# Characterization and management of genetic diversity in *Phytophthora sojae* populations

by

**Silvina Stewart**

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Program of Study Committee:
Alison E. Robertson, Major Professor
Thomas Harrington
Silvia R. Cianzio
Edward Braun
Xiao-Bing Yang

Iowa State University

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### I.1 The host - soybean

Soybean *Glycine max* L. (Merr.) (Fabaceae: Phaseoleae) is a native legume to East Asia. It was domesticated in China (1550-1027 B.C.) and introduced to America in 1765 by Samuel Bowen, a sailor who had visited China (36). It is considered the number one oilseed crop produced and consumed in the world today. About 50 countries in the world grow soybean, but during the past half century, the U.S. has been the world's leading producer (90). In 2000, U.S. accounted for 45-50% of the world's soybean production and $6.66 billion in soybean and product exports (41, 90). More than 2.5 billion bushels of soybeans were harvested from almost 73 million acres of cropland that year. The acreage in 2000, was roughly equivalent to that of corn grown for grain in the U.S. (41). In 2009 soybean was considered the second most important crop in U.S. after corn, with an estimated 3.4 billion bushels harvested, which was worth 31 billion dollars (42).

Soybean is grown in the eastern half of North America, from the Gulf of Mexico coast to the southern part of Canada, however, 82% of the total production occurs in the North-Central states (90). Within this region, Iowa and Illinois have the greatest harvested area and production (90).

### I.2 The pathogen – *Phytophthora*

*Phytophthora* is an Oomycete which belongs to the Kingdom Stramenopila, and is therefore more closely related to diatoms, brown and golden brown algae than to true fungi. It is a diploid organism, whose life cycle includes both sexual and asexual reproduction. The feature that gives the group its name is its sexually derived, thick-walled, persistent oospores which in *P. sojae* are accomplished mainly by selfing (33, 46). The genus name *Phytophthora* meaning 'plant destroyer' was given by Anton de Bary in 1876 when he described the potato late blight pathogen. Interaction between *Phytophthora sojae* Kaufmann & Gerdemann (syn. *Phytophthora megasperma* Drecsh. f. sp. *glycinea* Kuan & Erwin) and soybean causes an important disease called Phytophthora stem and root rot (PRR) that limits soybean production worldwide.

#### I.2.1 Classification and morphology of *Phytophthora sojae*

*Phytophthora* resembles fungi in its morphology, since it has thread like mycelium. However, *Phytophthora* and all other oomycetes differ from true fungi in many physiological traits that separate them into different kingdoms. As a group, oomycetes are associated with aquatic habitat, their cell wall is composed of glucan and cellulose, instead of chitin as fungi. Their mycelium is coenocytic, having no-septa or divisions as in true fungi. Their vegetative stage is diploid, as opposed to haploid true fungi.

After the first *Phytophthora* was discovered in 1876 other species were gradually described. It was not until 1931 that *P. megasperma* (Drechsler) was described. In 1963, Waterhouse proposed a key to classify species which divided the genus into six groups

(87). *Phytophtora sojae*, at that time synonymous with *P. megasperma* var *sojae*, was included in group V (22).

*Phytophthora sojae* has nonpapillate ovoid, ellipsoid or obpyriform sporangia, ranging from 23-88 x 16-52 µm (23). It has globose oogonia that are more than 30 µm in diameter, with both paragynous and amphigynous antheridia. Clamydospores are not readily formed and its optimum growth temperature is between 20-25 ºC. Oospores range from 19-38 µm in diameter and are formed in infected tissue (23, 36). Oospore germination is not synchronous and may continue for 30 days or more, and it can be direct or indirect by producing sporangia (23). The optimum temperature for formation and germination of oospores is 24°C (22, 23). Because of the continuous range in oogonial size between isolates from different hosts, oogonial sizes as well as other characteristics are questioned as taxonomic criteria. Species identification is now significantly aided by single-strand conformational polymorphism analysis of PCR-amplified ribosomal DNA internal transcriber spacer 1 for DNA fingerprinting (29).

### I.3 The disease – Phytophthora stem and root rot

Phytophthora stem and root rot was first observed in Indiana in 1948 and in Ohio in 1951. The first comprehensive report of this disease by Kaufmann and Gerdemann was published in 1958 (45, 69). The center of origin of the pathogen is unresolved. One hypothesis states that it coevolved with wild and domesticated soybean in East Asia, while alternatively the second one proposes host jump from species of lupine native to North America (32). Although it has a very narrow host range *P. sojae* has been reported

to infect *Lupinus angustifolius*, *L. luteus* and *L. albus* (34). Despite the proposed Asian origin, a center of diversity for this pathogen seems to be the American Midwest (32).

### I.3.1 Economic importance

*Phytophthora sojae* can infect soybeans at all growth stages, causing pre- and post-emergence damping-off, root and stem rot. PRR causes plant losses and severe yield losses in susceptible cultivars, being most severe in poorly drained clay soils that are readily flooded. It is considered the second most important disease affecting yield of soybean in the U.S., after soybean cyst nematode (93). When comparing resistant and susceptible near-isolines of soybean, the susceptible averaged 65 to 93% less yield than the resistant isoline in fields with high disease pressure (34). Annual losses resulting from PRR in North America are approximately $10^9$ kg of soybean and losses up to 1-2 billion dollars have been estimated worldwide (32).

### I.3.2 Disease symptoms

Seed rot and flaccid taproots on seedlings are typical symptoms of pre- and post-emergence damping-off. In older plants, symptoms are characterized by wilting, brown girdling lesions that extend up the stem, and ultimately plant death (69). PRR symptoms are dependent on the resistance level of the cultivar, and can range from asymptomatic infection to stunted, chlorotic, and wilting plants.

### I.3.3 Disease cycle and favorable conditions

The genus *Phytophthora* has complex life cycles, which involve many kinds of spores. Oospores (thick wall resting spores) are the outcome of sexual reproduction, produced by mating of a female organ called oogonium, which is fertilized by a male organ called antheridium. When oospores germinate, the inner wall is absorbed and the germ tube produces either a sporangium or mycelium (36). Sporangia are also capable of direct germination (functioning as conidia) or indirectly liberating zoospores. Ten to thirty zoospores are formed by compartmentalization of the mature multinucleated sporangium (84). They are wall-less motile asexual spores, with two types of flagella (tinsel and whiplash). They can swim short distances on flooded soil surfaces or through soil pores, but they are disseminated mainly by moving flood water (36). When they reach the root of susceptible host plants, they dock or encyst. Encystment of zoospores is a process involving detachment of flagella and secretion of glucoproteins and molecules to form a primary cell wall (22, 85). Once attached to the root, the cyst germinates, penetrates the host and infects. *Phytophthora sojae* is considered an hemibiotrophic plant pathogen (32), acting as a biotroph at early stages of infection and later transitioning into a necrotroph. Once inside the root cortex, the pathogen colonizes the root tissue intercellularly, hyphae invade throughout the cortex and penetrate into the stele. In older plants, this colonization results in lateral and taproot rot and lesions that extend up the stem. Within these lesions, the pathogen undergoes its sexual cycle forming oospores (homothallic or self-fertile), which are eventually deposited in the soil when the plant senesces. Large number of oospores are formed in the roots and stems of susceptible

cultivars and can persist for long periods of time in soil (34). Oospores are considered the primary source of inoculum throughout the growing season (34).

As a known water mold, saturated soil conditions and temperatures of 25 to 30 ºC favor oospore germination and production of asexual sporangia (34). PRR is favored by heavy, tightly compacted, clay soils that are subject to saturation and flooding (34, 36). Water-motile, bi-flagellated zoospores released from sporangia are considered the primary infective propagule and are attracted to genistein and other isoflavonoids secreted by soybean roots (32, 34). Like many soilborne pathogens, propagules are formed and released within the soil matrix and therefore have limited means of dispersal (8).

## II. Soybean-*Phytophthora* interactions

### II.1 Pathogenic variability

Interaction between the pathogen and host in the *P. sojae*-soybean system follows the gene for gene concept proposed by Flor (27), which assumes that for each gene for resistance in the host there is a corresponding avirulence gene in the pathogen. The resistance expression (incompatibility) requires an interaction between a gene for resistance in the soybean plant (*Rps*) and a gene for avirulence in *P. sojae*, therefore implies induced resistance. Induced resistance is the result of activation of a defense mechanism in response to infection which serves to inhibit or suppress disease development (57). In other words, a gene for avirulence in the pathogen codes for an elicitor that directly or indirectly interacts with the product of the corresponding gene for resistance in the plant causing a hypersensitive reaction (63). Susceptibility

(compatibility), on the other hand, is regarded as a passive (non-induced) response due to the absence of genes for resistance and/or avirulence (57). This concept explains the genetic basis of the host specificity exhibited by physiological races or pathotypes in the *P. sojae*-soybean system. There are 14 known *Rps* genes in soybean that confer resistance to PRR (19, 32), although today we know that there are probably more plant genes involved in 'gene-for-gene' resistance then just the *R* gene itself (63) .

Variability in *P. sojae* has been assessed traditionally through a virulence test using a differential set. Seven to 14 soybean lines, each with one resistance gene (*Rps*) to *P. sojae* and a universal susceptible are used to characterize *P. sojae* races or pathotypes (15, 27). More than 55 described races of *P. sojae* have been identified on the basis of compatible (susceptible) and incompatible (resistant) reactions on differential lines (15, 17). A race number was given to a pathotype of *P. sojae* with a previously described virulence formula. As new virulence gene combinations or pathotypes were continuously emerging in the pathogen (1, 18, 43, 52, 53, 67, 71, 96) the old race classification system was discouraged (13). Potentially there can be $2^{14}$ or 16,384 ($2^{N}$ where N = no. *Rps* genes) possible virulence combination or races, leading researchers to describe pathotypes rather than continue naming new races. Presently, pathotypes or virulence formulas are used to describe virulence patterns based on reactions on a differential set. Knowing the pathotype is far more informative since it indicates which *Rps* genes are compatible with the isolate (66). There are now more than 200 known pathotypes of this pathogen (17), suggesting high genetic variation with respect to virulence in the natural populations.

Molecular data, such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPDs) markers, have not correlated with the virulence/pathotypes of the isolates, thus there is no relationship between the two (28, 30, 55). Researchers continue to try and find molecular techniques that could substitute for the use of phenotype using the differential set to pathotype (28, 56). Many of the avirulence genes in *P.sojae*, such as *Avr*1a and *Avr*3a (64, 65), *Avr*3c (12), *Avr*4/6 (20) have been recently sequenced, and could potentially be used in the near future to directly identify the pathotype of new field isolates.

### II.2 Diversity

The rise of new races with the introduction of resistant cultivars seems to be too rapid to be accounted for by chance mutation, dispersal, and subsequent natural reselection by the resistant soybean cultivar (22). Hobe (38), suggested that a wide range of races may exist in the natural wild-type population that may be expressed when a resistant cultivar is introduced (38).

#### II.2.1 Diversity over time

Since 1948, when the first *P. sojae* race was identified, the number of races worldwide has increased dramatically. Many pathotype/races have been documented in surveys in U.S. States, such as Arkansas, Illinois, Indiana, Iowa, Michigan, Minnesota, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin and in countries such as Argentina, Australia, Canada and China (5, 6, 10, 24, 43, 44, 47, 50, 53, 58, 59, 67, 83,

89, 96, 97). Generally, there is a predominance of one or two pathotypes in an area, with several other pathotypes making up small percentage of the total population.

Surveys within a region over time indicate that pathotype proportions change after a few years. Between 1978 and 1980, race 7 was the most prevalent in Ohio followed by races 9 and 3 (71). A subsequent survey in the same area conducted 10 years later revealed races 1, 3, 4, 7 and 8 as most frequent (70), while between 1997 and 1999, 34 additional pathotypes were reported and races 1 and 25 followed by 3 and 4 were predominant (18). In Australia, prior to 1989 only races 1 and 15 were found, however, race 4 has become predominant since then (67). In Iowa, a survey conducted from 1966 to 1973 indicated race 1 was the only race present (80), later from 1991 to 1994 race 3 predominated (96), and race 3 was replaced by race 25 and 35 in a survey in 2001-2002 (61). Similar findings have been reported in Illinois (50), Indiana (1), and Michigan (44).

The complexity of *P. sojae* pathotypes seems to be increasing with time (18). Almost 20% of the isolates surveyed in Michigan had virulence to 10 or more *Rps* genes (44). Similarly, average complexity, indicated by the number of *Rps* genes with which an isolate has a susceptible interaction, has increased from 3.1 in the early 1990s to 5.9 in the late 1990s in Ohio (18).

### II.2.2 Diversity within a field

In Arkansas (43) and Australia (67) isolates of several pathotypes were identified in fields that had been used as breeding nurseries for *P.sojae*-resistant soybeans. Different and more complex races were found at these sites compared to commercial soybean fields, i.e. up to 13 different pathotypes were found at one site. Fifty four and 56 different

pathotypes were detected from two intensively sampled commercial fields in Ohio (18), while 11 and 18 were found in two commercial fields in Iowa (66). Interestingly, as many as four pathotypes were detected in some of the soil sub-samples, indicating that a single soybean plant may be physically and spatially subjected to infection by more than one pathotype (66).

### II.2.3 Diversity within a plant

Since a soybean plant could be surrounded by at least 4 different pathotypes of *P. sojae* in the field (66), the potential exists for a plant to be infected by more than one pathotype of the pathogen. Indeed, infection by more than one isolate has been demonstrated experimentally 'in planta', when true heterokaryons from co-inoculations of isolates with different drug resistant markers (7, 51). Moreover, true outbreeding (sexual recombination) after co-culturing in vitro was demonstrated to occur in *P.sojae* using genetic markers (88). Under optimal in vitro conditions with both isolates present in close proximity, the outcrossing rate is less than 5% (88). The frequency at which outcrossing occurs in the field is yet to be determined. Presumably outcrossing might occur within a plant lesion when two or more isolates co-infect and most likely the frequency is far less than the 5% observed in vitro.

### II.2.4 Diversity within an isolate

The pathogen has the ability to differentiate during asexual reproduction. Single zoospore isolates derived from a single zoosporangium have displayed molecular polymorphism and variation in their virulence (9,37). Differentiation during asexual

reproduction in *P.sojae* has been known since 1962. The actual mechanisms by which this happens has been hypothesized but yet remains to be proven (9).

## III. Genetic mechanisms of creating diversity

'The kind of life cycle and mating system of the pathogen affects the opportunities and limitations for genetic diversity and evolution' (2).

### III.1 Sexual reproduction

In heterothallic *Phytophthora* species, stimulation of sexual reproduction by substances originating in the opposite mating type has been demonstrated by the use of a polycarbonate membranes (48). The sex hormone produced by type A1 isolates of such *Phytophthora* spp. can induce sexual reproduction of A2 type isolates but not type A1, and the opposite also holds true (49). When homothallic species of *Phytophthora* are paired with heterothallic species on opposites sides of the membrane, both were able to induce oospore formation, suggesting that homothallism may also be controlled by hormones (49). Thus, heterothallic *Phytophthora* spp. are actually homothallic but require a hormone produced by the opposite mating type for induction of gametangia, whereas induction by exogenous hormones is not needed for sexual reproduction of homothallic species. Nevertheless, homothallic species such as *P. sojae* can outcross (86), thus the amount of inbreeding cannot necessarily be predicted on the basis of hetero- and homothallism. Self fertilization reduces the amount of heterozygosity by one half in every generation, so by the seventh generation of self fertilization only one percent of the original heterozygosity will remain (33). Consequently, almost no

heterozygosity is predicted in homothallic species of *Phytophthora* that have selfed for more than a few generations.

In the 1960s and independently, scientists from four different institutes reported that sterols were required for sexual reproduction in *Phytophthora* (49). Soon after, however, experiments showed that although sterols stimulated sexual reproduction in homothallic species, they did not have the same effect on heterothallic *Phytophthora* species.

*Phytophthora sojae* has been shown to be auxotrophic for sterol (60), the lack of synthesis of this compound in the pathogen has made it nutritionally dependent on the host during infection. Sitosterol is the major sterol in soybean shoots and it has been found to be essential for growth and sexual reproduction of this pathogen (54).

### III.2 Other specific mechanisms of creating diversity in pathogens

**Heterokaryosis** is the condition in which, as a result of fertilization or anastomosis, hyphae cells contain two or more nuclei that are genetically different (2). This mechanism has been proven to occur in *P.sojae* (7, 51). **Vegetative compatibility** is the process by which hyphae from two different colonies fuse or anastomose and allow exchange of genetic material (2). **Heteroploidy** is the existence of cells, tissues, or whole organisms with different number of chromosomes per nucleus than the normal complement number. Heteroploids can be haploids, diploids, triploids, tetraploids or aneuploids (have one, two or more extra chromosomes or missing chromosomes) (2). **Mitotic recombination** is the process by which recombination happens in somatic cells during mitosis and can cause changes to homozygosity at heterozygous loci (33).

**Parasexualism** is the process by which genetic recombination can occur within fungal heterokaryons when the two nuclei fuse within the hyphae (2).

### III.3 Mechanisms of creating genetic diversity within and among populations

It is important to consider the genetic structure of a pathogen population, defined as the amount and distribution of genetic variation within and among populations (55), when deploying effective control strategies (11, 55). A pathogen with high genetic variation has a high evolutionary potential and is more likely to quickly adapt to new conditions such as a resistant host or changing environment (55). Population genetics considers the factors that determine evolution of a population and tries to answer question such as, how do populations change over time, and what causes the change? How much genetic variation is there in natural populations, and how does the amount of genetic variation affect the rate of evolution (35).

There are seven basic evolutionary processes that may act on a population. The first and most fundamental is **mutation**. Mutations occur spontaneously in nature in all living organisms. A mutation is a more or less abrupt change in the genetic material of an organism, which can be then transmitted to the progeny. Mutations represent changes in the sequence of bases in the DNA either through substitution of one base pair for another or through addition or deletion of one or many base pairs. On average, one mutation occurs for every million copies of a gene per generation and is the ultimate source of genetic variation (55). Without mutation no evolution could occur (2, 35).

**Recombination** is the second source of genetic variation. It creates new combinations of alleles, and less often new alleles. Recombination occurs primarily

during sexual reproduction, when different genes or different alleles of the same gene are recombined during meiosis as a result of genetic crossing over. New combinations of alleles can lead to new phenotypes. Recombination occur rarely during mitotic cell division that occurs during normal growth of the individual (2, 35).

**Natural selection** is Darwin's idea that individuals with heritable favorable variations survive and reproduce at higher rate than other individuals in the population, thus leaving more offspring and therefore increasing these variations in next generations (35). Selection is a directional process by which the fittest variants in a particular environment increase their frequency in the population, whereas the less fit variants decrease their frequency (2). **Fitness** is defined as any new trait that gives the individual an advantage to live in a particular environment compared to other individuals.

