV field than in the HV history field. In Ivanhoe, only 12% of the isolates in the largest cluster (40 of 139 strains) were from fields with no HV history, and the majority of strains were from fields with a cultivation history of vetch and quite similar in their genetic makeup. Moreover, 8 of the 9 strains not belonging to any cluster were from no HV history field, further supporting our finding of greater diversity in the no history fields as compared to the field with HV history at this site. As previously mentioned, across all site-fields specificity of the different HV genotypes with particular strains was not pronounced. For example, a small number of strains isolated from across genotypes Iran 1, Afghanistan 2 and USA-MD2 were found to be 100% similar in Cedar Grove, and others isolated from USA-NE and Greece were also found to be 100% similar in Graham.

4. Discussion

Our results showed high Rlv diversity between sites, demonstrated by others to often result from prevailing site-specific environmental variables imposing general genetic adaptations on soil rhizobia (Bernal and Graham 2001; Farooq and Vessey 2009; Mutch et al. 2003; Tian et al. 2007; Yang ChengYun et al. 2008). There was great variability in soil phosphorus (P) content and soil pH among the 3 sites. The importance of soil P on legume-rhizobia symbiosis is well understood (Leite Silva et al. 2010; Wielbo and Skorupska 2008; Zaman-Allah et al. 2007), and soil P status has been shown to specifically affect diversity of Rlv in the field (Labidi et al. 2003). As well, there is a

large body of literature on the effect of soil pH, particularly acidity, on the diversity of rhizobia populations (Aarons and Graham 1991; Andrade et al. 2002a; Bala et al. 2001). Changing soil pH through liming can modify existing rhizobia populations (Andrade et al. 2002a), and high acid conditions have been found to select for strains that are tolerant to high acid conditions (Bala et al. 2001). Consequently, as strains become more suited to a given environment, they also become distinct from strains in other environments resulting in increased diversity between sites. Our research, in combination with previous findings, suggests that soil characteristics present in a site may have influenced population structure of resident Rlv between sites sampled in this study.

With the exception of the Ivanhoe site, there was greater rhizobia diversity in fields with histories of HV cultivation than in fields without history. Although studies have shown past cultivation of legume species to increase the population size of compatible rhizobia (Andrade et al. 2003; Chemining'wa and Vessey 2006), this is the first report documenting the effect of legume cultivation history on genetic structure of compatible rhizobia. Field inoculation with compatible rhizobia is known to increase the genetic diversity resident rhizobia (de Fatima Loureiro et al. 2007) likely by introducing new rhizobia strains into the environment with the potential for transfer of genetic material between strains. Several studies have suggested horizontal gene transfer to be a major factor contributing to rhizobia diversity (Aoki et al. 2010; Barcellos et al. 2007). The higher diversity observed in our field with HV history that had received past rhizobia inoculation (Cedar Grove) relative to the paired fields with no vetch history, may possibly be attributed to introduction of Rlv through inoculation. However, this

idea is contrary to our finding that the field with HV cultivation history field in Graham, with no history of inoculation, was also found to have a greater diversity of rhizobia strains than the paired no HV history field, suggesting that it is the presence of hairy vetch roots, or perhaps strains introduced to the field inadvertently on the legume seed at planting, that serve to increase diversity in this field rather than inoculation alone. There is no identified reason why horizontal gene transfer between existing strains cannot also occur in fields with no HV history (Zhang et al. 2001), and the mechanism through which legume cultivation increases diversity requires further attention.

The high diversity in the Ivanhoe field that had never been planted to hairy vetch was unexpected, particularly since this field had the lowest pH, which often is linked to reduced diversity as compared to similar fields with higher pH values. The Ivanhoe field with no HV history had never been cultivated. Studies have reported high levels of diversity in uncultivated sites under native vegetation compared to cropped areas (Hungria et al. 2006). Some agricultural management practices such as tillage have been shown to reduce the diversity of resident populations of rhizobia relative to untilled fields (Ferreira et al. 2000; de Fatima Loureiro et al. 2007). We have shown here that in two of the three sites, past use of hairy vetch increased genetic diversity of Rlv. Additionally, we observed extremely low diversity of rhizobia in the Cedar Grove field with no history of hairy vetch, a field that had been intentionally planted to grass for over 20 years, with almost all Rlv isolates showing great similarity in their genetic makeup. The low pH of the Cedar Grove field could also result in selection of particular strain types adapted to acidic conditions, (Andrade et al. 2002b; Hungria and Vargas

2000; Zahran 1999). Our findings are consistent with Hungria et al. (2006), emphasizing that the rhizobia diversity in uncultivated soils may either be maintained or increased by agricultural practices such as no-till and crop rotations including a legume host, or reduced by practices such as tillage and monoculture.

Our results indicate HV genotype to be less important in determining diversity of associated rhizobia than site or a field having a history of vetch planting. In general, legume species in the tribe viciae are known to be specific in their symbiosis, and are particularly capable of selecting preferred rhizobia strains from diverse populations in the soil (Laguerre et al. 2003). Faba bean species (*Vicia faba*), another host species for Rlv, is more discriminative than pea in strain selection, such that it is consistently nodulated by Rlv *nod* type *g* (Laguerre et al. 2003). Pea genotype has also been suggested to influence diversity of Rlv isolates (Depret and Laguerre 2008). Our observed lack of diversity in Rlv as related to host genotype may have been related to the use of BOX-PCR technique for characterization, a less discriminatory technique as compared to the use of gene-specific primers. Using REP-PCR techniques, (Laguerre et al. 2003) showed that vetches are less discriminative of Rlv genomic backgrounds than pea or faba bean. It is possible that the use of techniques targeting more specific functional symbiotic genes such as *nif* and *nod* genes could reveal more information on rhizobia diversity associated with the distinct hairy vetch genotypes.

5. Conclusions

In this study we evaluated genetic structure of rhizobia isolated from fields with and without history of HV cultivation. Ten different HV genotypes were used as trap host for resident Rlv across paired fields from three farms. Our results showed that strain diversity depended greatly on site conditions, with greater diversity observed between sites than by field management of vetch history. Past history of hairy vetch cultivation was found to increase diversity of Rlv. In general there appears to be little distinction between the Rlv trapped by different HV genotypes, particularly where initial soil rhizobial diversity is low. However, techniques that evaluate diversity of symbiotic genes such as *nif*, *nod* and *fix* genes could be used to gain more information on the influence of host genotypes. Overall, understanding how previous cultivation of hairy vetch has important and far-reaching economic and ecological implications for farmers interested in using hairy vetch as a winter annual cover crop, as strain genetic structure can ultimately affect the amount of N_2 fixed by cover crops used across agroecosystems.

Table 3-1: Oligonucleotides used as PCR primers

Primer	5' - 3' Nucleotide sequence	Target gene	Reference
nifH Forward	GCTGCCTATGCAGACGATG	*nifH*	Kaschuk et al. 2006
nifH Reverse	TTACTGGCTTTCATTTGGC	*nifH*	Kaschuk et al. 2006
nodC Forward	GCTGCCTATGCAGACGATG	nodC	Sarita et al. 2005
nodC Reverse	GGTTACTGGCTTTCATTTGGC	nodC	Sarita et al. 2005
BOX-A1R	CTACGGCAAGGCGACGCTGACG	DNA located between BOX sequences	Kaschuk et al. 2006

Table 3-2: Number of rhizobia isolates obtained from nodules of HV genotypes inoculated with soil dilutions from field with and without hairy vetch cultivation history from Graham, Cedar Grove and Ivanhoe

	Graham		Cedar Grove		Ivanhoe		
	+HV	-HV	+HV	-HV	+HV	-HV	Total
Turkey 1	6	3	6	5	3	3	26
Turkey 2	5	9	9	6	6	6	41
Iran 1	5	3	6	6	3	0	23
USA-NE	11	12	12	12	11	9	67
Afghanistan 1	5	9	8	9	9	7	47
Iran 2	7	12	10	12	11	12	64
Afghanistan 2	11	12	8	12	9	7	59
Greece	6	6	2	6	3	3	26
USA-MD 1	10	10	12	12	12	6	62
USA-MD 2	10	10	9	10	12	6	57
Total	76	86	82	90	79	59	472

