

# Diversity of field isolates of *Sinorhizobium meliloti* nodulating alfalfa

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## INTRODUCTION

Most alfalfa seed is treated with a rhizobial inoculant consisting of one or more strains of *Sinorhizobium meliloti* before planting to enhance nodulation of seedlings. However, little is known about the persistence of inoculated strains through time as alfalfa plants develop and interact with indigenous rhizobial strains in soil. There is also a paucity of information on genetic and phenotypic diversity of *S. meliloti* strains in the U.S.

One strategy for increasing alfalfa forage yields, particularly in less fertile sites, is selection and use of highly competitive and efficient nitrogen fixing strains of rhizobia. To develop inoculants, a greater understanding of the basis of field competitiveness of rhizobia is needed.

The availability of the complete genome sequence for a large number of *S. meliloti* strains can be leveraged to identify the bacterial genes contributing to field competitiveness. Recently, type four secretion system (T4SS) genes were identified in *S. meliloti* that may play a role in host specificity (1).

## OBJECTIVES

1. Evaluate the genetic diversity of alfalfa-associated rhizobia from two field sites in Minnesota that had not been planted with alfalfa for over 30 years.
2. Determine how bacteria isolated from field grown plants are related to bacteria used for seed inoculation.
3. Measure the frequency of the T4SS and functional characteristics of isolated rhizobia.

## MATERIALS AND METHODS

### Sample Collection

- Soil cores of whole alfalfa plants were excavated from University of Minnesota Long Term Agricultural Research (LTAR) plots in Lamberton and Waseca, MN representing Year 1 (6-months-old) and Year 2 (18-months-old) plants. We collected 8 cores per plot, with 4 replicate plots.
- DKA44-16RR seeds that generated Year 1 and Year 2 plants were grown in sterile vermiculite in a growth chamber.

### Isolation of Rhizobia

- 48 nodules were collected from the combined 8 cores.
- Nodules were surface sterilized, crushed and plated on TY agar amended with Congo Red and grown at 28°C.
- A total of 562 verified *S. meliloti* strains were recovered from field grown plants. 53 strains were recovered from growth chamber grown plants.

### Using PCR to Explore Rhizobial Diversity

- Single colonies were screened via PCR using *Sinorhizobium*-specific primers.
- Isolates were screened for the T4SS using PCR for the *virD* gene.
- Repetitive Extragenic Palindromic (REP)-PCR was used to generate "fingerprints" of 158 field isolates and 53 seed coat isolates (Figure 1) using the BOXA1R primer.
- Isolates were confirmed to be *Sinorhizobium meliloti* by 16S rRNA sequencing.

### Characterizing the Functional Capacity of Rhizobial Isolates

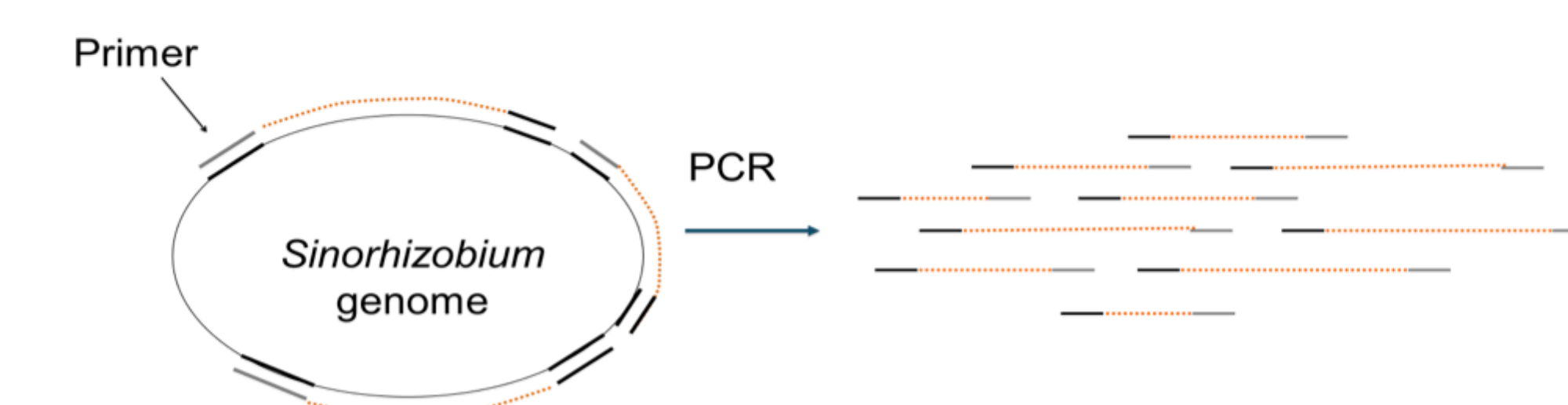
- All isolates confirmed as *S. meliloti* were assayed for indole-3-acetic acid (IAA) production and for solubilizing phosphate.