**Genetic drift** is defined as the occurrence of random effects in a population that affect the survival of various genetic traits in subsequent generations (2). Random changes in the frequency of alleles from generation to generation; especially in small populations, can lead to the elimination of a particular allele by chance alone. Consequently, the larger the population size, the higher the probability that variants will be present (55).

**Gene flow** is the process by which certain alleles (genes) or individuals (genotypes) move from one population to another geographically separated population (35, 55). The extent of gene flow is very important with pathogens; it can tell us how far new mutant alleles can spread. Pathogens that have limited means of dispersal, such as soil-borne fungi and Oomycetes, have lower levels of gene flow and are less diverse than those with long distance aerial dispersal, such as rust and smuts, may have high levels of

gene flow which can encompass entire continents (2, 55). In the long term, gene flow makes populations more similar to one another, while the opposite effect is attained with genetic drift, which tends to cause isolated populations to diverge from one another.

Another factor affecting genetic variation in a population is **nonrandom mating**. This occurs when mating individuals are genetically related to one another, thus mating is not at random. Reproduction in fungi and Oomycetes is quite complex, in many cases an individual mycelium can produce sex organs of both kinds, which go through the processes of sexual fusion and produce viable zygotes (46). This condition is described as homothallism, and is opposite to heterothallism, the condition in which a different mating type is needed to reproduce sexually. Sexual reproduction will introduce more genetic variation to a population if the genomes which meet, and are reassorted during meiosis, come from different randomly chosen individuals (46). The opposite, asexually reproducing populations display low genetic and genotypic diversity with increased levels of clonality.

In the absence of all the processes described above, allele frequencies and genotype frequencies would remain constant in a population, consequently, there would be no evolution and the population would be in **Hardy-Weinberg equilibrium (**HWE). The HWE law is the population genetic principle that predicts constant allele and genotype frequencies. HWE serves as a kind of null hypothesis in population studies and tells us what to expect if all assumptions are met. It is the starting point of any population study. If we sample a population and find that the genotype frequencies deviate from the HWE predictions, then we can conclude that at least one of the assumptions (no mutation,

no recombination, no natural selection, no genetic drift, no gene flow, random mating) is violated and that at least one evolutionary process is operating (35).

A framework to predict the evolutionary potential of a pathogen has been proposed based on the analysis of the genetic structure (55). Based on this framework, pathogens that have mixed reproductive systems, high potential for gene flow, large effective population sizes, and high mutation rates pose the greatest risk of breaking down resistance on their hosts. *Phytophthora sojae* has been classified as a medium risk pathogen because it has the potential to outcross, but has limited potential for gene flow being soilborne. Also it is effectively a monocyclic disease, so there is only one chance to develop new pathotypes during the growing season, giving it a lower effective population size (55).

### III.4. Determining genetic diversity in a population

In the 1980s, it became possible to manipulate and analyze DNA and apply these techniques to population genetics. Initially, restriction enzymes were used to analyze pieces of DNA, later sequences were analyzed directly (35).

There are two common ways of quantifying the amount of genetic variation in a diploid population: one is the proportion of polymorphic loci and the other is average heterozygosity. An allele is defined as an alternative form (alternative DNA sequence) of a gene that occupies the same locus (position) on a chromosome. A locus is monomorphic when there is only one allele in the population and polymorphic when there is more than two alleles in the population. This type of genetic variation is studied using molecular markers which are less affected by environmental factors and are

considered selectively neutral. Neutral markers are the result of random mutations that have no effect on the organism fitness, but can help us to infer patterns and levels of gene flow among different populations.

Restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPDs) markers have been previously used to study diversity in *P.sojae* (28, 30, 56). Both types of markers seemed to cluster isolates into four distinct groups, some groups being comparable among markers while others are not. The distribution of RFLP markers among isolates suggests that new races of *P.sojae* have arisen both by progressive mutation and by infrequent outcrossing. It also suggests that all isolates studied are produced by rare outcrosses between representatives of four genotypes designated as progenitor isolates (28). Low level genotypic diversity using RFLPs was observed in Australian *P.sojae* population when compared to the U.S. population, suggesting a single introduction of race 1 of the pathogen to Australia (21). Moreover, all races found in Australia belong to RFLP group 1 which includes race 1, and thus provides evidence that these Australian races could have evolved locally through mutations from an original introduced genotype (21). On the contrary, in South America RAPD detected high level of variability even among isolates from the same geographic region (30). In addition, isolates evaluated in the studies above could not link RFLPs nor RAPDs to pathotype or geographic region (14).

Microsatellites markers, also known as simple sequence repeats (SSRs), are polymorphic DNA loci consisting of a repeated nucleotide sequence. The repeat sequence can be 2 to 7 base pairs long. SSRs usually represent a single locus (locus-specific) since primers are designed based on the flanking sequences. Thus, different PCR product sizes

represent the different alleles in a locus, and they usually differ in length by a multiple of the repeat size. The number of repeat units (alleles) varies in a population, thereby creating multiple alleles for a microsatellite locus. It has been suggested that microsatellites are a better tool for studying population diversity than RFLP and RAPD. Although SSRs are neutral (like RFLPs and RAPDs) because they do not code for proteins, they are more highly variable, and are co-dominant enabling heterozygous individuals to be determined.

Twenty one polymorphic microsatellites markers were developed from *P.sojae* (isolate P6497) sequences obtained from Virginia Bioinformatic Institute (VBI) database and used in a preliminary study (14). Fifty three alleles were identified among 33 *P.sojae* isolates, with an average of 2.5 alleles per locus. All alleles deviated significantly from HWE and the observed heterozygosity (Ho) was as low as expected for a homothallic soilborne species, with an overall mean of 0.015 (14).

# IV. Management

## IV.1 General

### IV.1.1 Effect of tillage on PRR

Tillage and tiling both help to promote soil drainage which consequently reduces the *P.sojae* infection period. Tillage also helps to bury oospores deeper in the soil profile (34). Vertical distribution of the pathogen in the soil may be directly related to distribution of soybean residues (91). Recovery of *P. sojae* near the soil surface from no-till fields was 3 to 4 times greater than conventional-till fields at the same depth, suggesting greater risk of damping-off (91). The effect of tillage on the distribution of *P.*

*sojae* in the soil profile depends on the soil texture; conservation tillage on silt loam and loam soils bear more *P.sojae* in the top 15 cm compared with conventional tilled fields, however on sandy loam soils, *P.sojae* numbers are greater in the top 15 cm of conventional tilled fields (92).

### IV.1.2 Effect of fungicides on PRR

Most of the fungicides that are used to control soybean diseases act on the sterol synthesis, such as ergosterol needed for membrane structure and function on fungi. Thus, these fungicides have no effect on Oomycetes due to their difference in cell wall components, which are mainly glucans and cellulose. On the contrary, benzenoid fungicides such as metalaxyl that act by inhibiting protein and nucleic acid synthesis, particularly RNA production, have a direct effect on Oomycetes. Metalaxyl applied in-furrow or as seed treatment has improved plant emergence and increased yields of susceptible and low partial resistant cultivars in disease-conducive environments (4, 34, 72). On the contrary, neither treatment had an effect on the yield of partially resistant soybean cultivars (4, 34). Later studies have however demonstrated that seed treatments can have a significant impact on emergence and yield of partial resistant cultivars if conditions are conducive for the disease during seed germination until first unifoliate leaves are visible (16). Seed treatment were considerably less effective than high rates of soil- applied metalaxyl (72). Schmittenhemer and van Doren hypothesized that ‘Combinations of high tolerance, tile drainage, complete tillage, and metalaxyl seed treatment would be as effective as multirace resistance for Phytophthora control’ (72).

### IV.1.3 Effect of inorganic elements on PRR

Alternative disease management strategies may include use of inorganic elements known to suppress disease. It has been demonstrated that Ca considerably suppresses incidence of PRR (75, 77). Sixteen days after inoculation incidence of the disease in the cultivar Sachiyutaka went from 90% to 29%, 4.2%, 2.1%, 2.1% and 0% in the presence of 0.4, 4,10,20 and 30mM of CaCl2, respectively (75). Commercial formulations of Ca, had control efficiency that ranged between 37.1-98.6 %, with Ca(COOH)2-A being the most effective formulation (76).

### IV.1.4 Effect of crop rotation on PRR

Rotation of soybeans and corn is not an effective way to manage damping off caused by *P. sojae* because oospores can survive in the soil for many years even in absence of soybeans (95). However, less stand and yield loss have been demonstrated when a resistant cultivar was sown in rotation with corn compared to soybean as monocrop (73).

Field studies have shown that after 5 years of monoculturing susceptible, tolerant and resistant cultivars, severe disease resulted on the sixth year on plots previously planted with susceptible and tolerant cultivars while, moderate disease resulted in those planted previously with resistant cultivars (3). This can be explained by the fact that more oospores are formed in susceptible and tolerant cultivars than in resistant ones (36). Oospores numbers in roots of seedlings grown hydroponically in the lab were less in tolerant than in susceptible cultivars (3). Other studies have observed equal number of oospores in taproots of both susceptible and tolerant cultivars (31).

### IV.1.5 Biological control

Several *Actinomycetes* (26), *Hyphochytrium catenoides* (26, 40), and *Bacillus cereu*s (62) have been shown to be effective as biocontrol agents against *P.sojae*, improving both establishment and yield of soybean. Antibiotic–producing *Streptomyces* isolates have also been shown to have inhibitory capabilities against *P.sojae* in-vitro, the isolates significantly reduced PRR in sterilized vermiculite and natural infested field soil (94).

## IV.2 Resistance

The most effective way to manage PRR has been through the use of resistant cultivars with single resistance genes (*Rps*). Fourteen known resistant genes (*Rps*) in soybean confer resistance to PRR (19, 32). Several *Rps* genes, such as *Rps*1a, *Rps*1c and *Rps*1k have been incorporated into commercial cultivars (34), however, as in many other host-pathogen systems which are governed by a gene-for-gene system (18, 27) the pathogen adapts to the specific *Rps* resistant gene. The effectiveness of these genes has been lost progressively as new races/pathotypes of the pathogen have appeared. Prior to 1990, *Rps*1a and *Rps*1c had failed in the North Central regions of the Mid West and by the late 1990 there were reports of races causing disease on *Rps*1k. In Iowa, *Rps*1k is the most common resistance gene used at present, followed by *Rps*1c, but the increasing prevalence of race 25 (virulence formula 1a, 1b, 1c, 1k,7) (61) has prompted incorporation of *Rps*6 into germplasm for Iowa (66). Durability of *Rps* effectiveness in the field has been estimated in 8-15 years (13, 18).

### IV.3 Rotation of resistance genes

An alternative way to prolong the life of a resistance gene in the field is to generate disruptive selection by rotating major resistant genes through time and space or by growing mixtures of cultivars with different resistant genes (55). This strategy disrupts selection by favoring different variant alleles at different times and places, reducing the rate in which one particular variant increases its frequency. The strategy has not been widely adopted, but one successful example at commercial level has been the control of rice blast disease of rice. Disease-susceptible rice varieties planted in mixtures with resistant varieties had 89% greater yield and 94% less disease when compared to monoculture (98). Another experimental example was a 4-year cultivar rotation study to investigate effect of resistant gene deployment on the race structure of tobacco black shank pathogen, *Phytophtora nicotianae* (78, 79). The cultivar rotation studies were conducted in fields initially containing single or mixed races of the pathogen. In those fields where no race 1 was detected initially; disease incidence was high with the use of partially resistant cultivars and race 0 was the dominant race recovered. On the contrary, single-gene resistance was very effective in suppressing the disease, but race 1 was recovered after only one growing season. A rotation between cultivars with single-gene resistance and cultivars with high levels of partial resistance is proposed by the authors to provide not only a reduction in disease incidence but minimizing race shifts of the pathogen (78).

### IV.4 Tolerance or partial resistance

Tolerance or partial resistance (PR) is defined as 'the relative ability to survive root infection, either natural or artificial, without showing severe symptom development such as death, stunting, or yield loss' (86). PR does not exhibit the boom-and-bust cycle characteristic of major resistant genes (55). It is not based on receptor-elicitor recognition and thus works equally across all pathogen strains (55). This type of resistance is polygenic, and limits the lesion growth rate of the pathogen within the host tissue allowing the host to tolerate the infection better when compared to a fully susceptible host (17, 25, 82). Thus, tolerance should be more stable than single-gene resistance because of the lack of selection pressure imposed on the pathogen (74). It has been shown to be effective against all pathotypes of *P. sojae,* restricting the pathogens colonization of the plant tissue (34).

Cultivar Conrad, with high levels of PR was highly susceptible to infection by *P.sojae* from the day of planting until 5 days after planting (18), thus indicating that PR needs to become active in the plant before it starts being effective. PR becomes active after the first true soybean leaves emerge (VC growth stage), so seeds and seedlings of high PR cultivars are susceptible to disease at planting and during the early stages of growth.

The layer test is the greenhouse standardized method used to screen for partial resistance to *P. sojae* in soybean (17, 25, 43, 68, 81). In the layer test, an agar culture of the pathogen is placed at a certain distance below the seed at planting time, 3 to 4 weeks later incidence and severity are evaluated using a 1-10 scale (66).

## V. Project and research justification

Our specific objectives and the questions we want to address in this project are:

**Objective 1:** Assess genetic diversity of *Phytophthora sojae* in Iowa using microsatellite markers.

It has been suggested that there exists a large reservoir of genetic diversity in *P.sojae* populations (39), however, only few studies have tried to characterize it using genetic markers (28, 56). Contrary to previous studies, the proposed use of microsatellites will enable quantification of putative heterozygotes, which will ultimately lead to quantification of naturally occurring outcrossing.

Questions to be addressed are: how much genetic diversity is out there? Does *P.sojae* outcross in nature? Can infection by more than one pathotype occur *in planta*? Does outcrossing occur in the plant lesion or in the soil? Is there differentiation between field populations? How much gene flow is there between fields?

**Objective 2:** Monitor pathotype and genetic shifts in *Phytophthora sojae* populations with cultivar rotation.

Changes in the pathotype structure of the *P.sojae* populations have been attributed to deployment of specific resistant genes (43, 67, 71, 96). The use of single gene, complete resistance to race 0 of *Phytophthora nicotianae*, which causes black shank of tobacco, resulted in a rapid increase of race 1 of the pathogen in North Carolina. Rotation between cultivar with single-gene resistance and cultivars with high levels of partial resistance was shown to be an effective approach to managing black shank (78). Studies to test this hypothesis in *P. sojae*-soybean system have not been done.

Can continued use of a single *Rps* gene result in the selection of a new pathotype of *P. sojae*? How long does a pathotype-shift take? Can we slow down pathotype shifting by rotating cultivars with different *Rps* genes? Does the use of a partial resistance cultivar in the rotation help?

**Objective 3:** To assess a method to screen partial resistance to *Phytophthora sojae* in soybean.

There are several possibilities to change the way selection operates on a pathogen population. The most common alternative would be to pyramid several major resistant genes. Another option, already mentioned, would be to generate disruptive selection by rotating major genes or by growing mixture of cultivars. A third option would be the use of partial resistance. Partial resistant is more difficult to detect than major gene resistance, being one of the reasons that it is shaped by genotype-by-environment interactions. Only a few commercial cultivars with high levels of PR are currently available, mainly due to challenges faced by breeders in incorporating it into the desired germplasm. This objective tries to validate an easier, feasible, and more effective way to screen for PR.

Does the modified method to screen for partial resistant work? Is it a quantitative trait? Is it objective detected?

Phytophthora root and stem rot has been managed traditionally through breeding for resistance determined by single genes. Breeding efforts have focused on incorporating major *Rps* gene resistance into soybean lines even though the pathogen is rapidly and continuously evolving (5, 6, 10, 24, 43, 44, 47, 50, 53, 58, 59, 67, 83, 89, 96, 97), the

complexity of pathotypes is increasing (18, 43), and in spite the short durability of the *Rps* resistance genes in field conditions (13,18). If we want to deploy effective durable control strategies it is crucial to determine the amount and distribution of genetic variation within and among *P.sojae* populations. We need to define if *P.sojae* has a medium or high risk of breaking down host resistance based on its genetic variation and its evolutionary potential (54). And according to this risk, we need to improve or adapt management strategies to minimize losses due to PRR. It may be the time for breeders to switch from major gene resistance to partial resistance, however, for this to occurr and for this we need easy, feasible and effective ways to incorporate partial resistance into the desired germplasm, i.e. one of them being a reliable, and repeatable screening protocol.

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# Chapter 2: Microsatellite Markers and Pathotyping to Assess Diversity of *Phytophthora sojae* in Iowa

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S. Stewart, and A. E. Robertson

Department Plant Pathology, Iowa State University, Ames, IA 50011

Accepted for publication

Corresponding author: A.E. Robertson, alisonr@iastate.edu

## ABSTRACT

Stewart, S., and Robertson, A. E. 2011. Using microsatellite markers and pathotyping to assess diversity of *Phytophthora sojae* in Iowa. Phytopathology …..

The population genetic structure of 93 isolates of *Phytophthora sojae* collected from Iowa between 2008 and 2010 was characterized using eight polymorphic microsatellite markers (SSRs) and pathotyping. Forty MLGs (multilocus SSR genotypes) and 19 different pathotypes were found, with a genotypic and pathotype diversity (D) of 0.43 and 0.20, respectively. Low observed heterozygosity (*Ho*= 0.027) and high inbreeding coefficient (F=0.944) were found as expected in this homothallic species. Significant genetic and pathotype differentiation ($\Theta_{RT}$ = 0.574 and $\Theta_{RT}$ = 0.558) was found among seven fields, indicating restricted gene flow among fields (*Nm*= 0.29). Moreover, 10 and 19% of the genetic and pathotype variation in the 7-field population, respectively, was attributed to differences between multiple isolates recovered within a single plant. Thirty five percent of the plants from which multiple isolates were recovered yielded 2 or 3 MLGs, while 18% yielded two pathotypes. Furthermore, at least one of the

seven populations of *P. sojae* recovered from a commercial soybean field showed some level of outcrossing.

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*Phytophthora sojae* Kaufmann & Gerdemann (syn. *Phytophthora megasperma* Drecsh. f. sp. *glycinea* Kuan & Erwin), which causes Phytophthora root and stem rot, can infect soybeans at all growth stages, causing pre- and post-emergence damping-off, root and stem rot. It is considered the second most important disease affecting yield of soybean in the USA, after soybean cyst nematode (50). Total estimated reduction due to PRR for 5 of the top soybean-producing countries during 1998 was 1.27 million metric tons. Countries included were Argentina, Canada, China, Italy and US (49).