Table 3-3: Isolates of Rlv that did not PCR-amplify the *nifH* gene

Location	Field History	Genotype group	Genotype
Graham	+HV	2	USA-NE
Graham	+HV	3	Afghanistan 1
Graham	+HV	5	USA-MD 1
Graham	+HV	5	USA-MD 1
Graham	-HV	5	USA-MD 1
Graham	-HV	5	USA-MD 1
Graham	-HV	5	USA-MD 2
Graham	-HV	5	USA-MD 2
Cedar Grove	+HV	2	USA-NE
Cedar Grove	+HV	3	Afghanistan 1
Cedar Grove	+HV	3	Iran 2
Cedar Grove	+HV	4	Afghanistan 2
Cedar Grove	+HV	5	USA-MD 2
Cedar Grove	+HV	5	USA-MD 2
Cedar Grove	+HV	3	Iran 2
Cedar Grove	+HV	4	Afghanistan 2
Cedar Grove	+HV	4	Greece
Cedar Grove	-HV	1	Turkey 1
Cedar Grove	-HV	2	USA-NE
Ivanhoe	+HV	1	Turkey 1
Ivanhoe	+HV	1	Turkey 2
Ivanhoe	+HV	1	Turkey 2
Ivanhoe	+HV	2	Iran 1
Ivanhoe	-HV	1	Turkey 1
Ivanhoe	-HV	2	USA-NE
Ivanhoe	-HV	3	Iran 2
Ivanhoe	-HV	4	Afghanistan 2
Ivanhoe	-HV	4	Afghanistan 2
Ivanhoe	-HV	5	USA-MD 2
Ivanhoe	-HV	5	USA-MD 2
Ivanhoe	+HV	1	Turkey 1
Ivanhoe	-HV	2	USA-NE
Ivanhoe	-HV	3	Afghanistan 1
Ivanhoe	-HV	3	Iran 2
Ivanhoe	+HV	3	Afghanistan 1
Ivanhoe	+HV	4	Afghanistan 2
Ivanhoe	+HV	4	Afghanistan 2
Ivanhoe	+HV	4	Afghanistan 2
Ivanhoe	-HV	3	Afghanistan 1
Ivanhoe	-HV	4	Afghanistan 2
Ivanhoe	-HV	4	Afghanistan 2
Ivanhoe	-HV	4	Afghanistan 2

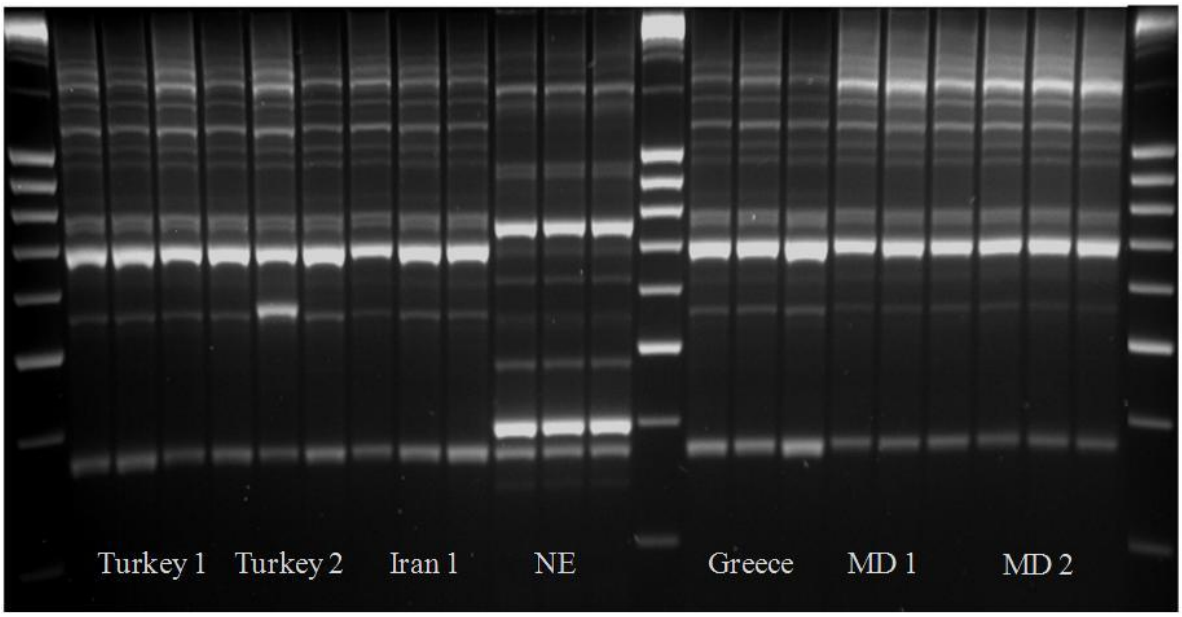


Figure 3-1a. Gel image of BOX-PCR products isolated from nodules of distinct hairy vetch genotypes inoculated with soil dilutions form a no hairy vetch history field from Cedar Grove