## REFERENCES

1. Sugawara, M., Epstein, B., Badgley, B.D., Unno, T., Xu, L., Reese, J., et al. (2013). Comparative genomics of the core and accessory genomes of 48 *Sinorhizobium* strains comprising five genospecies. *Genome Biology* 14:R17.

## Repetitive Extragenic Palindromic (REP) PCR "Fingerprinting"

1. Rep-PCR primers bind to repetitive sequences throughout the genome. Fragments are amplified via PCR.

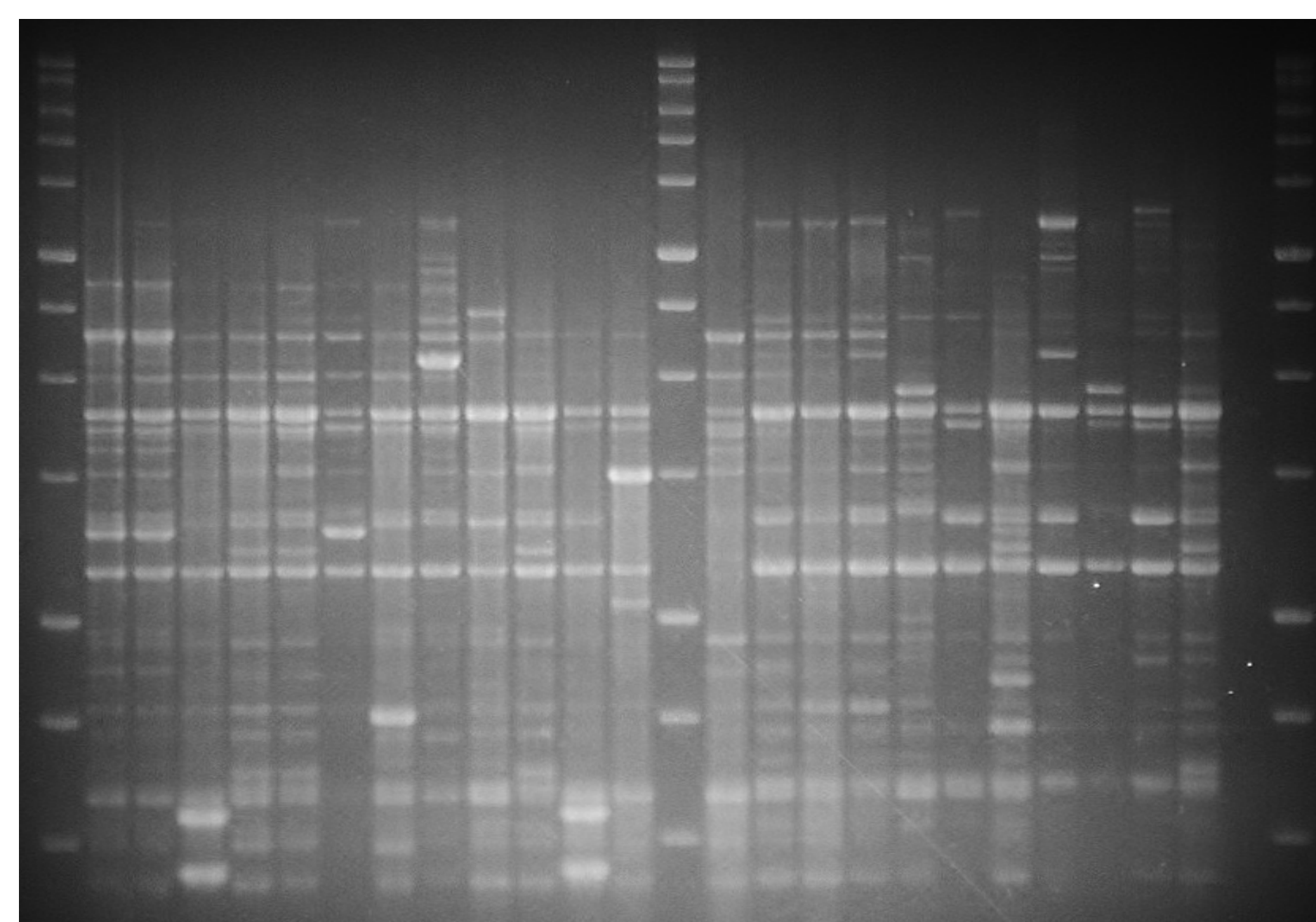


2. Fragments are separated by size using gel electrophoresis. A Rep-PCR fingerprint is created using band size and intensity. Unique "Fingerprints" are generated for each isolate.