*Phytophthora sojae* is an Oomycete which belongs to the kingdom Stramenopila, and is more closely related to diatoms, brown and golden brown algae, than to true fungi. It is a diploid organism, whose life cycle includes both sexual and asexual reproduction. Saturated soil conditions and temperatures of 25 to 30 ºC favor oospore germination and production of asexual sporangia (23). Water-motile, bi-flagellated zoospores released from sporangia are considered the primary source of inoculum, and are attracted to isoflavones secreted by soybean roots (21). Like many soil borne pathogens, *P. sojae* has limited means of dispersal and thus its genetic flow is thought to be limited.

The most effective way to manage Phytopththora root and stem rot has been through planting of resistant cultivars with single resistance genes (*Rps*). Fifteen known resistance genes (*Rps*) in soybean confer resistance to *P. sojae* (11, 21,44). Several genes, such as *Rps*1a, *Rps*1c and *Rps*1k have been incorporated into commercial cultivars (23), however, as in many other host-pathogen systems which are governed by a gene-for-gene

system (18) the pathogen adapts to the specific *Rps* resistant gene in the host. Thus, the effectiveness of *Rps* genes is eroded progressively as new races/pathotypes of the pathogen have appeared. Prior to 1990, soybean cultivars with *Rps*1a and *Rps*1c developed Phytophthora root and stem rot in the North Central regions of the Midwest and by the late 1990 there were reports of races causing disease on plants with the *Rps*1k gene (23). Durability of *Rps* effectiveness in the field has been estimated in 8-20 years (10, 23).

Variability in *P. sojae* has been assessed traditionally through virulence tests using a soybean differential set for pathotype characterization consisting of 7 to 14 soybean lines, each carrying one *Rps* gene and a universal susceptible (9, 18). Prior to the year 2000, 55 races were officially described based on 8 differentials (*Rps*1a, *Rps*1b, *Rps*1c, *Rps*1d, *Rps*1k, *Rps*3a, *Rps*6, and *Rps*7) (23). Due to increasing pathogen diversity assigning race numbers has become cumbersome and currently, direct virulence formula (indicating the *Rps* gene in soybean that the isolate is able to infect) is used to designate pathotype. There are more than 200 known pathotypes of this pathogen (10), suggesting high genetic variation with respect to virulence in natural populations. Despite the obvious value of pathotype data, their use to assess genetic variation in plant pathogens has several limitations; lines have to be common among labs to be able to compare the data (32), assays are subject to environmental variation (20,32), and it does not provide any information on how the pathogen changes are occurring within a population.

During the past two decades, DNA-based genetic markers have become widely used to analyze the genetic structure of plant pathogen populations. Genetic markers are less affected by environmental factors and are considered selectively neutral. They are

the result of random mutations that have no effect on the organism fitness, but can help researchers infer patterns and levels of gene flow among different populations (26). Consequently, neutral loci can reveal the general history of a population, whereas individual virulence loci only reveal a unique story or their history under selection. Genetic markers are also used to study the genetic structure of a population and how it is affected by the relative contribution of sexual and asexual reproduction within each generation. Organisms that reproduce asexually may exhibit a high degree of clonality, with few genotypes present at high frequencies, while sexually reproducing organisms often have a high degree of genotypic diversity (4).

Restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPDs) markers were previously used to study diversity in *P. sojae* (19, 33). Both types of markers clustered isolates into four distinct groups, with some groups being comparable among markers and others not. The distribution of RFLP alleles among isolates suggested that new races of *P. sojae* were result of both by progressive mutation and by infrequent outcrosses. Foster et al. (19) also suggested that all 48 isolates studied were produced by rare outcrosses between representatives of four genotypes designated as progenitor isolates (19). Another study which used similar RFLPs, identified low level genotypic diversity in a population of *P. sojae* from Australia when compared to a population of the pathogen from the U.S., suggesting a single introduction of one isolate of the pathogen (14). Moreover, all races found in Australia belonged to RFLP group 1, which includes race 1, and thus provided evidence that the races could have evolved locally through mutations from the original introduced genotype (14).

Microsatellite markers, also known as short simple tandem repeats (SSRs), are polymorphic DNA loci consisting of a repeated nucleotide sequence. The repeat sequence can be 2 to 7 base pairs long. The number of repeat units varies within individuals of a population, thereby creating multiple alleles for a microsatellite locus. It has been suggested that microsatellites are a better tool for studying population diversity. Like RFLPs and RAPDS, they are selectively neutral, however, SSRs are more variable, they amplify single loci, and more importantly are co-dominant so heterozygote individuals in a diploid organism can be easily determined (40). Twenty one polymorphic microsatellites were developed from sequence data of *P. sojae* race 2 obtained from VBI database (7), and represented 14 super-contigs (51). Fifty three alleles were identified among 33 isolates of *P. sojae* in a preliminary study, with an average of 2.5 alleles per locus (7). All alleles deviated significantly from Hardy-Weinberg equilibrium (HWE) and the observed heterozygosity was low, as expected for a homothallic soil borne species, with an overall mean of 0.015 (7). Predictions have been made that there should be almost no heterozygosity in homothallic *Phythophthora* populations (22). Self-fertilization reduces the amount of heterozygosity by one-half in every generation, thus less than 1% of the original heterozygosity should remain after only seven generations (22). The objective of this study was to determine the level of genetic diversity of a population of *P. sojae* from Iowa using SSR analysis and pathotype characterization.

## MATERIALS AND METHODS

***P. sojae* isolates.** A total of 93 pure isolates of *P. sojae* from Iowa were evaluated in this study. The collection included 82 isolates recovered from either symptomatic

plants or soil samples collected from fields with a history of PRR from 13 fields in Iowa between 2008 and 2010. The other 11 isolates were previously recovered from plant and soil samples collected from 10 counties in Iowa in 2001(34), and had been stored in sterile water in the laboratory (Table 1). Seedlings of the cultivar Sloan were used to bait *P. sojae* from soil samples (12, 39). The pathogen was recovered from stem lesions on PBNIC medium (12). *P. sojae* isolates were confirmed by their growth pattern on PBNIC (12) and by morphology of sporangia. A subset of 6 isolates were further confirmed to be *P. sojae* by sequence data of the internal transcribed spacer (ITS) region of ribosomal DNA amplified with universal forward ITS1 and reverse ITS4 primers (30).

**Purification and storage of isolates.** Purification of *P. sojae* isolates was done using Chen-Zentmeyers' salt wash solution technique (39). Briefly, mycelium from the perimeter of newly grown cultures was transferred with a sterile scalpel to a 60x15 mm plastic Petri plate containing 15 ml Lima bean broth (50 g frozen Lima beans in 1000 ml distilled water). After 48 to 72 hours at room temperature and 12 hour light cycle, the LBB was poured off and replaced by Chen-Zentmeyers' salt wash solution (0.01 M $Ca(NO_3)_2.4H_2O$, 0.004 M $MgSO_4.7H_2O$, 0.005M $KNO_3$, 0.02 mg/l $FeSO_4.7H_2O$, pH 7.0). Fifteen minutes later, this salt solution was replaced with new salt solution. A third wash was done using sterile water. Five to 12 hours later, sporangia and zoospores were formed (39). Mono-zoosporic isolates were obtained by spreading 200 µl of the suspension onto water agar plates and transferring single germinated zoospores to dilute V8 medium under a stereo microscope after 24 hours. Purified isolates of *P. sojae* were stored in sterile distilled water at room temperature until further use.

**Pathotype characterization.** Pathotypes of *P.sojae* were determined with the hypocotyl inoculation technique using a 15 cultivar differential set (6, 9, 39). Standard soybean varieties, each with a specific *Rps* gene, were grown in trays in the greenhouse. The varieties used were: L88-8470 (*Rps*1a, Muckden source), L77-1863 (*Rps*1b, Hanga source*)*, Williams 79(*Rps*1c), L99-3312 or PI 103091 (*Rps*1d), Williams 82 (*Rps*1k), L82-1449 (*Rps*2, CNS source*)*, L83-570 (*Rps*3a), L91-8347 (*Rps*3b), L92-7857 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Altona or L89-1581 (*Rps*6), L93-3258 or Harosoy (*Rps*7), PI 399073 (*Rps*8) and Williams (susceptible).

Soybean seeds were grown potting mix (mixture soil, sand and vermiculite) in trays in the greenhouse. Ten 7-day-old seedlings of each differential were inoculated by making a slit in the hypocotyl with a syringe filled with a slurry of a 7-10 day old culture of each isolate and placing approximately 0.2 to 0.4 ml of slurry into the slit (12, 39). A plastic covering was placed over tray for 12-16 hours to prevent the inoculum from drying. Seven to 10 days after inoculation, PRR incidence was evaluated. Plants that died or developed distinct symptoms of PRR were classified as susceptible, while resistant plants developed a hypersensitive reaction (slight necrotic lesion around the wound). The differential was considered susceptible when at least 7 out of 10 seedlings were susceptible. The test was repeated at least twice for each isolate. Isolates from 2008 to 2010 were characterized for virulence as soon as they were purified, while the virulence formula determined in 2001 was used for the 11 older isolates (34).

**DNA extraction.** Eight to ten square pieces (5 $mm^2$) of *P. sojae* grown on diluted V8 agar were transferred to 250 ml flasks containing 50 ml of V8 broth (40 ml of V8 juice, 0.6 g CaCO3, 0.2 g Bacto yeast extract, 1 g sucrose, 0.01 g cholesterol in 1000 ml

of distilled water). Flasks were placed on shaker at 100-120 rpm at room temperature for 4-7 days. Mycelium was harvested by vacuum aspiration through no. 1 filter paper, frozen with liquid nitrogen and ground using a sterile mortar and pestle. DNA was extracted immediately using a modification of the cetyltrimethylammonium bromide (CTAB) procedure (8, 41). Briefly, the dried powder mycelium was placed in plastic 50 ml centrifuge tube with 10 ml of extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.0064 M ethylenediaminetetraacetic acid (EDTA), pH 7.5, 0.017 M sodium bisulphate) and vortexed. Ten ml of lysis buffer (0.2 M Tris, 0.064 M EDTA, pH 7.5, 2.0 M NaCl, 2% CTAB and 60 µl of 5% sarkosyl (5 g N-lauryl sarcosine per 100 ml H2O)) was added to the tubes, which were vortexed, and then incubated at -80°C for 15 min, followed by 65°C for 15 min. This step was repeated twice, with the final incubation at 65°C lasting 45 min. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube, mixed gently and then the tubes were centrifuged for 10 min at 2000 rpm. The aqueous phase in the tube was transferred to a new tube and then one volume of chloroform:isoamyl alcohol (24:1) was added, followed by centrifugation and transfer to a new tube. DNA was precipitated by the addition of one volume of cold isopropanol and incubation at -20°C overnight. After centrifugation, the supernatant was discarded, the pellets dried at room temperature, and then resuspended in 200 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Two µl of pancreatic RNase A (0.01 µg/µl) was added to the DNA solution, which was incubated at 37°C for an hour, then the solution was transferred to a 1.5 ml tube and the alcohol washes were repeated. After the isopropanol was poured off, the pellets were washed in buffer (76% Ethanol, 0.8 M NaOAc, pH 7.0) and rinsed (76% Ethanol, 0.026 M NH4OAc). The DNA was then resuspended in TE and

its concentration measured using a Nanodrop™ ND-1000 Spectrophotometer. DNA was extracted from all isolates as soon as they were purified. Concentrations were adjusted to 100 ng/µl and stored a -20 ºC for SSR analysis.

**Molecular genotypes.** Twenty five microsatellite primer pairs (PS01, PS04, PS05, PS06, PS07, PS10, PS12, PS16, PS17, PS18, PS19, PS20, PS24, PS25, PS27, PS29, PS30, PS33, PS36, PS37, PS38 (7) and S38-39, S64-65, S68-69 and S74-75 (7, 38) were used to screen 64 *P. sojae* isolates for amplification ease and polymorphism on 4% agarose gels. Based on amplification success and the highest number of alleles (band sizes) encountered per locus, eight SSRs were selected for further studies. Forward fluorescent primers for Ps01, Ps16, Ps24, and Ps 33 were labeled with 6-FAM dye and Ps05, Ps10, PS12, and Ps 29 with HEX dye. Amplification was performed in a 96-well Eppendorf Mastercycler® thermal cycler (Hamburg, Germany) in a 15.0 µl volume with 200 µM dNTP mixture, 2.5 mM $MgCl_2$, 1X Go Taq® Hot Start Colorless Master Mix buffer, 0.08 units Go Taq® Hot Start DNA polymerase (Promega Inc., Madison, WI), 0.45 µM of each primer, and 1µl of DNA template. The thermal cycler was programmed for an initial step at 85 ºC for 2 min, denaturalization step at 94 ºC for 95 s, then 24 cycles at 52 ºC for 1 min, 72 ºC for 72 s, 94 ºC for 30 s, then 52 ºC for 1 min and 72 ºC for 30 min. Five standard isolates from other labs, an isolate of *P. cactorum* and a *Pythium* isolate were included in the runs. A 96-capillary Applied Biosystem 3730 Genetic Analyzer was set up to run samples labeled with these dyes and a GeneScan™ 500 ROX ™ size standard (Applied Biosystem, Foster City, CA) was used. GeneMapper® Software 4.0 (Applied Biosystem, Foster City, CA) was used to size the alleles to the nearest base pair.

**Data analyses.**

**Genetic and pathotype diversity within Iowa.** The total data set comprising 93 isolates recovered from 25 fields in Iowa was used in this analysis. Different eletropherogram peaks (fragment sizes) resulting from a microsatellite marker were considered different alleles. For the purpose of the pathotype analysis, each soybean differential having its respective *Rps* gene interacting with *P. sojae* was considered as a locus (avirulence locus) with two possible interaction outcomes: compatible (1) or incompatible (0).

The number of SSR alleles per locus, observed heterozygosity (analogous to gene diversity) (*Ho*), unbiased expected heterozygosity calculated on a single locus basis (*UHe*), Hardy-Weinberg equilibrium (HWE) for each locus, and Fixation Index (F) were calculated using population genetic software GenAlEx 6 (35) for raw and clone-corrected data. The data was clone-corrected by removing isolates that had the same multilocus genotype (MLG) with the same pathotype within a field, these isolates now represented a *genotype* and were given an identification roman number from I to XXXXVI (Table 4). Pairwise linkage disequilibrium (LD) among SSR clone-corrected data was tested using GENEPOP version 4.0 (37), and significance levels were evaluated using Markov-chain randomization procedure (25) with 10,000 dememorization steps, 500 batches and 5,000 iterations per batch. Genotypic and pathotype diversity (D) were calculated as the number of MLG and the number of pathotypes, respectively, divided by the number of samples. Index of association ($I_A$) was calculated for the whole data set and the clone corrected population using MultiLocus V1.3b (1).

Correlation of individual by individual genetic distance matrices was calculated using co-dominant (genetic) and binary (pathotype) options in software GenAlEx 6 (35). Mantel test for matrix correspondence was used to test statistical relationship between the two distances (42).

A dendrogram comparing the relatedness among 97 isolates (93 Iowa and four standards from other labs) was created based on Nei's genetic distance, using UPGMA (unweighted pair group method) in PHYLIP version 3.69 using GENDIST and NEIGHBOR (17).

**Genetic and pathotype diversity among fields**. Subsets of the isolates were used to analyze this objective. Subset A included 80 isolates of *P. sojae* recovered from soybean plants collected in 2008 to 2010 from 11 fields, while subset B included 68 isolates collected in 2008 to 2010 from seven fields from which multiple isolates of *P. sojae* were recovered from single plants.

Genetic and pathotype divergence using genotypic and binary distance among seven fields was assessed using $\Theta_{st}$ (47). Analysis of molecular variance (AMOVA) (16) was performed among those seven fields and among multiple isolates recovered from 17 plants within those seven fields using software GenAlEx 6 (35). Significant levels were evaluated using 999 permutations.

Index of association ($I_A$) was calculated for all isolates of *P.sojae* recovered from seven fields and also for each field individually using MultiLocus V1.3b (1). Multilocus genotypic diversity was estimated for each field population using Stoddart and Taylor's *G* index (43). In order to compare indexes using population of different sizes, *G* was

scaled by the expected number of genotypes for the smallest population size (24) with rarefaction curves using Analytical Rarefaction version 1.3 (28).

Correlation of individual by individual genetic distance matrices, for the overall isolates in the seven fields, was calculated using co-dominant (genetic) and binary (pathotype) options in software GenAlEx 6 (35). Mantel test for matrix correspondence was used to test statistical relationship between the two distances using the same software.

A dendrogram comparing the relatedness among the seven populations of *P. sojae* was created based on allele frequencies and Neis's genetic distance, using UPGMA (unweighted pair group method) with bootstrap values calculated on 100 UPGMA trees using SEQBOOT, GENDIST, NEIGHBOR and CONSENCE in PHYLIP version 3.69 (17).

Relatedness among SSR genotypes and among pathotypes of *P .sojae* from the seven fields was examined through network analysis with NETWORK 4.6.0.0 software under default parameter settings. The constraint for the branching structure was relaxed for construction of median-joining networks (2) for SSR genotypes, as well as for construction of reduced median networks (3) for the pathotypes.

**Genetic diversity within a plant.** A subset of 23 isolates recovered from the seven fields mentioned above was considered for this objective. Out of the 17 plants where we recovered more than one isolate, only six plants had different genotypes and pathotypes within them. Thus, a total of 23 isolates recovered from those six plants were used in this objective.

SSR genotypes and pathotypes relatedness among 23 isolates of *P.sojae* recovered from six plants were examined through network analysis with NETWORK 4.6.0.0 software under default parameter settings. The constraint for the branching structure was relaxed for construction of median-joining networks (2) for SSR genotypes, as well as for construction of reduced median networks (3) for the pathotypes.

## RESULTS

**Genetic diversity within Iowa.** A total of 93 mono-zoosporic isolates of *P. sojae* were recovered from soybean plants and soil samples collected from commercial fields in Iowa (Table 1). Each of the eight SSRs amplified one or two bands per primer pair per isolate. All of the SSRs were polymorphic, with 2 to 8 alleles observed per locus across the population (Table 2). Forty two alleles were identified in the 93 isolates obtained from Iowa, and in 4 additional standard isolates from two labs, the average was 5.25 alleles/locus. Considering solely the 93 isolates from Iowa, 33 alleles were identified with an average of 4.12 alleles/locus.