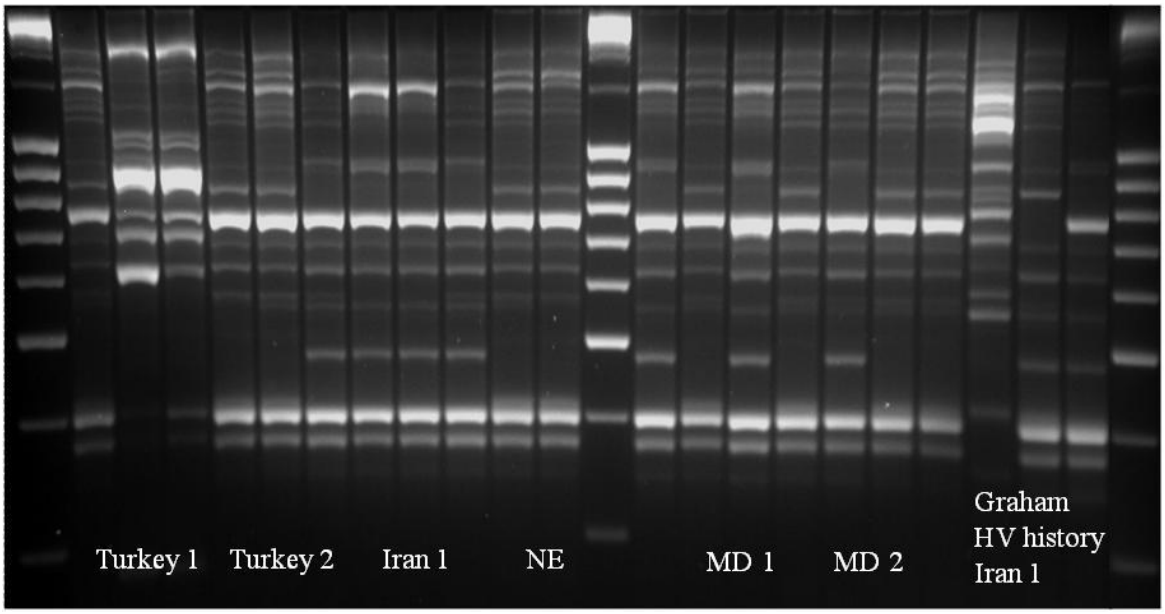
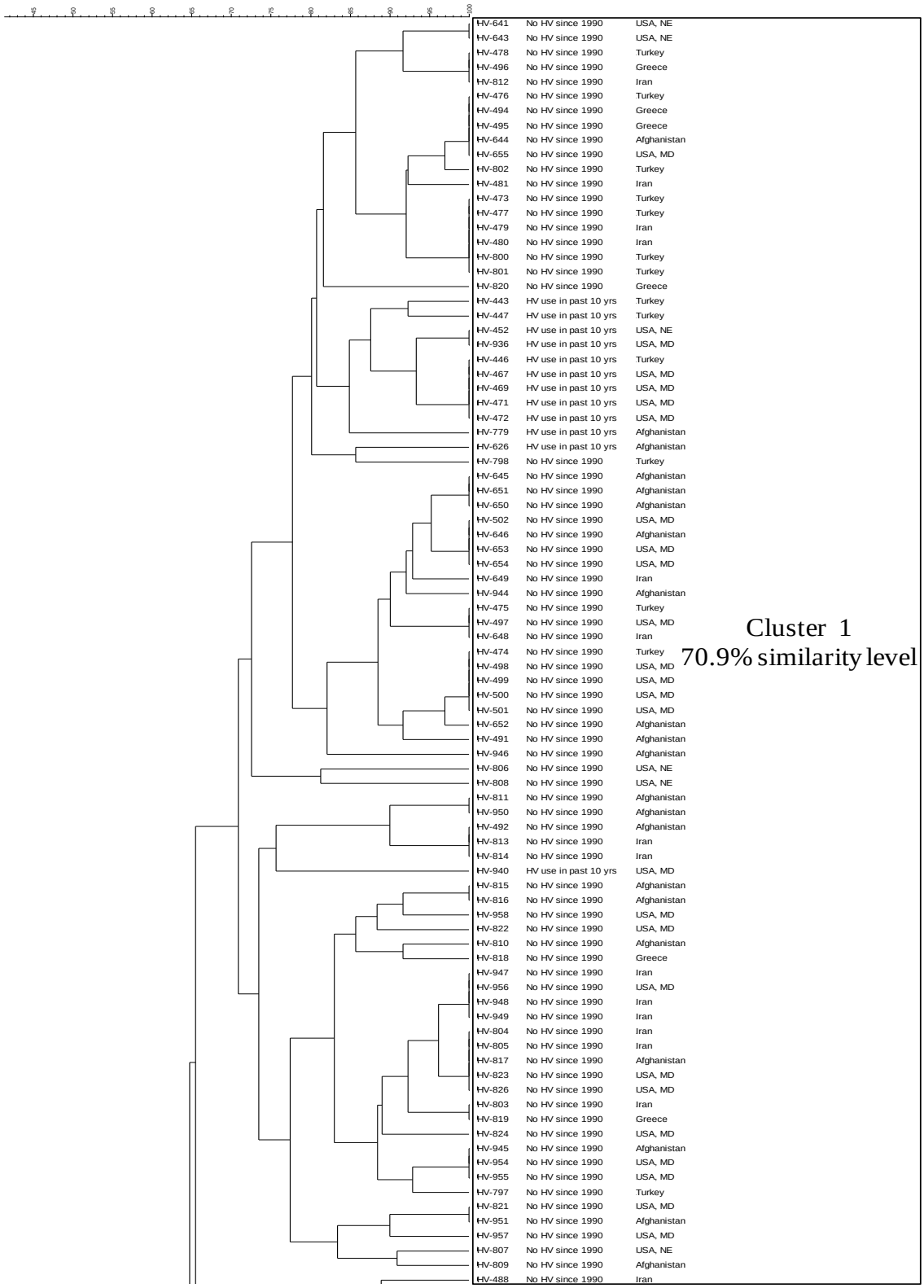


Figure 3-1b. Gel image of BOX-PCR products isolated from nodules of distinct hairy vetch genotypes inoculated with soil dilutions form a field with hairy vetch history from Cedar Grove

Figure 3-2. Dendrogram of rhizobia strains isolated from ten hairy vetch genotypes inoculated with Cedar Grove soil from fields with and without a history of hairy vetch cultivation.


HV-641 No HV since 1990 USA, NE
HV-643 No HV since 1990 USA, NE
HV-478 No HV since 1990 Turkey
HV-496 No HV since 1990 Greece
HV-812 No HV since 1990 Iran
HV-476 No HV since 1990 Turkey
HV-494 No HV since 1990 Greece
HV-495 No HV since 1990 Greece
HV-644 No HV since 1990 Afghanistan
HV-655 No HV since 1990 USA, MD
HV-802 No HV since 1990 Turkey
HV-481 No HV since 1990 Iran
HV-473 No HV since 1990 Turkey
HV-477 No HV since 1990 Turkey
HV-479 No HV since 1990 Iran
HV-480 No HV since 1990 Iran
HV-800 No HV since 1990 Turkey
HV-801 No HV since 1990 Turkey
HV-820 No HV since 1990 Greece
HV-443 HV use in past 10 yrs Turkey
HV-447 HV use in past 10 yrs Turkey
HV-452 HV use in past 10 yrs USA, NE
HV-936 HV use in past 10 yrs USA, MD
HV-446 HV use in past 10 yrs Turkey
HV-467 HV use in past 10 yrs USA, MD
HV-469 HV use in past 10 yrs USA, MD
HV-471 HV use in past 10 yrs USA, MD
HV-472 HV use in past 10 yrs USA, MD
HV-779 HV use in past 10 yrs Afghanistan
HV-626 HV use in past 10 yrs Afghanistan
HV-798 No HV since 1990 Turkey
HV-645 No HV since 1990 Afghanistan
HV-651 No HV since 1990 Afghanistan
HV-650 No HV since 1990 Afghanistan
HV-502 No HV since 1990 USA, MD
HV-646 No HV since 1990 Afghanistan
HV-653 No HV since 1990 USA, MD
HV-654 No HV since 1990 USA, MD
HV-649 No HV since 1990 Iran
HV-944 No HV since 1990 Afghanistan
HV-475 No HV since 1990 Turkey
HV-497 No HV since 1990 USA, MD
HV-648 No HV since 1990 Iran
HV-474 No HV since 1990 Turkey
HV-498 No HV since 1990 USA, MD
HV-499 No HV since 1990 USA, MD
HV-500 No HV since 1990 USA, MD
HV-501 No HV since 1990 USA, MD
HV-652 No HV since 1990 Afghanistan
HV-491 No HV since 1990 Afghanistan
HV-946 No HV since 1990 Afghanistan
HV-806 No HV since 1990 USA, NE
HV-808 No HV since 1990 USA, NE
HV-811 No HV since 1990 Afghanistan
HV-950 No HV since 1990 Afghanistan
HV-492 No HV since 1990 Afghanistan
HV-813 No HV since 1990 Iran
HV-814 No HV since 1990 Iran
HV-940 HV use in past 10 yrs USA, MD
HV-815 No HV since 1990 Afghanistan
HV-816 No HV since 1990 Afghanistan
HV-958 No HV since 1990 USA, MD
HV-822 No HV since 1990 USA, MD
HV-810 No HV since 1990 Afghanistan
HV-818 No HV since 1990 Greece
HV-947 No HV since 1990 Iran
HV-956 No HV since 1990 USA, MD
HV-948 No HV since 1990 Iran
HV-949 No HV since 1990 Iran
HV-804 No HV since 1990 Iran
HV-805 No HV since 1990 Iran
HV-817 No HV since 1990 Afghanistan
HV-823 No HV since 1990 USA, MD
HV-826 No HV since 1990 USA, MD
HV-803 No HV since 1990 Iran
HV-819 No HV since 1990 Greece
HV-824 No HV since 1990 USA, MD
HV-945 No HV since 1990 Afghanistan
HV-954 No HV since 1990 USA, MD
HV-955 No HV since 1990 USA, MD
HV-797 No HV since 1990 Turkey
HV-821 No HV since 1990 USA, MD
HV-951 No HV since 1990 Afghanistan
HV-957 No HV since 1990 USA, MD
HV-807 No HV since 1990 USA, NE
HV-809 No HV since 1990 Afghanistan
HV-488 No HV since 1990 Iran
Cluster 1
70.9% similarity level