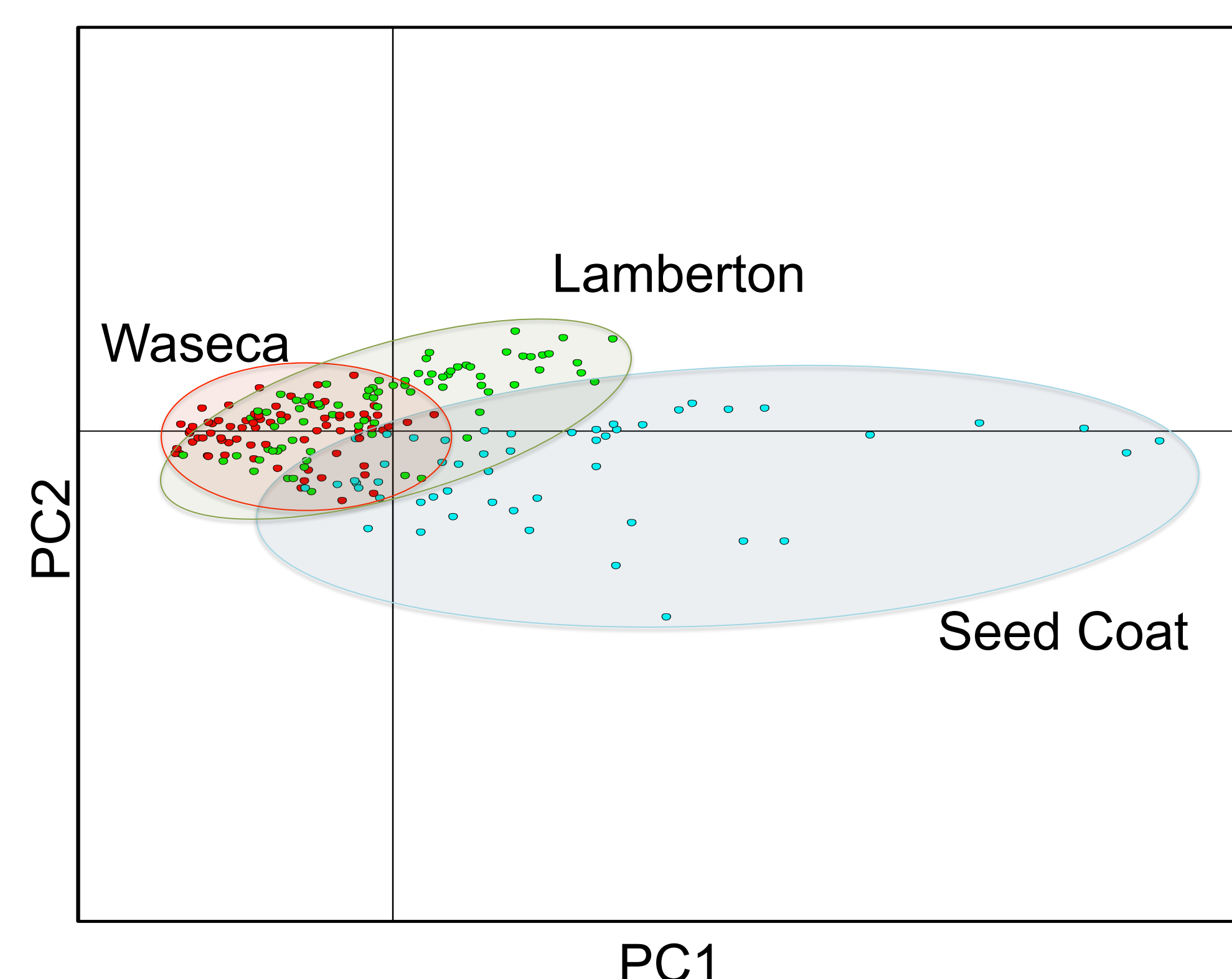


**Figure 1.** *Sinorhizobium meliloti* isolates were subjected to Repetitive Extragenic Palindromic PCR (REP-PCR). This technique creates unique fingerprint patterns from the fragments amplified during the PCR cycle.