Forty MLGs were identified within the 93 isolates from Iowa, with 25 of them being unique genotypes to a single isolate. The remaining 15 MLGs included 2 to 7 isolates each. This population had a genotypic diversity (D) of 0.43. Using clone-corrected data, 29% of the 8 loci pairwise comparisons (eight out of 28) displayed significant linkage disequilibrium (LD), and only two loci (PS05 and PS12) of the eight selected were completely independent from the other loci.

Within the 93 isolates from Iowa, 19 pathotypes were identified resulting in a pathotype diversity (D) of 0.20. Eleven of the identified pathotypes were unique to a

single isolate. The pathotypes ranged from being virulent on one *Rps* gene represented by virulence formula 7 (formally race 1), to being virulent on six *Rps* genes represented by virulence formulas 1a,1b,1c,1k,3b,7; 1a,1b,1c,1d,1k,7; and 1a,1b,1d,1k,6,7 (Table 4). Of the 93 Iowa isolates, 98% were virulent on *Rps* 7, followed by 62% on *Rps* 1b and *Rps* 1k. No isolates compatible on *Rps* 2, 3c, 4, 5 and 8 were recovered (Fig. 1).

After clone-correction, a total of 46 different *genotypes* were identified (Table 4). Clone-correction reduced the number of isolates by half, and altered the statistic estimates. The observed heterozygosity (*Ho*) increased from 0.027 to 0.047 (Table 3). The mean *F* over all loci was greater than 0.9 indicating high inbreeding in *P. sojae*. The index of association ($I_A$) for the non-corrected data was almost 2-fold that of the clone corrected data and significant, indicating the population is not in random mating conditions. Accordingly, all loci deviated significantly from HWE (Table 3).

The correlation coefficient between genetic distance matrix (genotypes) and binary distance matrix (pathotype) was low ($r = 0.379$), although significant ($P=0.001$). This, indicates a relationship between the MLGs assessed with the eight SSRs and pathotypes characterized among the 93 isolates from Iowa.

Analysis of the 97 isolates (93 from Iowa and the four standards from other labs) using Nei's genetic distance and UPGMA, showed five distinct groups. In general isolates tended to group primarily by field (Fig. 2).

**Genetic diversity among fields.** Three of the 11 fields from which more than one isolate of *P. sojae* was recovered shared identical *genotypes*. Within each single field, one to five MLGs were found that differed by a maximum of seven loci and up to four alleles per locus. Furthermore, one to five pathotypes also were found within each single field,

which differed in up to four avirulence genes. When both MLG and pathotype data were combined, up to six different *genotypes* were identified within an individual field (Fig. 3).

AMOVA showed significant genetic divergence among isolates of *P. sojae* from seven fields and among the isolates recovered from 17 plants within the seven fields, $\Theta_{RT}$ = 0.574 (P=0.001) and $\Theta_{PR}$ = 0.755 (P=0.001), respectively. Thirty two percent of the AMOVA's estimated variance was due to differences among plants, while 57% was due to differences among fields. The seven populations were significantly differentiated from each other, with moderate to very high differentiation, ranging from $\Theta_{PT}$ of 0.15 to 0.90 (Table 5). As expected with this level of differentiation, the corresponding migration estimates, $Nm$=0.29, suggested low gene flow between populations.

The AMOVA also showed significant pathotype divergence among isolates of *P. sojae* recovered from the same seven fields and among the isolates recovered from the 17 plants collected from those fields ($\Theta_{RT}$ = 0.558 (P=0.001) and $\Theta_{PR}$ = 0.564 (P=0.001), respectively). Twenty five percent of the AMOVA's estimated variance was due to differences among plants, while 56% was due to differences among fields. Not all field populations were significantly differentiated from each other when considering pathotype data. Five of the 21 pairwise comparisons were not differentiated from each other (Table 5).

Within the seven soybean fields from which isolates of *P. sojae* were recovered, the overall observed heterozygosity was 0.018, ranging from 0.000 to 0.050 among fields. Heterozygote deficiencies was observed in the overall seven field population, as the mean unbiased expected heterozygosity ($UH_e$) was ten-fold that of the observed heterozygosity ($H_o$) (Table 6). Heterozygote deficiencies were also observed at the field

level in the individual populations of *P. sojae* in each field (Table 6). The mean index of association for the seven fields combined was high and significant ($I_A$=1.35, P<0.001) indicating that non-random mating or asexual reproduction had occurred. However, the observed $I_A$ for the population of *P. sojae* recovered from field 2010 was low and non-significant ($I_A$= 0.08, P= 0.41) (Table 6), which indicates that this population is sexually outcrossing. Genotypic diversity (*G*) was estimated with rarefaction to be able to compare populations with different sampling sizes. The mean *G* across the seven populations (n=68) was very high (G=3.6), considering that the maximum value is 4.0 (all MLGs differ from each other). For individual populations, however, *G* ranged from 1.9 and 4.0 (Table 6).

The UPGMA analysis showed population 1011 with a long branch length, closely related to population 2010 (Fig. 4). Bootstrap supports and separates populations 1011, 2010 and 1012 from the rest, as well as it supports a high relatedness among populations 2011 and 2001 (Fig 4). Relationship with geographical location of the field can also be seen; thus field 2011 and 2001 are from Story County, field 1019 and 1005 are from Monroe County, and fields 1011 and 1012 are both from Polk County. Only field 2010 from Story County, did not relate to the geographical location with the other two fields from Story County, but then it was the only field that showed evidence of outcrossing (Table 6).

Network analysis can show the relatedness among MLGs graphically by distance (Fig. 5a). The closer two MLGs are to each other, the more alleles they share. The MLGs identified in population of *P. sojae* recovered from field 1011 appear to be closely related, and also to the MGLs identified in population of *P. sojae* recovered from field

1012. These two fields appear to be differentiated from each other and from the rest of the populations of *P. sojae* recovered from the fields sampled, showing low genotypic diversity (*G*=2.0 and 2.2, respectively) (Table 6). Even though high genotypic differentiation was found among populations of *P. sojae* among fields using AMOVA analysis, the populations of *P. sojae* recovered from fields 2001 and 1019 shared some identical MLGs with field 2011. On the contrary, MLGs within the population of *P. sojae* recovered from field 2011 appear to be not related to each other, as well as the MLGs of the populations of *P. sojae* recovered from fields 1005 and 1019. The MLGs of *P. sojae* recovered from field 2010 had the highest diversity (*G*= 4), and every MLG in this population of *P. sojae* was different from the other.

Similarly, network analysis can be used to view the relatedness among pathotypes (Fig 5b). The closer two pathotypes are to each other, the more avirulence genes they share. Only one pathotype was found within the population of *P. sojae* recovered from fields 1011, 2010 and 1019 (Fig 5b, Table 6). Moreover, the pathotype identified in the population of *P. sojae* recovered from fields 1011 and 2010 was identical. Pathotypes detected in the population of *P. sojae* recovered from field 1012 appeared to be closely related to each other. On the contrary, pathotypes detected in the population of *P. sojae* recovered from field 2011 seem unrelated to each other and more related to pathotypes detected in the population of *P. sojae* recovered in other fields (Fig. 5b).

The correlation coefficient between genetic distance matrix (genotypes) and binary distance matrix (pathotype) among isolates of *P. sojae* recovered from the seven commercial soybean fields was moderate (r = 0.468), although significant (P=0.001),

suggesting a relationship between the MLGs assessed by the eight SSRs used in this study and the pathotypes characterized among 68 isolates of *P. sojae* from Iowa.

Although greater diversity in the population of *P.sojae* in Iowa was revealed by SSRs compared to pathotypes (24 MLGs compared to 11 pathotypes, Table 6), similar patterns can be visualized in the network diagrams (Fig 4a and b). All isolates of *P. sojae* recovered from fields 1011, 2010 and one isolate recovered from field 2011 grouped together in both diagrams. Isolates of *P. sojae* recovered from field 2011 were mostly unrelated in both diagrams, while the MLGs detected in the population of *P. sojae* recovered from field 1011 show strong relatedness and were all a single pathotype.

**Genetic diversity within a plant.** The AMOVA analysis determined that 10 and nineteen percent of the estimated variance among genotypes and pathotypes detected in the populations of *P. sojae*, respectively, were due to within-plant variations. Consequently, variation among MLGs and pathotypes between isolates of *P. sojae* recovered from a single plant was observed. Of the 17 plants from which multiple isolates of *P. sojae* were recovered, more than one MLG was detected from six plants, and more than one pathotype was detected from three plants.

The relatedness between MLGs of the multiple isolates of *P. sojae* recovered from the six individual plants is shown graphically by distance in the network analysis (Fig. 6a). Two isolates of *P. sojae* recovered from a single plant in field 2010 each had a different MLG, however, these isolates were more related to each other than to any other isolate in the diagram. Fifteen isolates of *P. sojae* recovered from three plants (no. 10, 11 and 13) in field 1012 grouped together; two MLGs were identified in isolates of *P. sojae* recovered from plant no. 11 and 13, and three from plant no. 10. Two of the isolates of *P.*

*sojae* had MLGs that were identical despite being recovered from different plants in that field (1012). Three isolates of *P. sojae* recovered from one plant in field 1019 each had its own MGL, two MGLs were closely related, but the third MGL was not.

Overall observed heterozygosity from the six plants from which multiple isolates of *P. sojae* were recovered was *Ho*= 0.049 (ranging 0.00 to 0.167), which was much higher than the observed heterozygosity in the general population of *P. sojae* (*Ho*=0.027, Table 3) and the overall combined population of *P. sojae* recovered from the seven fields (*Ho*=0.018, Table 6). This was expected since only plants where multiple MLGs were recovered were analyzed. The highest observed heterozygosity (*Ho*= 0.167) was found among three isolates of *P. sojae* recovered from plant 11 in field 1019 (Fig. 5a) that had various heterozygote loci (Table 7).

Pathotype relatedness among multiple isolates of *P. sojae* recovered from three single plants is shown graphically by distance in the network analysis (Fig. 6b). In each of the three plants (no. 11, 20 and 25), two pathotypes of *P. sojae* were recovered. Pathotypes recovered from plant no. 20 in field 2001 were closely related to each other but unrelated to the rest of the pathotypes in the diagram (Fig. 6b).

Two SSR genotypes and two pathotypes were detected in isolates of *P. sojae* recovered from plant no. 11 collected from field 1012 (Fig. 6a and b).

## DISCUSSION

**Genetic diversity within Iowa.** We considered each 14 soybean differentials having its respective *Rps* gene interacting with *P. sojae* as a 'locus', and used the virulence formula of the pathogen as a binary system giving each 14 interactions a '1' if

compatible and a '0' if incompatible. This assumption might someday be proven incorrect, especially taking into account that only ten single dominant avirulence genes in *P. sojae* have been genetically identified or mapped (21,46), and that it has been demonstrated that avirulence genes *Avr*4 and *Avr*6 are actually a single gene and can trigger a hypersensitive response in the presence of either *Rps*4 or *Rps*6 (13).

This is the first study to extensively evaluate the diversity of a population of *P. sojae* using SSR analysis and pathotyping. We detected 40 MLGs and 19 different pathotypes from a total of 93 isolates from Iowa. These were fewer alleles than were determined in this previous report using 33 isolates from Ohio by Dorrance and Grunwalt (2009), however the average number of alleles per locus (4.12) and the average observed heterozygosity ($H_o$= 0.027) was higher than in that report (7). Our observed heterozygosity is low, although not unexpected since *P. sojae* is homothallic and thus inbreeds. In fact, the calculated inbreeding coefficient (*F*) of 0.944 further supports our results and expectations. Under complete selfing the observed heterozygosity should be near 0, and *F* should be near 1 (22). These statistics concur with reports of heterozygosity in other homothallic species of *Phytophthora*, such as *P. boehmeriae*, *P. citricola*, and *P. katsurae* (22). Moreover, a statistically significant index of association ($I_A$) and the fact that all loci identified in our study deviated from Hardy-Weinberg equilibrium further concur with a non-random mating organism.

Previous work by Foster et al. (1994), clustered isolates in 4 groups by genetic similarities using RFLPs (19). We obtained DNA from 3 of these groups from the researchers's lab with the objective of relating the two studies. DNA from isolate P6497 (R2) belonging to Group I, P7076 (R19) belonging to Group II and P7074 (R17)

belonging to Group III were included in the dendrogram analysis. Allele frequency detected by the 8 SSR loci grouped isolate R17 by itself and isolates R2 and R19 together, thus SSRs clustered Foster's Groups I and II together in the same group. The dendrogram in our study suggest little relation between the two types of markers, the RFLPs used by Foster et al. (1994) and the SSR used in our study.

**Genetic diversity among fields**. The genetic diversity of populations of *P. sojae* from seven fields in Iowa was compared. The genetic and pathotype differentiation determined for the seven fields ($\Theta_{RT}$ = 0.574 and $\Theta_{RT}$ = 0.558, respectively), in conjunction with low migration estimates (*Nm*= 0.29), revealed limited movement and limited gene flow of *P. sojae.* This is expected as *P. sojae* is a soil borne pathogen, and dispersal of individuals among population is limited. Nevertheless, it is interesting to note that some SSRs and pathotypes were common among fields. Conversely and in spite of the limited means of dispersal of this pathogen, we also observed moderate to very high genotypic diversity (*G*) of the endemic *P. sojae* population in the fields sampled (*G* ranged from 1.9 to 4). The overall index of association in seven fields ($I_A$ =1.35, P<0.001) indicated non-random mating, although at least one of the seven fields seemed to have some degree of outcrossing. Evidence of outcrossing in the laboratory conditions has also been demonstrated when two genetically different isolates formed hybrid oospores after co-culturing in vitro (48). In this case, under optimal in vitro conditions and both isolates present in close proximity, the outcrossing rate was less than 5% (45). In our study, 5.9% of the isolates showed evidences of outcrossing if we consider that one out of the seven fields (4 isolates from field 2010 out of a total 68 isolates) was randomly mating as demonstrated by a low and non-significant $I_A$ (Table 6).

The UPGMA and the NETWORK analysis both showed similar patterns. Some populations of *P. sojae* appear genetically distant from the rest (1011 and 2010), while others appear to be closely related to each other (2011 and 2001) in both types of analysis. The UPGMA analysis also showed some relation to geographical location.

The correlation coefficients between genotype and pathotype genetic distances were low (whole data set) to moderate (subset isolates from seven fields), but highly significant. This indicates a relationship between the MLGs assessed with the eight SSRs and pathotypes characterized from Iowa. Larger data sets using these same SSRs including also more pathotypes would be needed to confirm this relationship.

Overall results indicate that the population of *P. sojae* in Iowa is diverse, with restricted gene flow among fields. Similarly, the population structure of *Phytophthora capsici* was found to be highly diverse and differentiated among sites in New York (15), while in coastal Peru populations of this same pathogen seem to be clonal (29). More studies have to be done outside Iowa to be able to compare population structures in *P. sojae* in different regions. Our findings, nevertheless, are consistent with *P. sojae* dispersal mechanism, which primarily includes movement of surface water and soil particles, and no aerial dispersal.

**Genetic diversity within a plant**. Previous studies examining the diversity of *P. sojae* in intensively sampled fields showed many pathotypes of the pathogen could exist in a single field (10, 36). Genotype and pathotype diversity, however, have not been demonstrated to occurr within a naturally-infected plant.

Co-infection of soybean by more than one isolate of *P. sojae* has been demonstrated in greenhouse experiments when two drug resistant mutants of *P. sojae*

were inoculated onto susceptible soybean plants (31). To our knowledge, these observations have never been reported in nature. In our study, we were able to recover multiple isolates of *P. sojae* from a single PRR-symptomatic plant in the field, where isolate from a single plant belonged to either two or three MLGs, or two pathotypes. Since oospores are formed within the plant lesion (22, 27), it is possible that this is where outcrossing may occur. Thus when two or more isolates co-infect a plant and form lesions in close proximity to each other, the possibility of outcrossing within the lesion increases.

Different analysis (Network, UPGMA by field and by individual isolate, AMOVA) showed that Iowa's *Phytophthora sojae* population is differentiated by field, and although there are some isolates found in more than one field, the populations from different fields are considered different populations with low gene flow between them.

In this study, microsatellites as neutral loci revealed the general history of *P. sojae* population in Iowa as highly diverse although restricted movement and gene flow among fields. Pathoytpes, or avirulence loci as defined, revealed a unique story of their history under selection, although similar its interpretation is different; genetic diversity is the evolutionary potential of the pathogen in Iowa, while pathotype diversity may be a function of the specific cultivar used in a particular field or in fields in Iowa.