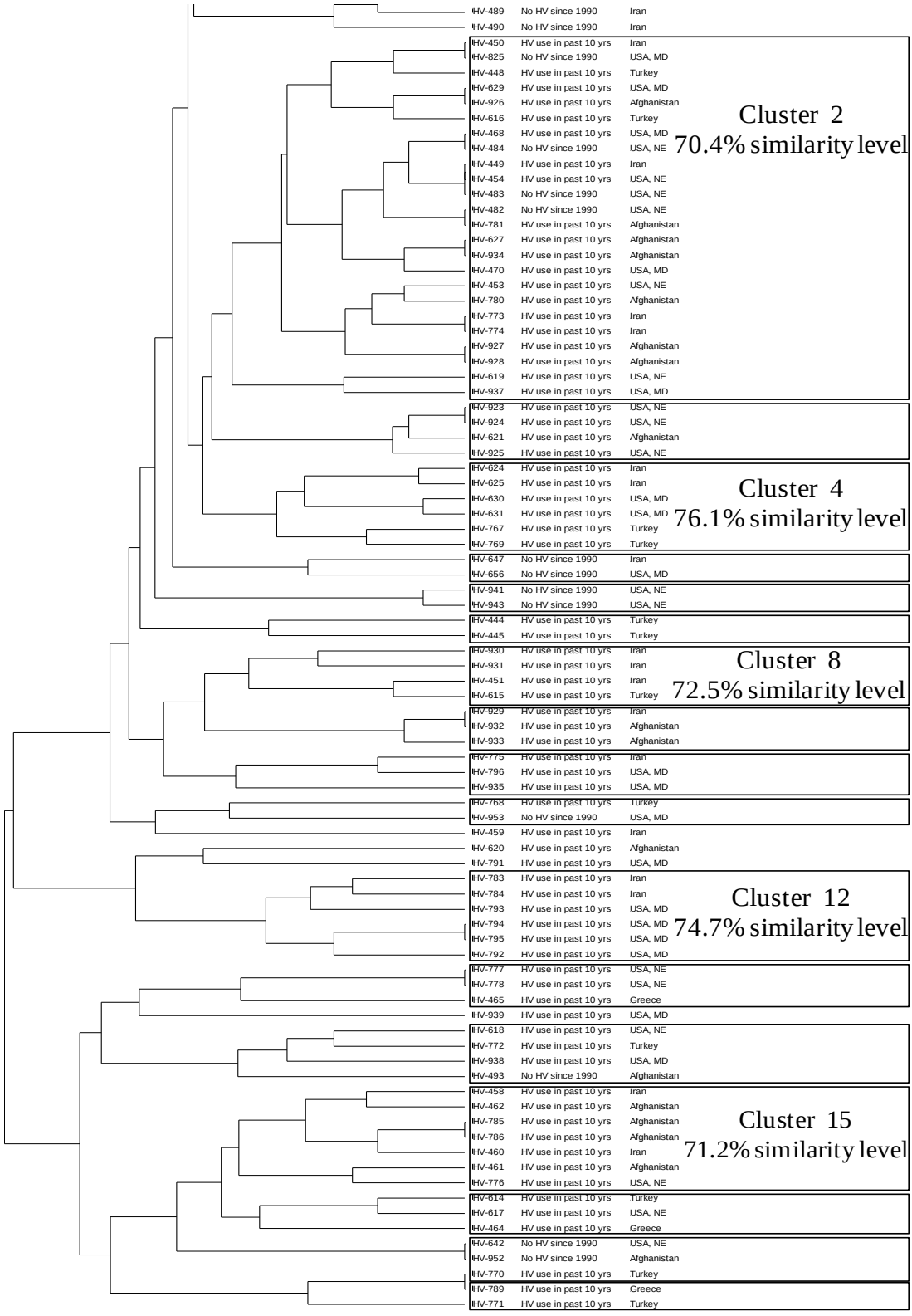
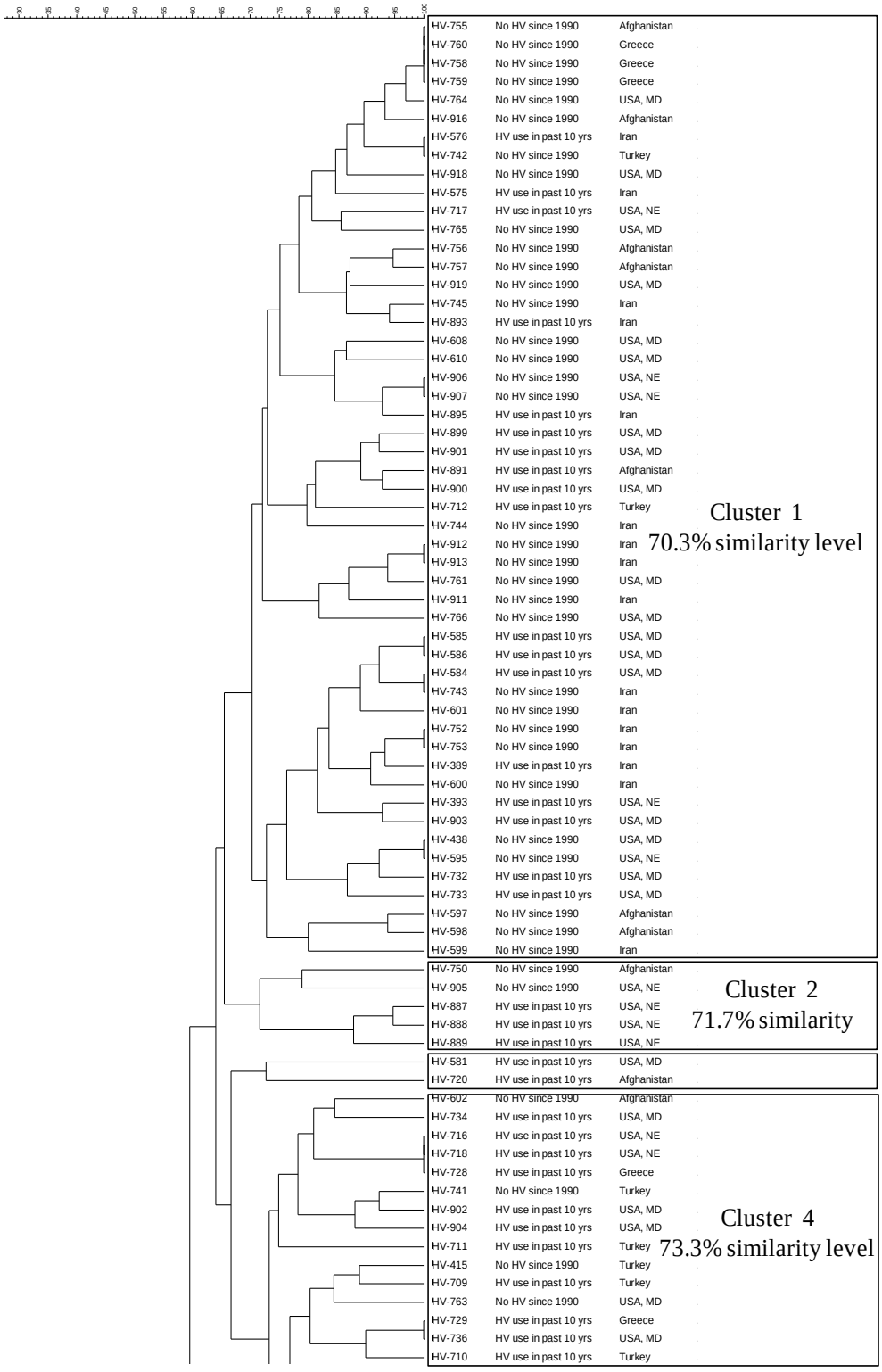