## RESULTS

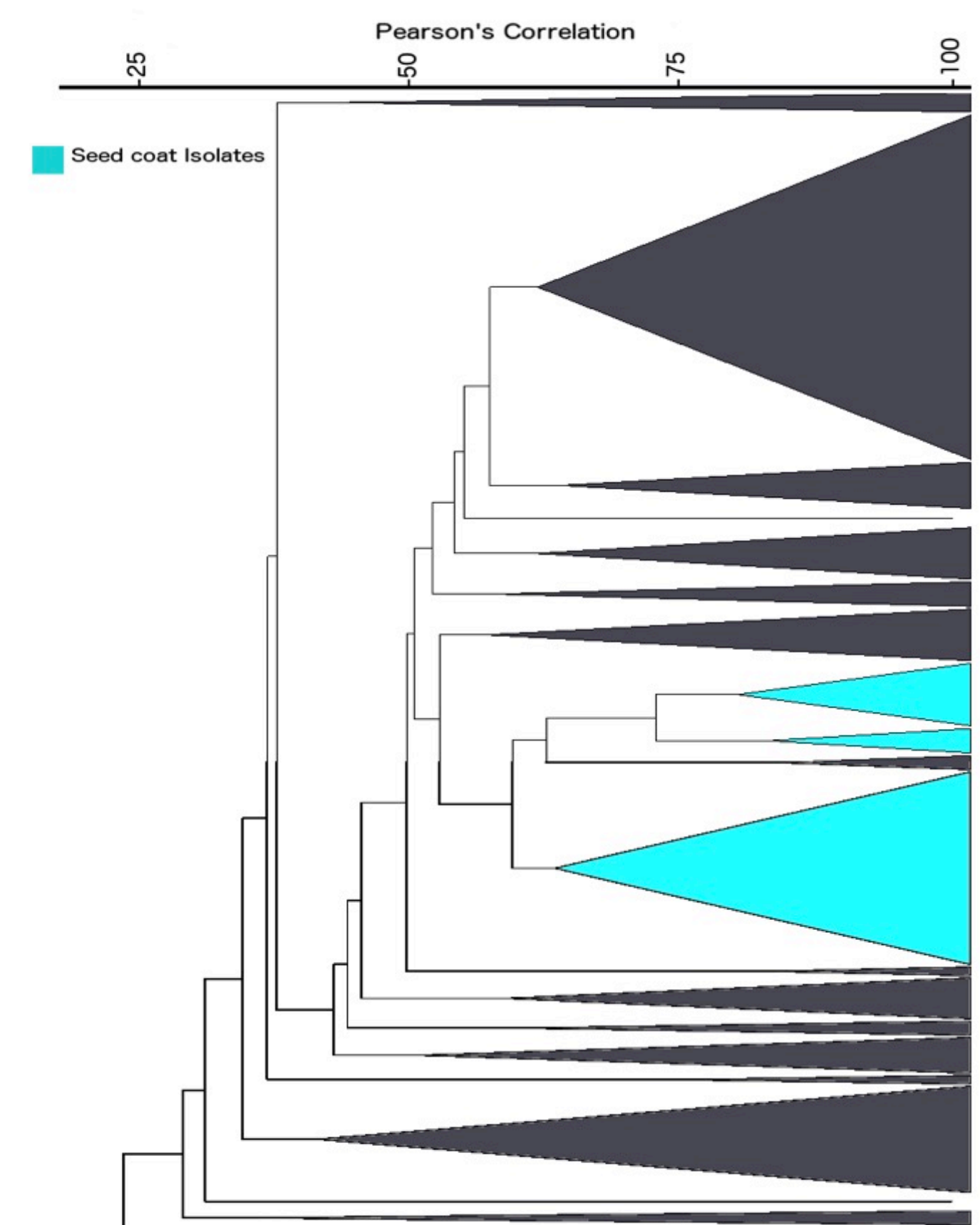


**Figure 2.** Agarose gel image of "fingerprint" fragments of *Sinorhizobium meliloti* isolates generated REP-PCR. External DNA standards (ladders) can be seen in the center and on either side. Negative controls without DNA were also run with each PCR assay.



**Figure 3.** Principal Component Analysis (PC1 and PC2) shows significant differences in rhizobia isolates between field sites and seed coat regardless of year. This PCA analysis is based on REP-PCR fingerprint patterns generated by the gel analysis software BioNumerics v.3.5 (Applied Maths, Sint-Martens-Latem, Belgium).

**Figure 4.** Dendrogram illustrating genetic diversity of alfalfa-nodulating *S. meliloti* based on REP-PCR fingerprinting. Field isolates do not cluster distinctly by location. Seed coat isolates form three clusters distinct from field isolates.



**Table 1.** Jackknife Maximum Similarities Analysis. Analysis describes the percentage of maximum similarity of each isolate's gel band pattern to that of a particular group (i.e. Lamberton, Waseca, Seed Coats) individually. Seed coat isolates were not similar to isolates from either field site.

	Lamberton	Waseca	Seeds
Lamberton	87.34	28.95	0
Waseca	11.39	67.11	0
Seeds	1.27	3.95	100

**Table 2.** Summary of functional characteristics of isolates used in REP-PCR fingerprinting.

Isolate Source	Total Isolates	T4SS	IAA Production Assay	Phosphate Solubility Assay
<b>Lamberton</b>				
2014	40	18	40	0
2015	40	15	40	7
<b>Waseca</b>				
2014	36	14	36	0
2015	40	11	40	0
<b>Seed coat</b>				
2014	24	0	24	0
2015	29	0	29	0

## CONCLUSIONS

- Alfalfa nodules from each field site harbored distinct rhizobial populations that were significantly different from seed-coat derived strains.
- There were no temporal differences in bacterial populations among 6-month-old and 18-month-old alfalfa plants.
- Populations at each site were highly diverse, with greater diversity at the Lamberton site.
- 33% of field isolates were positive for the *virD4* gene. Of the 53 strains from the seed inoculum, none had the T4SS genes.
- These results indicate that even in the absence of alfalfa, highly competitive indigenous populations of *S. meliloti*, some of which contained T4SS genes, replace the seed coat inoculum rhizobia as early as 6 months after planting.
- Plant growth promoting activity and competitiveness of field strains is currently being investigated.

## ACKNOWLEDGEMENTS

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