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Table 1. Isolates of *Phytophthora sojae* analyzed in this study.

| Isolate | Field | Plant | Recovered from | County, Iowa | Year |
|---|---|---|---|---|---|
| 1024 (CC5[a]) | 1 | - | Soil | Grundy | 2001 |
| 1025 (CE5[a]) | 2 | - | Soil | Jones | 2001 |
| 1026 (CE10[a]) | 3 | - | Soil | Johson | 2001 |
| 1027 (CW1[a]) | 4 | - | Soil | Greene | 2001 |
| 1028 (MaR25[a]) | 5 | - | - | - | 2001 |
| 1029 (NW5A[a]) | 6 | - | Soil | Sioux | 2001 |
| 1030 (POLK3-3[a]) | 7 | 1 | Plant | Polk | 2001 |
| 1031 (SC4A[a]) | 8 | - | Soil | Ringgold | 2001 |
| 1032 (SC8A[a]) | 9 | - | Soil | Lucas | 2001 |
| 1033 (SW1A[a]) | 10 | - | Soil | Adair | 2001 |
| 1034 (SW1B[a]) | 11 | - | Soil | Adair | 2001 |
| 1005.1 | 1005 | 2 | Plant | Monroe | 2008 |
| 1005.2 | 1005 | 3 | Plant | Monroe | 2008 |
| 1005.3 | 1005 | 3 | Plant | Monroe | 2008 |
| 1005.4 | 1005 | 3 | Plant | Monroe | 2008 |
| 1005.5 | 1005 | 4 | Plant | Monroe | 2008 |
| 1005.6 | 1005 | 4 | Plant | Monroe | 2008 |
| 1005.7 | 1005 | 4 | Plant | Monroe | 2008 |
| 1005.8 | 1005 | 4 | Plant | Monroe | 2008 |
| 1005.9 | 1005 | 4 | Plant | Monroe | 2008 |
| 1005.11 | 1005 | 5 | Plant | Monroe | 2008 |
| 1005.12 | 1005 | 5 | Plant | Monroe | 2008 |
| 1009.1 | 1009 | 6 | Plant | Story | 2008 |
| 1009.2 | 1009 | 6 | Plant | Story | 2008 |
| 1010.1 | 1010 | 7 | Plant | - | 2008 |
| 1010.2 | 1010 | 7 | Plant | - | 2008 |
| 1011.1 | 1011 | 8 | Plant | Polk | 2008 |
| 1011.2 | 1011 | 8 | Plant | Polk | 2008 |
| 1011.3 | 1011 | 8 | Plant | Polk | 2008 |
| 1011.4 | 1011 | 9 | Plant | Polk | 2008 |
| 1011.5 | 1011 | 9 | Plant | Polk | 2008 |
| 1011.6 | 1011 | 9 | Plant | Polk | 2008 |
| 1012.1 | 1012 | 10 | Plant | - | 2008 |
| 1012.2 | 1012 | 10 | Plant | - | 2008 |
| 1012.3 | 1012 | 10 | Plant | - | 2008 |
| 1012.4 | 1012 | 10 | Plant | - | 2008 |
| 1012.5 | 1012 | 11 | Plant | - | 2008 |
| 1012.6 | 1012 | 11 | Plant | - | 2008 |
| 1012.7 | 1012 | 11 | Plant | - | 2008 |
| 1012.8 | 1012 | 11 | Plant | - | 2008 |
| 1012.9 | 1012 | 11 | Plant | - | 2008 |
| 1012.11 | 1012 | 11 | Plant | - | 2008 |
| 1012.12 | 1012 | 11 | Plant | - | 2008 |
| 1012.13 | 1012 | 11 | Plant | - | 2008 |
| 1012.14 | 1012 | 11 | Plant | - | 2008 |
| 1012.15 | 1012 | 12 | Plant | - | 2008 |
| 1012.16 | 1012 | 12 | Plant | - | 2008 |
| 1012.17 | 1012 | 13 | Plant | - | 2008 |
| 1012.18 | 1012 | 13 | Plant | - | 2008 |
| 1014.1 | 1014 | 14 | Plant | Monroe | 2008 |
| 1014.2 | 1014 | 14 | Plant | Monroe | 2008 |

Table 1. Continued

| | | | | | |
|---|---|---|---|---|---|
| 1014.3 | 1014 | 14 | Plant | Monroe | 2008 |
| 1015.1 | 1015 | 15 | Plant | Woodbury | 2008 |
| 1019.1 | 1019 | 16 | Plant | Monroe | 2008 |
| 1019.2 | 1019 | 16 | Plant | Monroe | 2008 |
| 1019.3 | 1019 | 16 | Plant | Monroe | 2008 |
| 1019.4 | 1019 | 17 | Plant | Monroe | 2008 |
| 1019.5 | 1019 | 18 | Plant | Monroe | 2008 |
| 1019.6 | 1019 | 18 | Plant | Monroe | 2008 |
| 1019.7 | 1019 | 18 | Plant | Monroe | 2008 |
| 1019.8 | 1019 | 18 | Plant | Monroe | 2008 |
| 1019.9 | 1019 | 18 | Plant | Monroe | 2008 |
| 1019.11 | 1019 | 18 | Plant | Monroe | 2008 |
| 1023.1 | 1023 | - | Soil | Marshal | 2008 |
| 1023.2 | 1023 | - | Soil | Marshal | 2008 |
| 1023.3 | 1023 | - | Soil | Marshal | 2008 |
| 1023.4 | 1023 | - | Soil | Marshal | 2008 |
| 1023.5 | 1023 | - | Soil | Marshal | 2008 |
| 2001.1 | 2001 | 19 | Plant | Story | 2009 |
| 2001.2 | 2001 | 19 | Plant | Story | 2009 |
| 2001.3 | 2001 | 20 | Plant | Story | 2009 |
| 2001.4 | 2001 | 20 | Plant | Story | 2009 |
| 2001.5 | 2001 | 20 | Plant | Story | 2009 |
| 2001.6 | 2001 | 20 | Plant | Story | 2009 |
| 2001.7 | 2001 | 20 | Plant | Story | 2009 |
| 2001.8 | 2001 | 20 | Plant | Story | 2009 |
| 2001.9 | 2001 | 20 | Plant | Story | 2009 |
| 2001.11 | 2001 | 20 | Plant | Story | 2009 |
| 2001.12 | 2001 | 20 | Plant | Story | 2009 |
| 2001.13 | 2001 | 20 | Plant | Story | 2009 |
| 2002.1 | 2002 | 21 | Plant | Story | 2009 |
| 2010.1 | 2010 | 22 | Plant | Story | 2010 |
| 2010.2 | 2010 | 23 | Plant | Story | 2010 |
| 2010.3 | 2010 | 23 | Plant | Story | 2010 |
| 2010.4 | 2010 | 24 | Plant | Story | 2010 |
| 2011.1 | 2011 | 25 | Plant | Story | 2010 |
| 2011.2 | 2011 | 25 | Plant | Story | 2010 |
| 2011.3 | 2011 | 26 | Plant | Story | 2010 |
| 2011.4 | 2011 | 26 | Plant | Story | 2010 |
| 2011.5 | 2011 | 27 | Plant | Story | 2010 |
| 2011.6 | 2011 | 27 | Plant | Story | 2010 |
| 2011.7 | 2011 | 27 | Plant | Story | 2010 |
| 2011.8 | 2011 | 28 | Plant | Story | 2010 |
| R2-VT | P6497[b] | - | - | - | - |
| R19-VT | P7076[b] | - | - | - | - |
| R17-VT | P7074[b] | - | - | - | - |
| R25-OH | R25[c] | - | - | - | - |

[a] isolated by Xiaofan Niu in 2001 to 2002 (35). Old code in braquets.
[b] DNA from Bret Tyler's lab (19).
[c] DNA from Anne Dorrance's lab.

Table 2. Loci, primers, and alleles based on approximate band sizes as determined by GeneMapper analysis for 93 isolates from Iowa and four standards of *Phytophthora sojae* from other laboratories.

| Locus | GeneBank Accession no. | Sequence motif in isolate P6497[a] | Primer[b] | Label on forward primer | Actual size (bp) in isolate P6497 | Allele size (bp) |
|---|---|---|---|---|---|---|
| PS01 | EF667485 | $(GACACT)_{49}$ | PS01-F<br>PS01-R | 6 FAM | 419 | 281,287,293[c],419,425, 431,437,443 |
| PS05 | EF667486 | $(TCAG)_{34}$ | PS05-F<br>PS05-R | HEX | 263 | 263,307,335,339,343, 347,355 |
| PS10 | EF667489 | $(CAAAC)_{27}$ | PS10-F<br>PS10-R | HEX | 228 | 146,191,196,211,221, 251 |
| PS12 | EF667490 | $(GCTGTT)_{23}$ | PS12-F<br>PS12-R | HEX | 306 | 251,257,292,304,310, 316 |
| PS16 | EF667491 | $(ATTAT)_{20}$ | PS16-F<br>PS16-R | 6 FAM | 469 | 395,405,460,470,475 |
| PS24 | EF667495 | $(CT)_{16}+(CT)_3$ | PS24-R<br>PS24-F | 6 FAM | 252 | 236,252 |
| PS29 | EF667499 | $(TAC)_{15}$ | PS29-R<br>PS29-F | HEX | 273 | 249,270 |
| PS33 | EF667501 | $(AT)_{15}$ | PS33-R<br>PS33-F | 6 FAM | 267 | 250,262,264,266,268, 270 |

[a] '+' indicates that two simple repeats were separated by other bases.

[b] primer sequence detailed in Dorrance and Grundwald, 2009.

[c] alleles found only on standards from other laboratories (4 DNA samples from Tyler's lab in Virginia and 1 DNA sample from Dorrance's lab in Ohio).

Table 3. Genetic diversity across all isolates of *Phytophthora sojae* recovered from Iowa and clone corrected data.

| | **No. isolates** | **Observed heterozygosity (*Ho)*** | **Unbiased Expected heterozygosity (*UHe)*** [a] | **Hardy-Weinberg equilibrium** | **Fixation index (F)** [b] | **Index association ($I_{A)}$** |
|---|---|---|---|---|---|---|
| All isolates | 93 | 0.027 | 0.513 | All loci deviate (P<0.0001) [d] | 0.944 | 0.87 (P<0.001) [e] |
| Clone-corrected data | 46 | 0.047 | 0.544 | All loci deviate (P<0.0001) [d] | 0.907 | 0.48 (P<0.001) [e] |

[a] $UHe = (n/n-1)(1-\sum p^2_i)$ where $p$ is the frequency of the $i^{th}$ allele.

[b] $F = (H_e - H_o)/H_e$.

[c] probability calculated $Chi^2$ test.

[d] probability calculated with 1000 randomization; null hypothesis = $I_a$ does not differ from purely sexually outcrossing population.

Table 4. Pathotypes and microsatellites alleles for the 46 *genotypes* (individuals having a unique SSR genotype and pathotype within a field) identified in *Phytophthora sojae* population in Iowa.

| | No. | Pathotype virulence formula [a] | | | | | | | | | | | | | Microsatellite loci[b] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Genotypes*** | isolates | 1a | 1b | 1c | 1d | 1k | 2 | 3a | 3b | 3c | 4 | 5 | 6 | 7 | 8 | PS01 | | PS05 | | PS10 | | PS12 | | PS16 | | PS24 | | PS29 | | PS33 | |
| **I** | 3 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 262 | 262 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **II** | 2 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 262 | 262 | 191 | 191 | 304 | 304 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **III** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 262 | 262 | 191 | 191 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 250 | 250 |
| **IV** | 7 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 191 | 191 | 310 | 310 | 460 | 460 | 252 | 252 | 270 | 270 | 250 | 250 |
| **V** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | n/d[c] | n/d |
| **VI** | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **VII** | 2 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 221 | 221 | 251 | 310 | 470 | 470 | 236 | 252 | 249 | 249 | 250 | 250 |
| **VIII** | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 339 | 339 | 221 | 221 | 310 | 310 | 470 | 470 | 236 | 236 | 249 | 249 | 266 | 266 |
| **IX** | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 335 | 335 | 221 | 221 | 310 | 310 | 470 | 470 | 236 | 236 | 249 | 249 | 266 | 266 |
| **X** | 8 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 339 | 339 | 191 | 191 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 250 | 250 |
| **XI** | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 339 | 347 | 191 | 191 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 250 | 250 |
| **XII** | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 262 | 262 | 191 | 221 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 250 | 250 |
| **XIII** | 3 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 343 | 343 | 191 | 191 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 250 | 250 |
| **XIV** | 2 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 339 | 339 | 191 | 191 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 250 | 250 |
| **XV** | 2 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 343 | 343 | 191 | 191 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 250 | 250 |
| **XVI** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 262 | 262 | 191 | 191 | 310 | 310 | 405 | 405 | 236 | 252 | 270 | 270 | 250 | 250 |
| **XVII** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 281 | 281 | 262 | 262 | 191 | 221 | 251 | 251 | 405 | 405 | 236 | 252 | n/d | n/d | n/d | n/d |
| **XVIII** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 262 | 262 | 191 | 221 | 251 | 251 | n/d | n/d | 236 | 252 | n/d | n/d | 250 | 250 |
| **XIX** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 221 | 221 | 251 | 251 | 405 | 405 | 252 | 252 | 249 | 249 | 266 | 270 |
| **XX** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 221 | 221 | 251 | 251 | 405 | 405 | 236 | 236 | 249 | 249 | 266 | 270 |
| **XXI** | 4 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXII** | 6 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 262 | 262 | 191 | 191 | 316 | 316 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXIII** | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 262 | 262 | 221 | 221 | 310 | 310 | 470 | 470 | 252 | 252 | 270 | 270 | 266 | 266 |
| **XXIV** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 262 | 262 | 221 | 221 | 310 | 310 | 470 | 470 | 252 | 252 | 270 | 270 | 264 | 264 |

Table 4. Continued

| *Genotypes* | No. | Pathotypes | | | | | | | | | | | | | | PS01 | | PS05 | | PS10 | | PS12 | | PS16 | | PS24 | | PS29 | | PS33 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **XXV** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 262 | 262 | 221 | 221 | 310 | 310 | 405 | 405 | 252 | 252 | 249 | 270 | 250 | 250 |
| **XXVI** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 262 | 262 | 211 | 211 | 310 | 310 | 470 | 470 | 252 | 252 | 270 | 270 | 266 | 266 |
| **XXVII** | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 431 | 431 | 262 | 262 | 221 | 221 | 251 | 251 | 405 | 405 | 252 | 252 | 270 | 270 | 266 | 266 |
| **XXVIII** | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 339 | 339 | 221 | 221 | 251 | 251 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXIX** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 419 | 419 | 262 | 262 | 196 | 196 | 310 | 310 | 405 | 405 | 252 | 252 | 249 | 270 | 250 | 250 |
| **XXX** | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 419 | 419 | 339 | 339 | 191 | 191 | 251 | 251 | 405 | 405 | 252 | 252 | 270 | 270 | 266 | 266 |
| **XXXI** | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 281 | 281 | 262 | 262 | 191 | 221 | 251 | 251 | 405 | 405 | 252 | 252 | n/d | n/d | 250 | 250 |
| **XXXII** | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 281 | 281 | 343 | 343 | 221 | 221 | 251 | 251 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXIII** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 419 | 419 | 262 | 262 | 221 | 221 | 310 | 310 | 470 | 470 | 252 | 252 | 270 | 270 | 266 | 266 |
| **XXXIV** | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 339 | 339 | 221 | 221 | 251 | 251 | 470 | 470 | 252 | 252 | 249 | 249 | 250 | 250 |
| **XXXV** | 2 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 437 | 443 | 262 | 262 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXVI** | 9 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 339 | 339 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXVII** | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 339 | 339 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXVIII** | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 425 | 425 | 339 | 355 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXIX** | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 437 | 437 | 262 | 262 | 191 | 191 | 310 | 310 | 405 | 405 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXX** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 431 | 431 | 339 | 339 | 221 | 221 | 310 | 310 | 470 | 470 | 252 | 252 | 249 | 249 | 266 | 266 |
| **XXXXI** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 431 | 437 | 262 | 262 | 221 | 221 | 316 | 316 | 470 | 470 | 252 | 252 | 249 | 249 | 270 | 270 |
| **XXXXII** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 431 | 431 | 262 | 262 | 221 | 221 | 316 | 316 | 470 | 470 | 252 | 252 | 249 | 249 | 268 | 268 |
| **XXXXIII** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 431 | 431 | 262 | 262 | 221 | 221 | 310 | 310 | 470 | 470 | 252 | 252 | 249 | 249 | 268 | 268 |
| **XXXXIV** | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 287 | 287 | 335 | 335 | 191 | 191 | 251 | 251 | 470 | 470 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXXV** | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 287 | 287 | 335 | 335 | 191 | 191 | 251 | 251 | 470 | 470 | 252 | 252 | 270 | 270 | 250 | 250 |
| **XXXXVI** | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 431 | 431 | 262 | 262 | 221 | 221 | 310 | 310 | 470 | 470 | 252 | 252 | 249 | 249 | 270 | 270 |

[a] Pathotypes defined as virulence formula; compatible=1 or incompatible=0 on the 14 on soybean differentials for *P.sojae. Rps* genes are presented in descendent order; *Rps* 1a,1b,1c,1d,1k,2,3a,3b,3c,4,5,6,7,and 8.

[b]Estimated band sizes in bp based on GeneMapper® Software.

[c] n/d not determined

Table 5. Estimate of differentiation (theta, $\Theta_{PT}$) among seven soybean field populations of *Phytophthora sojae* from Iowa (N=68 isolates recovered from plants, 2008-2010) based on eight microsatellite loci (not shaded) and standard pathotype characterization (shaded).

| Population | 1005 | 1011 | 1012 | 1019 | 2001 | 2010 | 2011 |
|---|---|---|---|---|---|---|---|
| 1005 | | 0.779** | 0.698** | 0.121 | 0.508** | 0.749** | 0.185* |
| 1011 | 0.783** | | 0.874** | 0.000[a] | 0.762** | 0.000[a] | 0.301* |
| 1012 | 0.781*** | 0.883** | | 0.893** | 0.849** | 0.862** | 0.425** |
| 1019 | 0.267** | 0.728** | 0.716** | | 0.733* | 0.000[a] | 0.396* |
| 2001 | 0.490** | 0.900** | 0.859** | 0.501** | | 0.733** | 0.246* |
| 2010 | 0.668** | 0.703* | 0.857** | 0.575** | 0.844** | | 0.226 |
| 2011 | 0.151* | 0.657* | 0.702** | 0.170* | 0.206* | 0.496* | |

Microsatellite $\Theta_{PT}$ values below diagonal, pathotype $\Theta_{PT}$ values are shaded above diagonal.

Pathotype characterization was done using a standard set of soybean differentials (9).

Significant at probability * P≤0.05 and **P=0.001, value based on 999 permutations.

[a] $\Theta_{PT}$ equals zero because there is no variation within one of the two populations being compared.

Table 6. Genotypic diversity statistics for populations of *Phytophthora sojae* recovered from seven commercial soybean fields in Iowa (N=68 isolates from 2008-2010)

| ***Field Population ID*** | ***1005*** | ***1011*** | ***1012*** | ***1019*** | ***2001*** | ***2010*** | ***2011*** | ***Total*** |
|---|---|---|---|---|---|---|---|---|
| No. isolate | 11 | 6 | 17 | 10 | 12 | 4 | 8 | 68 |
| No. genotypes | 4 | 2 | 4 | 5 | 3 | 4 | 4 | 24 |
| No. pathotypes | 2 | 1 | 2 | 1 | 3 | 1 | 5 | 11 |
| *Ho* [a] | 0 | 0 | 0.015 | 0.050 | 0.031 | 0.031 | 0 | 0.018 |
| *UHe* [b] | 0.221 | 0.068 | 0.090 | 0.268 | 0.083 | 0.246 | 0.354 | 0.190 |
| *G* [c] | 2.3 | 2.0 | 2.2 | 2.6 | 1.9 | 4 | 3 | 3.6 |
| $I_A$ [d] | 1.93 | - [f] | 0.73 | 2.11 | 0.93 | 0.08 | 2.03 | 1.35 |
| *P* [e] | 0.001 | - | 0.003 | 0.001 | 0.027 | 0.410 | 0.001 | 0.001 |

[a] Observed heterozygosity.

[b] Unbiased expected heterozygosity; $UHe = (2n/2n-1)(1-\sum p^2_i)$ where $p$ is the frequency of the $i^{th}$ allele.

[c] Stoddart and Taylor's genotypic diveristy index (*G)* with rarefaction; minimum value of 1.0 (one genotype in the population) and a maximum value of 4.0 (all individuals with different genotypes).

[d] Index of association = $V_o/(V_e-1)$, where $V_o$ is observed and $V_e$ the expected variance of K , and K is equal to the no. of loci at which 2 individuals differ.