HV-489 No HV since 1990 Iran
HV-490 No HV since 1990 Iran
HV-450 HV use in past 10 yrs Iran
HV-825 No HV since 1990 USA, MD
HV-448 HV use in past 10 yrs Turkey
HV-629 HV use in past 10 yrs USA, MD
HV-926 HV use in past 10 yrs Afghanistan
HV-616 HV use in past 10 yrs Turkey
HV-468 HV use in past 10 yrs USA, MD
HV-484 No HV since 1990 USA, NE
HV-449 HV use in past 10 yrs Iran
HV-454 HV use in past 10 yrs USA, NE
HV-483 No HV since 1990 USA, NE
HV-482 No HV since 1990 USA, NE
HV-781 HV use in past 10 yrs Afghanistan
HV-627 HV use in past 10 yrs Afghanistan
HV-934 HV use in past 10 yrs Afghanistan
HV-470 HV use in past 10 yrs USA, MD
HV-453 HV use in past 10 yrs USA, NE
HV-780 HV use in past 10 yrs Afghanistan
HV-773 HV use in past 10 yrs Iran
HV-774 HV use in past 10 yrs Iran
HV-927 HV use in past 10 yrs Afghanistan
HV-928 HV use in past 10 yrs Afghanistan
HV-619 HV use in past 10 yrs USA, NE
HV-937 HV use in past 10 yrs USA, MD
HV-923 HV use in past 10 yrs USA, NE
HV-924 HV use in past 10 yrs USA, NE
HV-621 HV use in past 10 yrs Afghanistan
HV-925 HV use in past 10 yrs USA, NE
HV-624 HV use in past 10 yrs Iran
HV-625 HV use in past 10 yrs Iran
HV-630 HV use in past 10 yrs USA, MD
HV-631 HV use in past 10 yrs USA, MD
HV-767 HV use in past 10 yrs Turkey
HV-769 HV use in past 10 yrs Turkey
HV-647 No HV since 1990 Iran
HV-656 No HV since 1990 USA, MD
HV-941 No HV since 1990 USA, NE
HV-943 No HV since 1990 USA, NE
HV-444 HV use in past 10 yrs Turkey
HV-445 HV use in past 10 yrs Turkey
HV-930 HV use in past 10 yrs Iran
HV-931 HV use in past 10 yrs Iran
HV-451 HV use in past 10 yrs Iran
HV-615 HV use in past 10 yrs Turkey
HV-929 HV use in past 10 yrs Iran
HV-932 HV use in past 10 yrs Afghanistan
HV-933 HV use in past 10 yrs Afghanistan
HV-775 HV use in past 10 yrs Iran
HV-796 HV use in past 10 yrs USA, MD
HV-935 HV use in past 10 yrs USA, MD
HV-768 HV use in past 10 yrs Turkey
HV-953 No HV since 1990 USA, MD
HV-459 HV use in past 10 yrs Iran
HV-620 HV use in past 10 yrs Afghanistan
HV-791 HV use in past 10 yrs USA, MD
HV-783 HV use in past 10 yrs Iran
HV-784 HV use in past 10 yrs Iran
HV-793 HV use in past 10 yrs USA, MD
HV-794 HV use in past 10 yrs USA, MD
HV-795 HV use in past 10 yrs USA, MD
HV-792 HV use in past 10 yrs USA, MD
HV-777 HV use in past 10 yrs USA, NE
HV-778 HV use in past 10 yrs USA, NE
HV-465 HV use in past 10 yrs Greece
HV-939 HV use in past 10 yrs USA, MD
HV-618 HV use in past 10 yrs USA, NE
HV-772 HV use in past 10 yrs Turkey
HV-938 HV use in past 10 yrs USA, MD
HV-493 No HV since 1990 Afghanistan
HV-458 HV use in past 10 yrs Iran
HV-462 HV use in past 10 yrs Afghanistan
HV-785 HV use in past 10 yrs Afghanistan
HV-786 HV use in past 10 yrs Afghanistan
HV-460 HV use in past 10 yrs Iran
HV-461 HV use in past 10 yrs Afghanistan
HV-776 HV use in past 10 yrs USA, NE
HV-614 HV use in past 10 yrs Turkey
HV-617 HV use in past 10 yrs USA, NE
HV-464 HV use in past 10 yrs Greece
HV-642 No HV since 1990 USA, NE
HV-952 No HV since 1990 Afghanistan
HV-770 HV use in past 10 yrs Turkey
HV-789 HV use in past 10 yrs Greece
HV-771 HV use in past 10 yrs Turkey
Cluster 2
70.4% similarity level
Cluster 4
76.1% similarity level
Cluster 8
72.5% similarity level
Cluster 12
74.7% similarity level
Cluster 15
71.2% similarity level

Figure 3-3. Dendrogram of rhizobia strains isolated from ten hairy vetch genotypes inoculated with Graham soil from fields with and without a history of hairy vetch cultivation.


HV-755 No HV since 1990 Afghanistan
HV-760 No HV since 1990 Greece
HV-758 No HV since 1990 Greece
HV-759 No HV since 1990 Greece
HV-764 No HV since 1990 USA, MD
HV-916 No HV since 1990 Afghanistan
HV-576 HV use in past 10 yrs Iran
HV-742 No HV since 1990 Turkey
HV-918 No HV since 1990 USA, MD
HV-575 HV use in past 10 yrs Iran
HV-717 HV use in past 10 yrs USA, NE
HV-765 No HV since 1990 USA, MD
HV-756 No HV since 1990 Afghanistan
HV-757 No HV since 1990 Afghanistan
HV-919 No HV since 1990 USA, MD
HV-745 No HV since 1990 Iran
HV-893 HV use in past 10 yrs Iran
HV-608 No HV since 1990 USA, MD
HV-610 No HV since 1990 USA, MD
HV-906 No HV since 1990 USA, NE
HV-907 No HV since 1990 USA, NE
HV-895 HV use in past 10 yrs Iran
HV-899 HV use in past 10 yrs USA, MD
HV-901 HV use in past 10 yrs USA, MD
HV-891 HV use in past 10 yrs Afghanistan
HV-900 HV use in past 10 yrs USA, MD
HV-712 HV use in past 10 yrs Turkey
HV-744 No HV since 1990 Iran
HV-912 No HV since 1990 Iran
HV-913 No HV since 1990 Iran
HV-761 No HV since 1990 USA, MD
HV-911 No HV since 1990 Iran
HV-766 No HV since 1990 USA, MD
HV-585 HV use in past 10 yrs USA, MD
HV-586 HV use in past 10 yrs USA, MD
HV-584 HV use in past 10 yrs USA, MD
HV-743 No HV since 1990 Iran
HV-601 No HV since 1990 Iran
HV-752 No HV since 1990 Iran
HV-753 No HV since 1990 Iran
HV-389 HV use in past 10 yrs Iran
HV-600 No HV since 1990 Iran
HV-393 HV use in past 10 yrs USA, NE
HV-903 HV use in past 10 yrs USA, MD
HV-438 No HV since 1990 USA, MD
HV-595 No HV since 1990 USA, NE
HV-732 HV use in past 10 yrs USA, MD
HV-733 HV use in past 10 yrs USA, MD
HV-597 No HV since 1990 Afghanistan
HV-598 No HV since 1990 Afghanistan
HV-599 No HV since 1990 Iran
HV-750 No HV since 1990 Afghanistan
HV-905 No HV since 1990 USA, NE
HV-887 HV use in past 10 yrs USA, NE
HV-888 HV use in past 10 yrs USA, NE
HV-889 HV use in past 10 yrs USA, NE
HV-581 HV use in past 10 yrs USA, MD
HV-720 HV use in past 10 yrs Afghanistan
HV-602 No HV since 1990 Afghanistan
HV-734 HV use in past 10 yrs USA, MD
HV-716 HV use in past 10 yrs USA, NE
HV-718 HV use in past 10 yrs USA, NE
HV-728 HV use in past 10 yrs Greece
HV-741 No HV since 1990 Turkey
HV-902 HV use in past 10 yrs USA, MD
HV-904 HV use in past 10 yrs USA, MD
HV-711 HV use in past 10 yrs Turkey
HV-415 No HV since 1990 Turkey
HV-709 HV use in past 10 yrs Turkey
HV-763 No HV since 1990 USA, MD
HV-729 HV use in past 10 yrs Greece
HV-736 HV use in past 10 yrs USA, MD
HV-710 HV use in past 10 yrs Turkey
Cluster 1
70.3% similarity level
Cluster 2
71.7% similarity
Cluster 4
73.3% similarity level