[e] P robability of $I_A$ based on 1000 randomizations; null hypothesis = $I_A$ does not differ from purely sexually outcrossing population.

[f] $I_A$ cannot be calculated when K= no. of loci at which two individual differ is 1.

Table 7. Heterozygote loci (shaded) among 3 isolates recovered from plant no. 11 in field 1019.

| Isolate | Locus | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PS01 | PS05 | PS10 | PS12 | PS16 | PS24 | PS29 | PS33 |
| 1 | 281/281 | 262/262 | 147/221 | 251/251 | 405/405 | 236/252 | 270/270 | 250/250 |
| 2 | 425/425 | 262/262 | 221/221 | 251/251 | 405/405 | 252/252 | 270/270 | 264/266 |
| 3 | 425/425 | 262/262 | 221/221 | 251/251 | 405/405 | 236/236 | 270/270 | 264/266 |

Figure Legend

Fig 1. Percent of isolates of *Phytophthora sojae* (N=93) that were compatible on known resistant genes (*Rps)* to the pathogen in soybean .

Fig 2. Dendrogram of 97 *Phytophthora sojae* isolates (93 isolates from Iowa and 4 standards from other labs) generated by UPGMA (unweighted pair group method) based on allele frequency of 8 microsatellite loci. Isolates are designated by codes (Table 1), 4 isolates that are highlighted with rectangle are standards from other labs. Bar indicated Nei's genetic distance.

Fig 3. Number of genotypes and pathotypes of *Phytophthora sojae* (N=80 isolates) recovered from 11 commercial soybean fields in Iowa, 2008-2010.

Fig. 4. Dendrogram generated by UPGMA (unweighted pair group method) of seven populations of *Phytophthora sojae* (N=68 isolates from 2008-2010) based on allele frequency of eight microsatellite loci. Bootstrap values are shown along the branches. Populations are designated by numbers. Bar indicates Nei's genetic distance.

Fig. 5. Diagram of genotype and pathotype network showing relationships among populations of *P. sojae* from seven commercial soybean fields in Iowa (N=68 isolates from 2008-2010). Each circle represents a) microsatellite genotype and b) pathotype. The area in the circle is proportional to the relative frequency of the microsatellite genotype or pathotype among the 68 isolates of the pathogen sampled. Each field is represented by a different color.

Fig. 6. Diagram of genotype and pathotype network showing relationship among multiple isolates of *P. sojae* (N= 23 isolates from 2008-2010) recovered from single soybean plants. Each circle represents a) microsatellite genotype and b) pathotype. The area in the circle is proportional to the relative frequency of the microsatellite genotype and pathotype of the isolates recovered from a plant. Different plants are represented by shades of the same color. Colors denote the field (in legend) from which the plant was recovered.

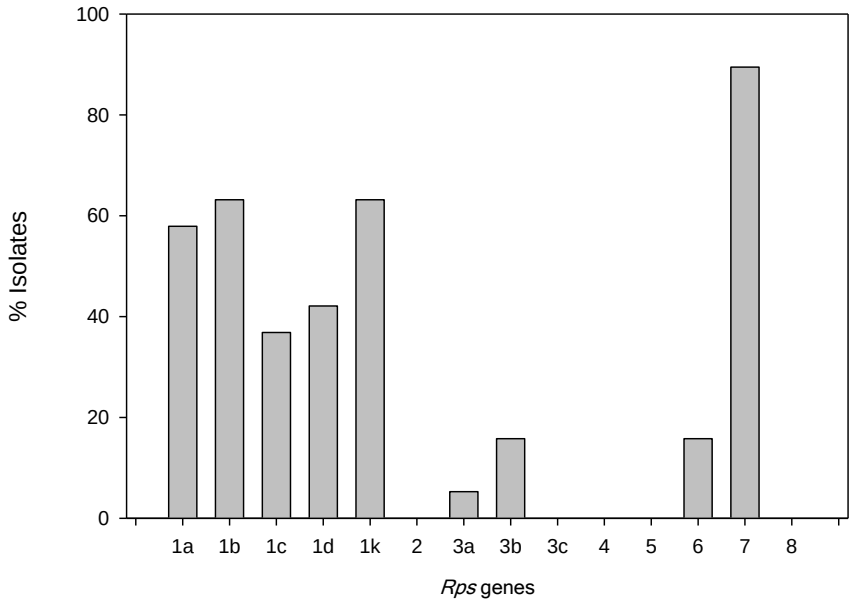
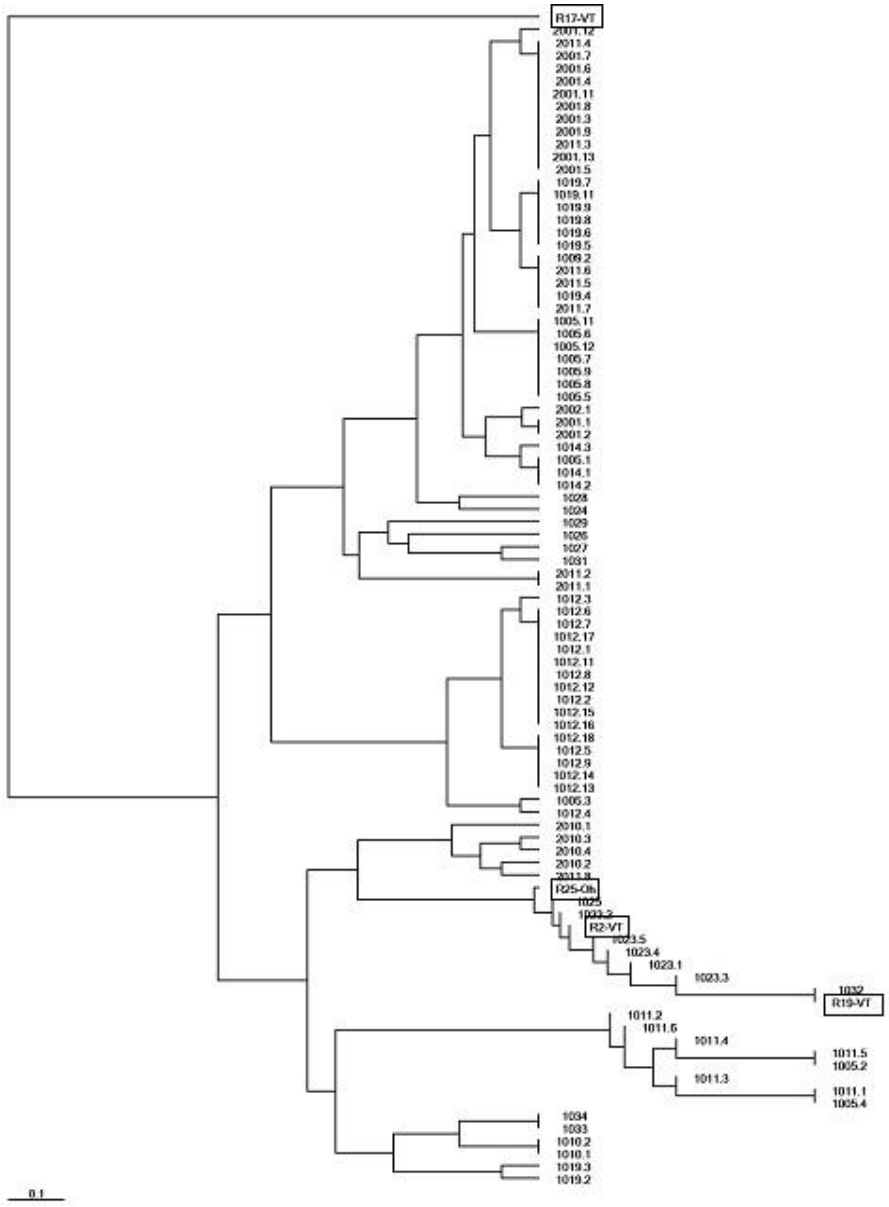

100
80
60
40
20
0
% Isolates
1a
1b
1c
1d
1k
2
3a
3b
3c
4
5
6
7
8
Rps genes

Fig.1


R17-VT
2001.12
2011.4
2001.7
2001.6
2001.4
2001.11
2001.8
2001.3
2001.9
2011.3
2001.13
2001.5
1019.7
1019.11
1019.9
1019.8
1019.6
1019.5
1006.2
2011.6
2011.5
1019.4
2011.7
1005.11
1005.6
1005.12
1005.7
1005.9
1005.8
1005.5
2002.1
2001.1
2001.2
1014.3
1005.1
1014.1
1014.2
1028
1024
1029
1026
1027
1031
2011.2
2011.1
1012.3
1012.6
1012.7
1012.17
1012.1
1012.11
1012.8
1012.12
1012.2
1012.15
1012.16
1012.18
1012.5
1012.9
1012.14
1012.13
1005.3
1012.4
2010.1
2010.3
2010.4
2010.2
R25-Ob
1025
1033.2
R2-VT
1023.5
1023.4
1023.1
1023.3
1032
R19-VT
1011.2
1011.6
1011.4
1011.5
1005.2
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1033
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1010.1
1019.3
1019.2
0.1

Fig. 2

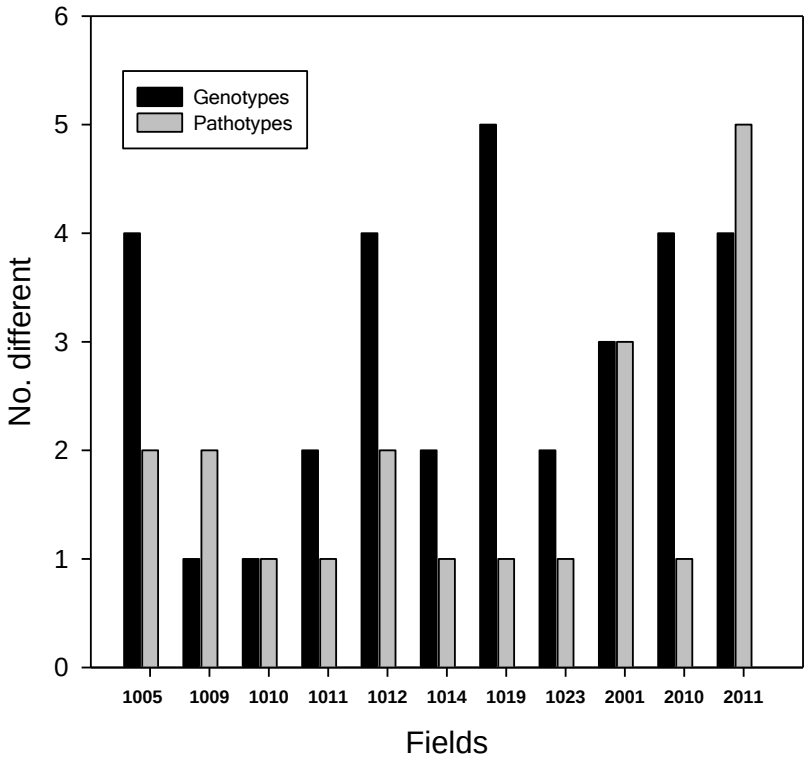

Genotypes
Pathotypes
No. different
0
1
2
3
4
5
6
1005
1009
1010
1011
1012
1014
1019
1023
2001
2010
2011
Fields

Fig. 3

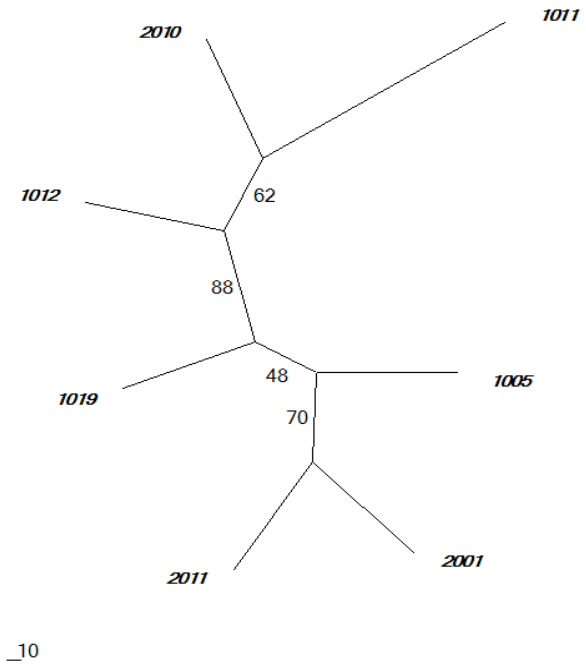
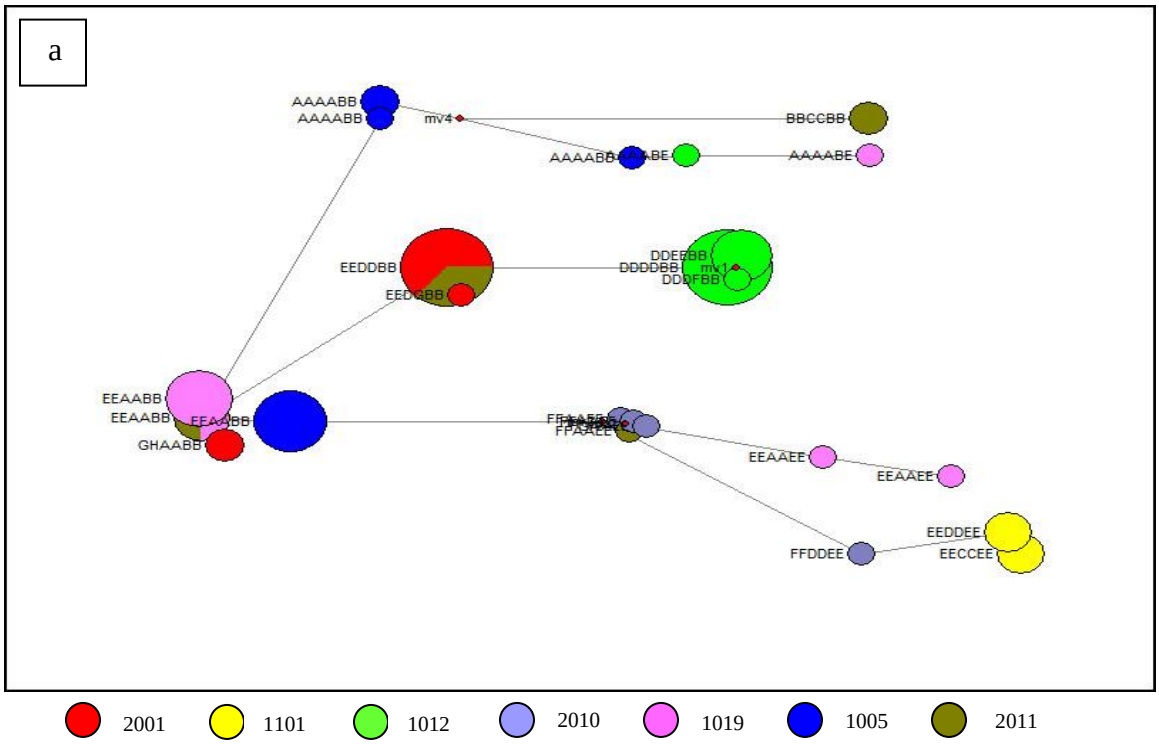



Fig. 4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AAAABB
mv4
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EEAABB
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2001
1101
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2010
1019
1005
2011

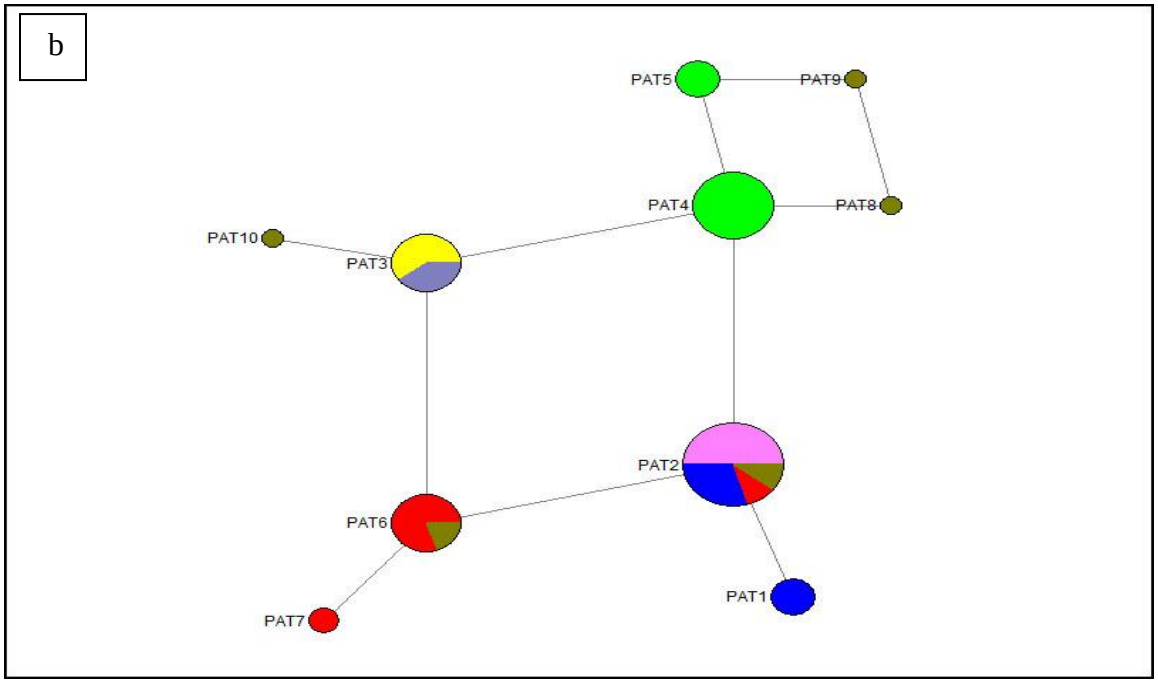
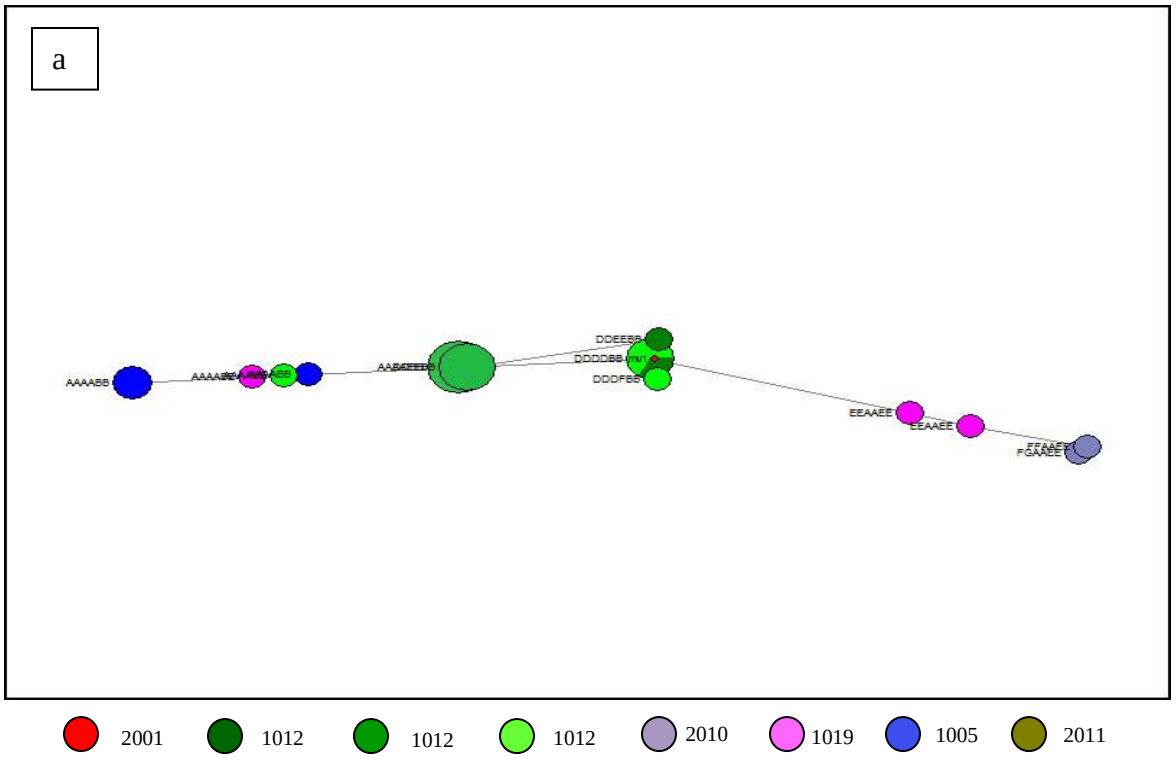

b
PAT5
PAT9
PAT4
PAT8
PAT10
PAT3
PAT2
PAT6
PAT1
PAT7

Fig. 5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2001
1012
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2010
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1005
2011