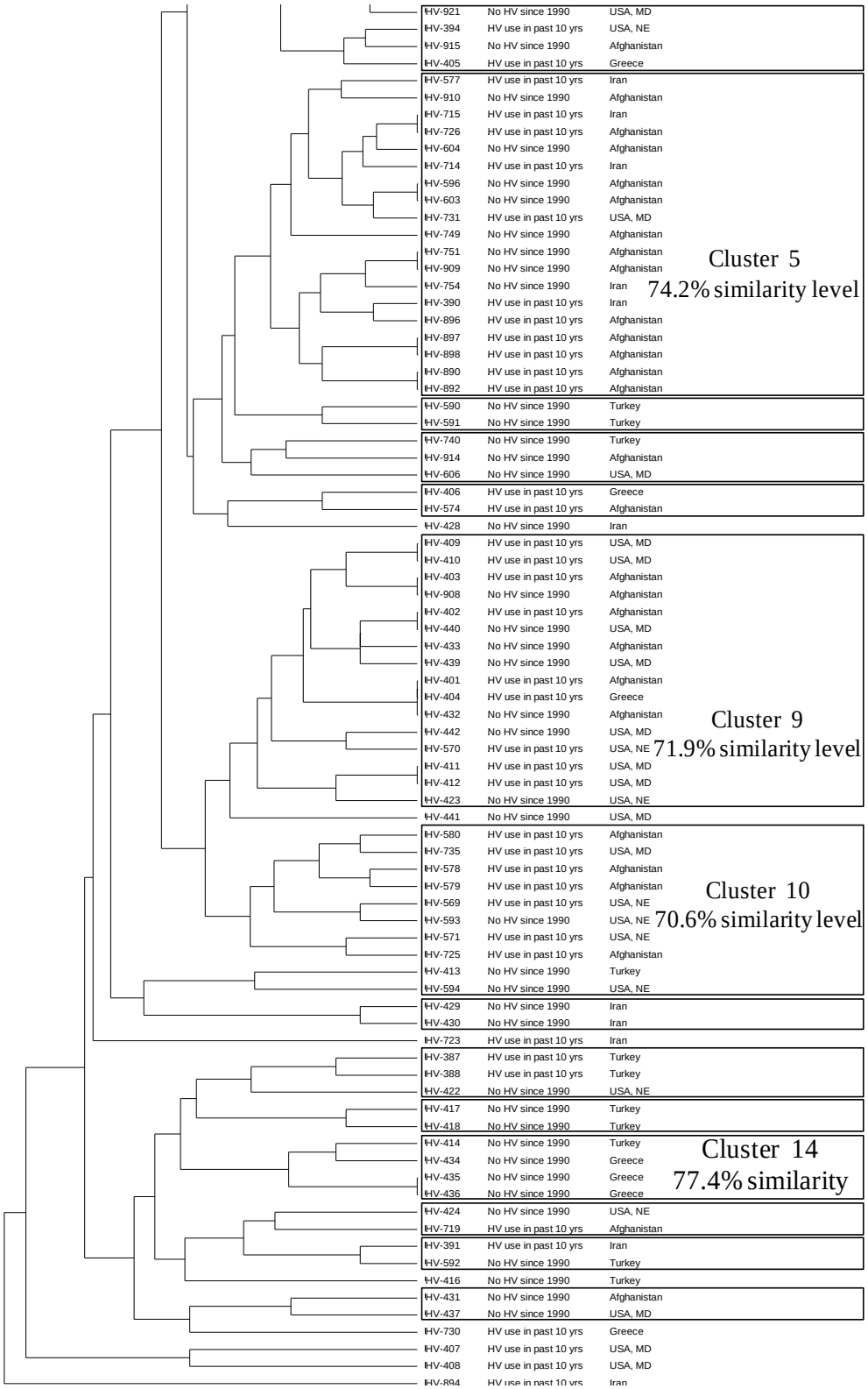
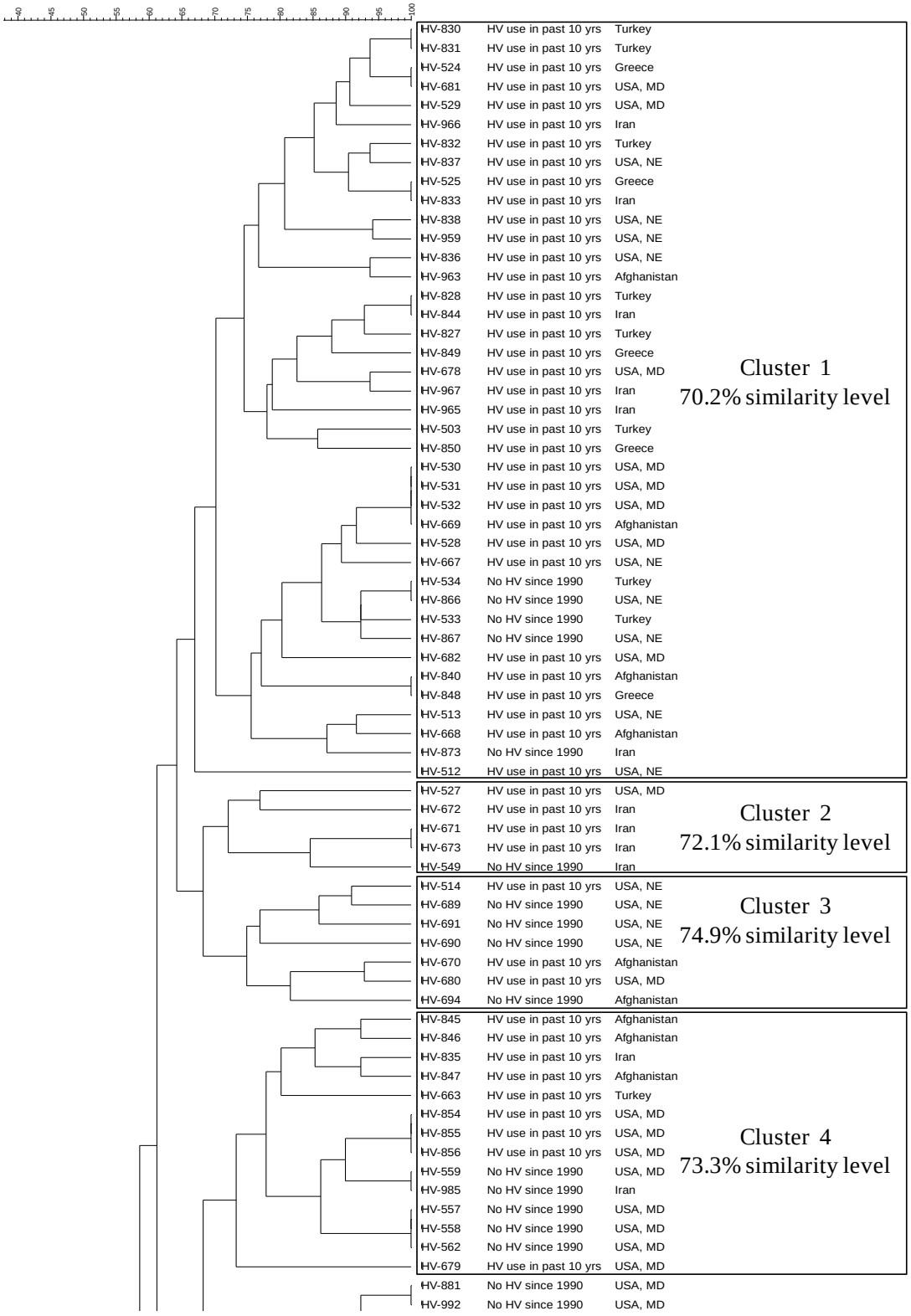
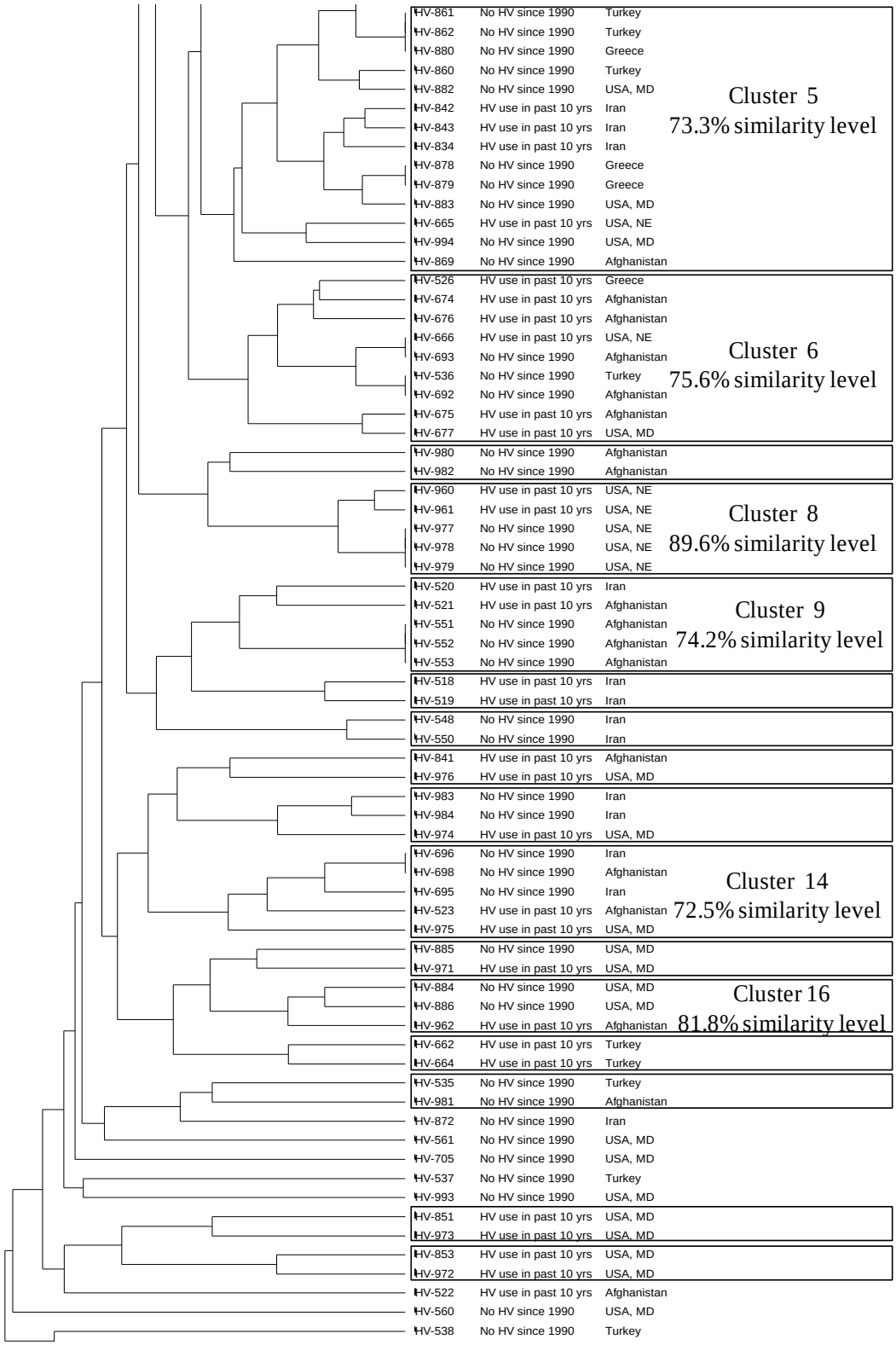

HV-921 No HV since 1990 USA, MD
HV-394 HV use in past 10 yrs USA, NE
HV-915 No HV since 1990 Afghanistan
HV-405 HV use in past 10 yrs Greece
HV-577 HV use in past 10 yrs Iran
HV-910 No HV since 1990 Afghanistan
HV-715 HV use in past 10 yrs Iran
HV-726 HV use in past 10 yrs Afghanistan
HV-604 No HV since 1990 Afghanistan
HV-714 HV use in past 10 yrs Iran
HV-596 No HV since 1990 Afghanistan
HV-603 No HV since 1990 Afghanistan
HV-731 HV use in past 10 yrs USA, MD
HV-749 No HV since 1990 Afghanistan
HV-751 No HV since 1990 Afghanistan
HV-909 No HV since 1990 Afghanistan
HV-754 No HV since 1990 Iran
HV-390 HV use in past 10 yrs Iran
HV-896 HV use in past 10 yrs Afghanistan
HV-897 HV use in past 10 yrs Afghanistan
HV-898 HV use in past 10 yrs Afghanistan
HV-890 HV use in past 10 yrs Afghanistan
HV-892 HV use in past 10 yrs Afghanistan
Cluster 5
74.2% similarity level
HV-590 No HV since 1990 Turkey
HV-591 No HV since 1990 Turkey
HV-740 No HV since 1990 Turkey
HV-914 No HV since 1990 Afghanistan
HV-606 No HV since 1990 USA, MD
HV-406 HV use in past 10 yrs Greece
HV-574 HV use in past 10 yrs Afghanistan
HV-428 No HV since 1990 Iran
HV-409 HV use in past 10 yrs USA, MD
HV-410 HV use in past 10 yrs USA, MD
HV-403 HV use in past 10 yrs Afghanistan
HV-908 No HV since 1990 Afghanistan
HV-402 HV use in past 10 yrs Afghanistan
HV-440 No HV since 1990 USA, MD
HV-433 No HV since 1990 Afghanistan
HV-439 No HV since 1990 USA, MD
HV-401 HV use in past 10 yrs Afghanistan
HV-404 HV use in past 10 yrs Greece
HV-432 No HV since 1990 Afghanistan
HV-442 No HV since 1990 USA, MD
HV-570 HV use in past 10 yrs USA, NE
HV-411 HV use in past 10 yrs USA, MD
HV-412 HV use in past 10 yrs USA, MD
HV-423 No HV since 1990 USA, NE
Cluster 9
71.9% similarity level
HV-441 No HV since 1990 USA, MD
HV-580 HV use in past 10 yrs Afghanistan
HV-735 HV use in past 10 yrs USA, MD
HV-578 HV use in past 10 yrs Afghanistan
HV-579 HV use in past 10 yrs Afghanistan
HV-569 HV use in past 10 yrs USA, NE
HV-593 No HV since 1990 USA, NE
HV-571 HV use in past 10 yrs USA, NE
HV-725 HV use in past 10 yrs Afghanistan
HV-413 No HV since 1990 Turkey
HV-594 No HV since 1990 USA, NE
Cluster 10
70.6% similarity level
HV-429 No HV since 1990 Iran
HV-430 No HV since 1990 Iran
HV-723 HV use in past 10 yrs Iran
HV-387 HV use in past 10 yrs Turkey
HV-388 HV use in past 10 yrs Turkey
HV-422 No HV since 1990 USA, NE
HV-417 No HV since 1990 Turkey
HV-418 No HV since 1990 Turkey
HV-414 No HV since 1990 Turkey
HV-434 No HV since 1990 Greece
HV-435 No HV since 1990 Greece
HV-436 No HV since 1990 Greece
Cluster 14
77.4% similarity
HV-424 No HV since 1990 USA, NE
HV-719 HV use in past 10 yrs Afghanistan
HV-391 HV use in past 10 yrs Iran
HV-592 No HV since 1990 Turkey
HV-416 No HV since 1990 Turkey
HV-431 No HV since 1990 Afghanistan
HV-437 No HV since 1990 USA, MD
HV-730 HV use in past 10 yrs Greece
HV-407 HV use in past 10 yrs USA, MD
HV-408 HV use in past 10 yrs USA, MD
HV-894 HV use in past 10 yrs Iran

Figure 3-4. Dendrogram of rhizobia strains isolated from ten hairy vetch genotypes inoculated with Ivanhoe soil from fields with and without a history of hairy vetch cultivation.