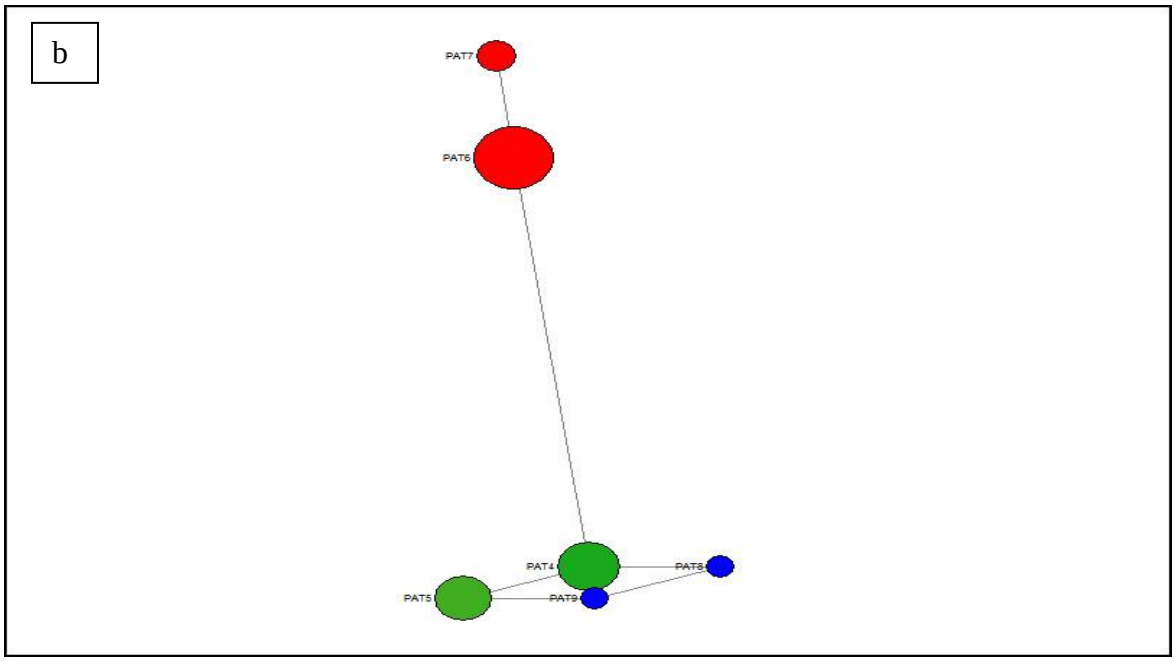
b
PAT7
PAT6
PAT4
PAT8
PAT5
PAT9

Fig. 6

# CHAPTER 3. PATHOTYPE AND GENETIC SHIFTS IN *Phytophthora sojae* UNDER SOYBEAN CULTIVAR ROTATION

A paper to be submitted to *Plant Disease*

S. Stewart, and A. E. Robertson

Department Plant Pathology, Iowa State University, Ames, IA 50011

Accepted for publication

Corresponding author: A.E. Robertson, alisonr@iastate.edu

## ABSTRACT

Stewart, S., and Robertson, A. E. 2011. Monitoring pathotype and genetic shifts in *Phytophthora sojae* with soybean cultivar rotation. Plant Dis.

Changes in pathotype structure of *Phytophthora sojae* populations have been attributed to deployment of specific resistant *Rps* genes. The pathogen quickly evolves and escapes detection by an *Rps* gene, affecting durability of *P. sojae*-resistant cultivars. To prolong the life of a resistance gene in the field, cultivars with major resistance genes could be rotated through time and space to generate disruptive selection in the pathogen population. The population structure of 121 isolates of *P. sojae* recovered from soil samples collected twice per growing season from a four-year six-cultivar-rotation experiment were assessed using eight microsatellite markers (SSRs) and pathotyping. The *P. sojae*-free site was inoculated with isolate PR1, race 1, in year one. A total of 14 pathotypes and 21 multilocus genotypes (MLG) were recovered over the four year experiment. Of the 121 isolates, only 49 % and 22 % had the same pathotype and MLG, respectively, as PR1. Ten new alleles were detected throughout the four year period, 31 % of the isolates had at least one new allele. We recovered new pathotypes and MGLs from rotation treatments at the second sampling date,

indicating *P. sojae* has the ability to evolve quickly. We found cultivar rotation affected the genetic structure of the *P. sojae* population, but pathotype shift was not a function of cultivar rotation.

Additional keywords: microsatellite, SSR, Phytophthora root and stem rot, soybean

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*Phytophthora sojae* is an oomycete that causes Phytophthora stem and root rot (PRR) on soybeans. The disease has been managed primarily through the use of resistant cultivars with single resistance genes (*Rps*). Fifteen known resistant genes (*Rps*) in soybean confer resistance to PRR (15, 22, 55), and some such as *Rps* 1a, *Rps* 1c and *Rps* 1k, have been incorporated into commercial soybean cultivars (24). Like many other host-pathogen systems which are governed by a gene-for-gene system (13, 21), *P. sojae* has adapted to specific *Rps* genes and consequently, the effectiveness of these genes has been lost progressively as new races/pathotypes of the pathogen have appeared. In the 1980s, *Rps*1a and *Rps*1c were the most common *Rps* genes incorporated into commercial soybean varieties in the North Central Regions to protect soybean from losses caused by PRR. When races of the pathogen compatible on these genes became prevalent (24), *Rps*1k became the most common resistant gene used in soybean varieties, but by the late 1990s there were reports of races of *P. sojae* causing disease on *Rps*1k. In Iowa, *Rps*1k is currently the most common resistance gene used, followed by *Rps*1c, but the increasing prevalence of race 25 (virulence formula 1a, 1b, 1c, 1k,7) (42) has prompted incorporation of *Rps*6 into germplasm for Iowa (45). Durability of *Rps* effectiveness in the field has been estimated at 8-20 years (8, 13, 24).

Variability in *P. sojae* has been assessed traditionally through a virulence test using a soybean differential set. Seven to 14 soybean lines, each with one resistance gene (*Rps*) to *P. sojae* and a universal susceptible are used to characterize *P. sojae* races or pathotypes (11, 21). More than 55 described races of *P. sojae* have been identified on the bases of compatible (susceptible) and incompatible (resistant) reactions on these differential lines (11, 12). Originally, a race number was given to a pathotype of *P. sojae* with a specific virulence formula, however, as new virulence gene combinations or pathotypes of the pathogen continued to emerge (1, 4, 5, 13, 19, 31-34, 36, 37, 40, 41, 46, 48, 57, 62-64) this race classification system was discouraged (64). Potentially, there are 32,768 ($2^N$ where N = 15 *Rps* genes) possible virulence combination or races, leading researchers to now describe pathotypes rather than continue naming new races. Since 1948, when the first *P. sojae* race was identified, the number of pathotypes has increased dramatically, there are now more than 200 known pathotypes of this pathogen (12). Complexity of *P. sojae* pathotypes also seems to have increased with time (13). For example, almost 20% of the isolates surveyed in Michigan in the mid-nineties had virulence to 10 or more *Rps* genes (32). These continuous changes in virulence of the pathogen are called pathotype (or race) shifts.

Another strategy that has been used in breeding programs to manage PRR is partial resistance. Partial resistance (PR) or tolerance is defined as 'the relative ability of the soybean plant to survive root infection, either natural or artificial, without showing severe symptom development such as death, stunting, or yield loss'(59). Partial resistance does not exhibit the boom-and-bust cycle characteristic of major resistance genes (38). It is polygenic, equally effective across all pathotypes (38) and limits the lesion growth rate of the pathogen within the host tissue consequently allowing the host to tolerate the infection better when

compared to a fully susceptible host (12, 20, 56). Thus, partial resistance should be more stable than single-gene resistance because of the lack of selection pressure imposed on the pathogen (50). Since PR is harder to breed for and introduce into desired germplasm, this strategy has not been as widely used as single gene resistance to reduce loses to PRR.

Changes in the pathotype structure of the *P. sojae* populations have been attributed to deployment of specific resistant genes (31, 46, 48, 63), which result in selection of a resistant pathotype. An alternative way to prolong the life of a resistance gene in the field is to generate disruptive selection by rotating major resistant genes through time and space (38) or, by rotating cultivars with single gene resistance with cultivars with high partial resistance, thus reducing selection pressure on the pathogen and extending the durability of a resistance gene. This strategy disrupts selection by favoring different pathoypes at different times and places, and therefore reducing the rate of which one particular pathotype increases its frequency within a population. In the tobacco (*Nicotianae tabacum*)- *Phytophthora nicotianae* pathosystem, rotation of single gene resistance and cultivars with high level of partial resistance resulted in a reduction in black shank disease incidence and also minimized race shifts of the pathogen (52).

The goal of this research was to determine if cultivar rotation could be used to effectively minimize pathotype shifts in a population of *P. sojae*, and thus prolong the life of a resistant soybean cultivar in the field. We monitored pathotype and genetic shifts in an inoculated population of *P. sojae* in a four year soybean-cultivar rotation experiment. We used conventional pathotype characterization (11) to detect shifts in pathotypes, and microsatellite marker (SSRs) analysis to monitor genotypic changes in the population.

## MATERIALS AND METHODS

**Site selection.** In 2007, 24 microplots (75 cm diameter) were established on a field site with no known history of soybean cultivation at the Iowa State University (ISU) Northern Research and Demonstration Farm, near Kanawha, Iowa. Soil samples from each microplot were collected when the microplots were established and were assayed three times to test for *P. sojae* using seedling baiting method (47). Furthermore, the presence or absence of *P. sojae* in each soil sample was also tested for using a *P. sojae*-primer specific (PSOJF1 and PSOJR1) PCR-based detection method (27) with DNA extracted from the soil with FastDNA spin kit for soil (MP Biomedicals, Carlsbad, Ca). DNA of *Phytophthora sojae*, *Pythium* sp. and *P. cactorum* were used as controls.

**Field experiment.** A soybean-cultivar rotation study was planted in the established microplots. The experiment consisted of six treatments arranged in a complete randomized block design with four repetitions, in a four year rotation (Table 1). Treatments included cultivars with different sources of resistance to *P. sojae*: susceptible cv. Sloan with no *rps* genes and low partial resistance (14), cv. Stine 2402 with no *rps* genes and moderate to high partial resistance (14), cv. 2834RR with *Rps*1k and low partial resistance (14), cv. Williams 79 with *Rps* 1c (11) and cv. L83-570 with *Rps* 3a (11). Sowing dates for the four year period varied from June 3$^{th}$ to June 17$^{th}$.

Each microplot was artificially inoculated in year 1 (2007) of the study with *P. sojae* PR1, which is virulent on *Rps* 7 (race 1) and had been grown for 10 days on sterile sorghum grain. Fifty cubic centimeters of PR1-infested sorghum was sprinkled directly on top of the sown soybean seeds (25 seeds per microplot). An additional 200 cm$^3$ of the inoculum was broadcast over the top of each microplot once the seeds had been covered with soil. In the

second year of the study (2008), each microplot was inoculated a second time with a slurry of 10 plates of PR1 grown on diluted V8 which had been blended with 1.0 L of sterile distilled water. At sowing, 50 $cc^3$ of the slurry was poured over the top of the sown seeds. In each year, after seedling emergence, microplots were thinned to 20 plants, and flooded 4 to 8 times throughout the season, depending on the year, to provide favorable soil conditions for infection of the soybean plants by *P. sojae*. In order to monitor pathotype and genetic changes in the PR1, soil samples (80 to 100 g) were collected from each microplot 2 to 3 weeks after planting and immediately prior to harvest. Soil samples were assayed to isolate *P. sojae* using seedling baiting method (47).

**Seedling baiting method.** Fifty to 60 $cm^3$ of soil from each plot at each sampling date were placed in a polystyrene 12 oz Dart® cup in a growth chamber adjusted to a 16 hour day and 8 hour night at 25° C. Six seeds of the susceptible soybean cv. Sloan were planted in each cup to bait *P. sojae* from the soil sample. Three to four days after planting, the pots were flooded for 24 hours. Thereafter, every three days, pots were watered with tap water by flooding the pot and letting the water drain from the bottom (8). Each soil sample was baited three times. When symptoms of PRR were evident on seedlings (brown hypocotyl lesion and/or collapsed hypocotyls), the plants were removed from soil, washed with running tap water, dried on sterile paper towel and placed under PBNIC agar in a Petri dish. Isolates of *P. sojae* were confirmed by their growth pattern (16) and morphology of sporangia (47).

**Purification and storage of isolates.** Purification of *P. sojae* isolates was done through the production of zoospores using Chen-Zentmeyers' salt wash solution technique (47). Briefly, mycelium from the perimeter of newly grown cultures were scraped with a sterile scalpel and transferred to a 60x15 mm plastic Petri plate containing 15 ml Lima bean

broth (LBB) (50 g frozen Lima beans in 1000 ml distilled water). After 48 to 72 hours at room temperature and 12 hour light cycle, the LBB was poured off and replaced by Chen-Zentmeyers' salt wash solution (0.01 M $Ca(NO_3)_2.4H_2O$, 0.004 M $MgSO_4.7H_2O$, 0.005M $KNO_3$, 0.02 mg/l $FeSO_4.7H_2O$, pH 7.0). Fifteen minute later, this salt solution was replaced with new salt solution. A third wash was done using sterile water. Five to 12 hours later, sporangia and zoospores were formed (47). Mono-zoosporic isolates were obtained by plating 200 µl of the suspension onto water agar plates and transferring single germinated zoospores under a stereo microscope after 24 hours. Purified isolates of *P. sojae* were stored in sterile distilled water at room temperature until further use.

**Pathotype characterization.** Pathotypes of *P .sojae* were determined with the hypocotyl inoculation technique using a 15 cultivar differential set (8, 11, 47). The following standard soybean lines or varieties, each having a specific resistant *Rps* gene were grown in trays in the greenhouse: L88-8470 (*Rps*1a), L77-1863 (*Rps*1b*)*, Williams 79 (*Rps*1c), L99-3312 or PI 103091 (*Rps*1d), Williams 82 (*Rps*1k), L82-1449 (*Rps*2*)*, L83-570 (*Rps*3a), L91-8347 (*Rps*3b), L92-7857 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Altona or L89-1581 (*Rps*6), L93-3258 or Harosoy (*Rps*7), PI 399073 (*Rps*8) and Williams (susceptible). Ten one-week old seedlings of each variety were inoculated by making a slit with a syringe in the hypocotyl and placing approximately 0.2 to 0.4 ml of a slurry 7-10 day old culture into the slit (16, 47). A plastic covering was placed over tray for 12-16 hours to keep the inoculum from drying. Seven to 10 days after inoculation, PRR incidence was evaluated. Susceptible plants died or developed distinct symptoms of PRR, while resistant plants developed a hypersensitive reaction (slight necrotic lesion around the wound). The soybean differential

was considered susceptible when at least seven out of 10 seedlings died. The test was repeated at least twice for each isolate.

**DNA extraction.** Eight to ten square pieces (5 $mm^2$) of *P. sojae* grown on diluted V8 agar were transferred to 250 ml flasks containing 50 ml of V8 broth (40 ml of V8 juice, 0.6 g CaCO3, 0.2 g Bacto yeast extract, 1 g sucrose, 0.01 g cholesterol in 1000 ml of distilled water). Flasks were placed on shaker at 100-120 rpm at room temperature for 4-7 days. Mycelium was harvested by vacuum aspiration through no. 1 filter paper, frozen with liquid nitrogen and ground using a sterile mortar and pestle. DNA was extracted using a modification of the cetyltrimethylammonium bromide (CTAB) procedure (10, 49). Briefly, the dried powder mycelium was placed in a plastic 50 ml centrifuge tube with 10 ml of extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.0064 M ethylenediaminetetraacetic acid (EDTA), pH 7.5, 0.017 M sodium bisulphate) and vortexed. Ten ml of lysis buffer (0.2 M Tris, 0.064 M EDTA, pH 7.5, 2.0 M NaCl, 2% CTAB and 60 µl of 5% sarkosyl (5 g N-lauryl sarcosine per 100 ml H2O)) was added to the tubes and vortexed, then the tubes were incubated at -80°C for 15 min, followed by 65°C for 15 min. This step was done twice, but the last incubation at 65°C lasted 45 min. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube, mixed gently and then centrifuged for 10 min at 2000 rpm. The aqueous phase was transferred to a new tube and then one volume of chloroform:isoamyl alcohol (24:1) was added, followed by centrifugation and transfer to a new tube. DNA was precipitated by the addition of one volume of cold isopropanol and incubation at -20°C overnight. After centrifugation, the supernatant was discarded, the pellets dried at room temperature, and then resuspended in 200 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Two µl of pancreatic RNase A (0.01 µg/µl) was added to the DNA solution, which was

incubated at 37°C for an hour, then the solution was transferred to a 1.5 ml tube and the alcohol washes were repeated. After the isopropanol was poured off, the pellets were washed in buffer (76% Ethanol, 0.8 M NaOAc, pH 7.0) and rinsed (76% Ethanol, 0.026 M NH4OAc). The DNA was then resuspended in TE and its concentration measured using a Nanodrop™ ND-1000 Spectrophotometer. DNA was extracted from all isolates as soon as they were purified. Concentrations were adjusted to 100 ng/µl and stored a -20 ºC for SSR analysis.