40 45 50 55 60 65 70 75 80 85 90 95 100
HV-830 HV use in past 10 yrs Turkey
HV-831 HV use in past 10 yrs Turkey
HV-524 HV use in past 10 yrs Greece
HV-681 HV use in past 10 yrs USA, MD
HV-529 HV use in past 10 yrs USA, MD
HV-966 HV use in past 10 yrs Iran
HV-832 HV use in past 10 yrs Turkey
HV-837 HV use in past 10 yrs USA, NE
HV-525 HV use in past 10 yrs Greece
HV-833 HV use in past 10 yrs Iran
HV-838 HV use in past 10 yrs USA, NE
HV-959 HV use in past 10 yrs USA, NE
HV-836 HV use in past 10 yrs USA, NE
HV-963 HV use in past 10 yrs Afghanistan
HV-828 HV use in past 10 yrs Turkey
HV-844 HV use in past 10 yrs Iran
HV-827 HV use in past 10 yrs Turkey
HV-849 HV use in past 10 yrs Greece
HV-678 HV use in past 10 yrs USA, MD
HV-967 HV use in past 10 yrs Iran
HV-965 HV use in past 10 yrs Iran
HV-503 HV use in past 10 yrs Turkey
HV-850 HV use in past 10 yrs Greece
HV-530 HV use in past 10 yrs USA, MD
HV-531 HV use in past 10 yrs USA, MD
HV-532 HV use in past 10 yrs USA, MD
HV-669 HV use in past 10 yrs Afghanistan
HV-528 HV use in past 10 yrs USA, MD
HV-667 HV use in past 10 yrs USA, NE
HV-534 No HV since 1990 Turkey
HV-866 No HV since 1990 USA, NE
HV-533 No HV since 1990 Turkey
HV-867 No HV since 1990 USA, NE
HV-682 HV use in past 10 yrs USA, MD
HV-840 HV use in past 10 yrs Afghanistan
HV-848 HV use in past 10 yrs Greece
HV-513 HV use in past 10 yrs USA, NE
HV-668 HV use in past 10 yrs Afghanistan
HV-873 No HV since 1990 Iran
HV-512 HV use in past 10 yrs USA, NE
HV-527 HV use in past 10 yrs USA, MD
HV-672 HV use in past 10 yrs Iran
HV-671 HV use in past 10 yrs Iran
HV-673 HV use in past 10 yrs Iran
HV-549 No HV since 1990 Iran
HV-514 HV use in past 10 yrs USA, NE
HV-689 No HV since 1990 USA, NE
HV-691 No HV since 1990 USA, NE
HV-690 No HV since 1990 USA, NE
HV-670 HV use in past 10 yrs Afghanistan
HV-680 HV use in past 10 yrs USA, MD
HV-694 No HV since 1990 Afghanistan
HV-845 HV use in past 10 yrs Afghanistan
HV-846 HV use in past 10 yrs Afghanistan
HV-835 HV use in past 10 yrs Iran
HV-847 HV use in past 10 yrs Afghanistan
HV-663 HV use in past 10 yrs Turkey
HV-854 HV use in past 10 yrs USA, MD
HV-855 HV use in past 10 yrs USA, MD
HV-856 HV use in past 10 yrs USA, MD
HV-559 No HV since 1990 USA, MD
HV-985 No HV since 1990 Iran
HV-557 No HV since 1990 USA, MD
HV-558 No HV since 1990 USA, MD
HV-562 No HV since 1990 USA, MD
HV-679 HV use in past 10 yrs USA, MD
HV-881 No HV since 1990 USA, MD
HV-992 No HV since 1990 USA, MD
Cluster 1
70.2% similarity level
Cluster 2
72.1% similarity level
Cluster 3
74.9% similarity level
Cluster 4
73.3% similarity level


HV-861 No HV since 1990 Turkey
HV-862 No HV since 1990 Turkey
HV-880 No HV since 1990 Greece
HV-860 No HV since 1990 Turkey
HV-882 No HV since 1990 USA, MD
HV-842 HV use in past 10 yrs Iran
HV-843 HV use in past 10 yrs Iran
HV-834 HV use in past 10 yrs Iran
HV-878 No HV since 1990 Greece
HV-879 No HV since 1990 Greece
HV-883 No HV since 1990 USA, MD
HV-665 HV use in past 10 yrs USA, NE
HV-994 No HV since 1990 USA, MD
HV-869 No HV since 1990 Afghanistan
Cluster 5
73.3% similarity level
HV-526 HV use in past 10 yrs Greece
HV-674 HV use in past 10 yrs Afghanistan
HV-676 HV use in past 10 yrs Afghanistan
HV-666 HV use in past 10 yrs USA, NE
HV-693 No HV since 1990 Afghanistan
HV-536 No HV since 1990 Turkey
HV-692 No HV since 1990 Afghanistan
HV-675 HV use in past 10 yrs Afghanistan
HV-677 HV use in past 10 yrs USA, MD
Cluster 6
75.6% similarity level
HV-980 No HV since 1990 Afghanistan
HV-982 No HV since 1990 Afghanistan
HV-960 HV use in past 10 yrs USA, NE
HV-961 HV use in past 10 yrs USA, NE
HV-977 No HV since 1990 USA, NE
HV-978 No HV since 1990 USA, NE
HV-979 No HV since 1990 USA, NE
Cluster 8
89.6% similarity level
HV-520 HV use in past 10 yrs Iran
HV-521 HV use in past 10 yrs Afghanistan
HV-551 No HV since 1990 Afghanistan
HV-552 No HV since 1990 Afghanistan
HV-553 No HV since 1990 Afghanistan
Cluster 9
74.2% similarity level
HV-518 HV use in past 10 yrs Iran
HV-519 HV use in past 10 yrs Iran
HV-548 No HV since 1990 Iran
HV-550 No HV since 1990 Iran
HV-841 HV use in past 10 yrs Afghanistan
HV-976 HV use in past 10 yrs USA, MD
HV-983 No HV since 1990 Iran
HV-984 No HV since 1990 Iran
HV-974 HV use in past 10 yrs USA, MD
HV-696 No HV since 1990 Iran
HV-698 No HV since 1990 Afghanistan
HV-695 No HV since 1990 Iran
HV-523 HV use in past 10 yrs Afghanistan
HV-975 HV use in past 10 yrs USA, MD
Cluster 14
72.5% similarity level
HV-885 No HV since 1990 USA, MD
HV-971 HV use in past 10 yrs USA, MD
HV-884 No HV since 1990 USA, MD
HV-886 No HV since 1990 USA, MD
HV-962 HV use in past 10 yrs Afghanistan
Cluster 16
81.8% similarity level
HV-662 HV use in past 10 yrs Turkey
HV-664 HV use in past 10 yrs Turkey
HV-535 No HV since 1990 Turkey
HV-981 No HV since 1990 Afghanistan
HV-872 No HV since 1990 Iran
HV-561 No HV since 1990 USA, MD
HV-705 No HV since 1990 USA, MD
HV-537 No HV since 1990 Turkey
HV-993 No HV since 1990 USA, MD
HV-851 HV use in past 10 yrs USA, MD
HV-973 HV use in past 10 yrs USA, MD
HV-853 HV use in past 10 yrs USA, MD
HV-972 HV use in past 10 yrs USA, MD
HV-522 HV use in past 10 yrs Afghanistan
HV-560 No HV since 1990 USA, MD
HV-538 No HV since 1990 Turkey

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