**Molecular genotypes.** Eight microsatellite primer pairs; PS01, PS05, PS10, PS12, PS24, PS29 and PS33 (9) were used to screen 121 isolates. Forward fluorescent primers for Ps01, Ps16, Ps24, and Ps 33 were labeled with 6-FAM dye, and Ps05, Ps10, PS12, and Ps 29 with HEX dye. Amplification was performed in a 96-well Eppendorf Mastercycler® thermal cycler (Hamburg, Germany) in a 15.0 µl volume with 200 µM dNTP mixture, 2.5 mM $MgCl_2$, 1X Go Taq® Hot Start Colorless Master Mix buffer, 0.08 units Go Taq® Hot Start DNA polymerase (Promega Inc., Madison, WI), 0.45 µM of each primer, and 1µl of DNA template. The thermal cycler was programmed for an initial step at 85 ºC for 2 min, denaturalization step at 94 ºC for 95 s, then 24 cycles at 52 ºC for 1 min, 72 ºC for 72 s, 94 ºC for 30 s, then 52 ºC for 1 min and 72 ºC for 30 min. A 96-capillary Applied Biosystem 3730 Genetic Analyzer was set up to run samples labeled with these dyes and a GeneScan™ 500 ROX ™ size standard (Applied Biosystem, Foster City, CA) was used. GeneMapper® Software 4.0 (Applied Biosystem, Foster City, CA) was used to size the alleles to the nearest base pair.

**Data analyses.** The number of SSR alleles per locus, observed heterozygosity (*Ho*), unbiased expected heterozygosity analogous to gene diversity was calculated on a single

locus basis (*UHe*), and Hardy-Weinberg equilibrium (HWE) for each locus were calculated using population genetic software GenAlEx 6 (44).

Index of association ($I_A$) was calculated using the genetic data for each treatment using MultiLocus V1.3b (2). Genotypic diversity was estimated for each treatment using Stoddart and Taylor's *G* index (51), however to be able to compare indexes using population of different sizes, *G* was scaled by the expected number of genotypes for the smallest population size (25) with rarefaction curves using Analytical Rarefaction version 1.3 (29).

Genetic and pathotype divergence using genotypic and binary distance among 6 treatments was assessed using $\Theta_{st}$ (60). Analysis of molecular variance (AMOVA) (17) was performed among the six treatments using software GenAlEx 6 (44). Significant levels were evaluated using 999 permutations. For the purpose of the pathotype AMOVA analysis, the interaction of *P. sojae* with the respective *Rps* gene of each soybean differential was considered a locus (avirulence locus) with two possible interaction outcomes: compatible (1) or incompatible (0).

A dendrogram comparing the relatedness among the six cultivar rotation treatments was created based on *P. sojae* allele frequencies and Neis's genetic distance, using UPGMA (unweighted pair group method) with bootstrap values calculated on 100 UPGMA trees using SEQBOOT, GENDIST, NEIGHBOR and CONSENCE in PHYLIP version 3.69 (18).

Correlation of individual by individual genetic distance matrices, for the overall isolates in the experiment, was calculated using co-dominant (genetic) and binary (pathotype) options in software GenAlEx 6 (44). Mantel test for matrix correspondence was used to test the statistical relationship between the two distances using the same software.

## RESULTS

No *P. sojae* was recovered by baiting three times the soil samples collected from each microplot immediately prior to the setup of the experiment. Amplification of a 130 bp band using the specific primers for *P. sojae* (PSOJF1 and PSOJR1) (27) occurred in soil samples from three of the 24 microplots, and the *P. cactorum* control. Amplification of a similar band in *P.sojae* and *P. cactorum* is demonstrating that the primers used were not *P. sojae*-specific. Additional baiting test were done on the three soil samples that tested positive by PCR, but we were never able to isolate *P. sojae*. No visible disease symptoms were observed throughout the four year study.

**Pathotypes.** A total of 121 isolates of *P. sojae*, belonging to 14 pathotypes, were recovered from soil samples collected from the microplots from 2007 through 2010 (Table 2). Forty nine percent of the isolates belonged to the original inoculated pathotype (race 1; vir 7) (Table 2), while the remaining isolates (51 %) were virulent on at least one additional *Rps* gene, with a maximum of 4 additional virulence genes in an isolate (Fig. 1). Of the 62 non-race 1 isolates, 55 grouped into seven pathotypes while six isolates had unique pathotypes. New pathotypes were recovered as early as the second sampling date (harvest of year 1) (Fig. 2).

Of the 121 isolates recovered, 120 were virulent on *Rps* 7, thus 99 % maintained the virulence of the original inoculated isolate (vir 7). More than 20 % were virulent on *Rps* 1a and 1c. No isolates compatible on *Rps* 1b, 3a, 3c, 4, 5, 6 and 8 were detected over the course of the study (Fig. 3).

The number of different pathotype recovered from the six treatments ranged from two to eight, and was lowest in treatments 4 and 6, and highest in treatments 3 and 5 (Fig. 1).

Only 1 and 2 additional non-race 1 pathotypes were recovered from treatment 4 and 6, respectively. Moreover, recovery of *P. sojae* as a measure of inoculum density was lowest in treatment 4 and 6 over the course of the experiment (Fig. 1).

AMOVA's estimated variance for pathotype divergence among treatments yielded 5%, and $\Theta_{PT}$ was low and non-significant ($\Theta_{PT}$=0.047, $P$=0.059). No statistical difference was detected among treatments since 95 % of the estimated variance was due to within treatment variations.

**SSR genotypes**. For each microsatellite marker, an eletropherogram peak with a different base pair size was considered an additional allele for that locus. Each of the eight SSRs amplified one or two alleles per locus per isolate. Six out of eight SSRs were polymorphic, with one to three alleles observed per locus in the population. A total of 19 alleles were identified within the 121 isolates, at an average of 1.8 alleles/loci (Table 3). Ten new alleles (different from the alleles in PR1) were detected throughout the four years (Table 3), and 31 % of the total isolates recovered had at least one new allele. A total of 21 multilocus genotypes (MLG) were identified within the 121 isolates from the experiment (Table 4), and 10 of these MLGs were unique genotypes. The remaining 11 MLGs groups included 2 to 38 isolates. Only 22% of the isolates recovered over the 4 year period had the same MLG as the original PR1 inoculated isolate, while 38% had at least one new allele.

The overall observed heterozygosis was 0.037 in the 121 isolates recovered from the soil throughout the duration of the experiment, but it ranged from 0.038 to 0.069 among treatments (Table 4). Heterozygote deficiencies were observed within all treatments, and the mean unbiased expected heterozygosis ($UH_e$) was almost seven-fold that of the observed ($H_o$) (Table 4). The *UHe,* analogous to gene diversity, was highest in treatments 4 and 5

(Table 4) meaning that two copies of a gene chosen at random in these treatments will have a higher probability of having different alleles than the rest of the treatments (39). Fixation index (F) across treatments was 0.86, and ranged from 0.80 to 0.92 among treatments. Moreover, all polymorphic loci deviated significantly from Hardy-Weinberg equilibrium when pooled across loci.

The mean index of association for the six treatments combined was moderate and significant ($I_A$=2.46, P<0.001) indicating that non-random mating or asexual reproduction had occurred, ranging from 0.97 to 3.32 among the six treatments (Table 4).

Genotypic diversity (*G*) was estimated with rarefaction to be able to compare treatments with different sample sizes. The number of isolates of *P. sojae* recovered was least in treatments 4 and 6 (Fig. 4). The *G* across the six treatments was high (*G*=4.6), considering that the maximum value is 7.0 (all MLGs differ from each other). For the individual treatments, however, *G* ranged from 3.7 to 4.7 (Fig. 4). Treatments 1 and 6 had the lowest *G* (3.7) and the least number of MLGs (four) detected (Fig. 4). The maximum number of MLGs per treatment was ten, which was observed in treatment 5 (Fig. 4).

The estimated variance for genotype divergence among treatments as calculated by AMOVA was 8 %, and $\Theta_{PT}$ was low but significant ($\Theta_{PT}$=0.075, *P*=0.044). Thus statistical differences were detected among treatments, even though 92 % of the estimated variance was due to within treatment variations. Only four out of 15 pairwise $\Theta_{PT}$ values among treatments were significant (Table 5). In fact, isolates of *P. sojae* recovered from soil that has been in a rotation alternating a susceptible soybean cultivar with a resistant one (Trt. 2) were genetically different from those that were recovered from soil that had been under a rotation with soybean cultivars with different resistant genes (Trt. 4) and under continuous partial

resistance (Trt 5). Furthermore, isolates of *P. sojae* recovered from the rotation of a soybean cultivar with the *Rps*1k gene and partial resistance (Trt 3) were genetically different from those isolates of *P. sojae* that were recovered from soil samples collected from both the continuous rotation of a soybean cultivar with partial resistance (Trt. 5) and the rotation that alternated single resistant genes (Trt. 4).

The UPGMA analysis divided treatments into three subdivisions supported by two high bootstrap values. Treatments 2, 3 and 6 grouped together, treatments 4 and 5 grouped together and treatment 1 was a group by itself. Relatedness among treatment 4 and 5 was very high, meaning that the genetic distance based on the allele frequency between isolates recovered from rotating soybeans with different resistant genes and continuous partial resistance was similar (Fig. 5).

The correlation coefficient between genetic distance matrix (genotypes) and binary distance matrix (pathotype) was low ($r = 0.232$) but significant ($P=0.001$), indicating a relationship between the genotypes assessed with the eight SSRs and pathotypes characterized among the 121 isolates recovered from the soil throughout the duration of the experiment.

**Segregation ratios for locus PS01.** The mean observed heterozygosis for the individual loci ranged from 0.000 to 0.267, and was highest for SSR PS01 (Table 6). This particular SSR resulted in an unusually high *Ho* (0.267) and low *F* (0.33) values for a selfing organism such as *P. sojae* which warranted further examination. Under complete selfing the *Ho* should be near 0 and *F* should be near 1 (23).

The PS01 locus was found to be heterozygous ‘EF’ in isolate PR1, which was used to inoculate the experiment. While the expected $F_2$ monogenic Mendelian segregation ratio is

1:2:1 for a diploid organism, the observed segregation for locus PS01 differed significantly ($\chi^2_{1:2:1}$= 52.6, *P*= 0.0000) in the population as a whole from the expected ratio (Fig. 6). Moreover, high and significant chi-square tests were also found among *P. sojae* populations within each year (data not showed). Segregation for PS01 differed significantly from the predicted ratio with an excess of 'EE' genotype and a deficiency of heterozygote 'EF' genotypes. The proportion of heterozygote isolates for PS01 locus recovered during the four year period was 14, 21, 26, and 28%, respectively from year 1 to 4. Interestingly, in the third year of the soybean-cultivar rotation, a third allele 'D' was determined for PS01 locus in three *P. sojae* isolates recovered from treatment 3 and 5; two isolates having an 'ED' genotype and one with a 'DD' genotype (Fig. 7). This allele has 49 6-bp repeats, while 'E' and 'F' have 50 and 51 6-bp repeats, respectively. Thus, the new allele had one or two 6-bp repeats less than the original 'EF' PR1 inoculated isolate. The corresponding pathotypes for the two heterozygotes isolates 'ED' was either virulent on *Rps* 7 (race1) or virulent on *Rps* 3b, 7 while the homozygote 'DD' was virulent on *Rps* 7.

**New alleles detected**. A total of ten new alleles were detected throughout the 4 years period, among six of the eight SSRs. Five new alleles were detected as early as the second year (Table 7). These five new alleles were all detected on isolate recovered from treatment 5, except for allele size 270 bp from SSR PS29 which was also detected on isolates recovered from treatment 2 on the second year (Table 7). The highest number of new alleles, as well as the highest number of isolates possessing new alleles were found in treatments 5 and 3 (Table 7).

## DISCUSSION

To our knowledge, this is the first study to examine the effect of deploying *Rps* genes and partial resistance on the population structure of *P. sojae.* It has long been suggested that resistance genes in the host exert selection pressure on pathogen populations which result in selection for virulent pathotypes in the pathogen (31, 46, 48, 63). In this study, we were able to show that the pathotypic and genotypic diversity of a population of *P. sojae* did change when rotations of different *Rps* genes and partial resistance were used, but the changes in the population were not necessarily a function of the type of resistance being deployed.

Although soil samples collected from three microplots prior to the start of the experiment were positive for *P. sojae* using PCR with *P. sojae*-specific primers, these primers also amplified a similar size band in the *P. cactorum* control, demonstrating that they are not *P. sojae*-specific. Additional seedling baiting tests were done on the three soil samples that had tested positive by PCR, but we were never able to isolate *P. sojae*. It is also possible, however, that the positive PCR were indeed *P. sojae*, but the pathogen was not viable.

**Pathotypes.** Although we detected variation in *P. sojae* pathotype profile, significant differences were not observed between treatments ($\Theta_{PT}$=0.047, $P$=0.059) but rather within treatments. In previous reports on the tobacco-black shank pathogen, *P. nicotianae* system (52), rotation between cultivars with single gene resistance and cultivars with high level of partial resistance provided not only significant reductions in disease incidence and inoculum density but also minimized race shifts in the pathogen. In our study on the soybean-*P. sojae* pathosystem, the effect of single gene resistance on race shifts in the pathogen was not as simple or delineated. However, there were some similarities detected between the two

pathosystems. In the tobacco-black shank pathosystem, low incidence of disease was observed and low inoculum density of *P. nicotianae* detected when continuous single gene resistance was deployed, and the population shifted from 100% race 0 at the beginning of the experiment to 76% race 1 at the end of the 4 year trial (52). In our study, we also recovered fewer isolates of *P. sojae* from the microplots in which continuous single gene resistance was deployed (Treatment 4 and 6), but we also detected the least race shift (Fig. 1). No race shift was observed when *P. parasitica var. nicotianae* was exposed to partial resistance deployed continuously (54), however, in our study, seven new pathotypes were observed when *P. sojae* was exposed to a continuous rotation of a cultivar with partial resistance. Thus, unlike Sullivan et al (2005 a), we were unable to demonstrate that pathotype shift in *P. sojae* is a function of cultivar rotation. This is probably not surprising since the soybean-*P. sojae* pathosystem is far more complex and diverse than tobacco-*P. nicotianae* pathosystem, with more than 200 known pathotypes in *P. sojae* (13) and 15 resistant genes identified in soybean (15, 22, 55) compared to four described races of *P. nicotianae* and two single resistant genes identified in tobacco (43, 53).

**SSR genotypes.** We detected genetic differentiation among treatments ($\Theta_{PT}$=0.075, $P$=0.044), indicating that populations of *P. sojae* recovered from some of the rotation treatments were genetically different from each other. We demonstrated with $\Theta_{PT}$ among treatments, that isolates of *P. sojae* recovered from rotations of susceptible cultivars with cultivars with either *Rps*1k resistance (treatment 2) or partial resistance (treatments 3) were different from isolates of *P. sojae* recovered from rotations of cultivars with different *Rps* genes (treatment 4) and from isolates recovered from continuous partial resistance (treatment

5). Similarly, the UPGMA analysis separated with high bootstrap the population of *P. sojae* recovered from treatment 4 and 5 from the rest of the treatments.

Recovery of isolates with different MLGs was greater under continuous rotation with partial resistance, which was also where genotypic diversity (*G*=4.7*)* was the highest and gene diversity (*UHe*=0.45) second highest. Moreover, this treatment was also where we recovered the highest number of isolates with new alleles, and also where we recovered five new alleles as early as the second year of the experiment. These data indicate that continuous rotation with partial resistance may not be a very effective way to prevent genetic shifts of the pathogen in the soybean-*Phytophthora sojae* system. Anderson (3) found that PRR disease severity can be similar after monoculture of partial resistant and susceptible cultivars. He also demonstrated that greater numbers of oospores can be produced in vitro on roots of partial resistant soybean seedlings cultivars than on susceptible cultivars, while less are produced in resistant cultivars. If genetic shifts were driven by the numbers of oospores produced (population size) more than by deployment of *Rps* genes, then this could explain higher number of MLGs and diversity found in the continuous partial resistant rotation in our experiment. Higher population sizes would enhance mutation/crossovers and increase gene and genotypic diversity.

**Segregation locus PS01.** The excess of genotype 'EE' for locus PS01 found in the total population may be explained by two processes; gene conversion or some kind of fitness advantage (survival, growth, aggressiveness, oospore production). High frequency of mitotic gene conversion has been previously demonstrated for *P. sojae* (6). This process rapidly converts heterozygous loci to homozygosis throughout vegetative growth, and is stimulated by sexual reproduction (6). A fitness advantage would be less likely because SSR markers

should be selectively neutral as they are derived from random mutations which should have no effect on the fitness of an organism (26). All the same, we cannot discard the possibility since pathogenic fitness advantages such as aggressiveness and survivability have been demonstrated for *P. nicotianae*. Race 0 isolates of *P. nicotianae* were more aggressive than race 1 isolates, incubation periods were shorter and root rot severity was greater with race 0 isolates. Furthermore, race 0 overwintered better than race 1 (53). Moreover, another study has related fitness to molecular markers (30). A RAPD marker putatively linked to a mefenoxam-resistance locus in *P. nicotianae* was capable of differentiating mefenoxam-resistant populations from sensitive populations. Fitness advantages have also been reported for certain races of *P. sojae* (42), significant differences among races were observed for colony diameter, zoospore production, and infection aggressiveness.

Hobe (1981) suggested that a wide range of *P. sojae* races may exist in a natural wild-type population and may only be detected when a resistant cultivar is introduced (28). In our study, no endemic population of *P. sojae* was present in the field site where we established the rotations. We introduced a single isolate of *P. sojae* of a known pathotype and recovered four pathotypes four and half months after inoculation, and a total of 14 pathotypes throughout the 4 year experiment (Fig. 3). Moreover, we recovered isolates belonging to two MLGs four and half month after inoculation, and 21 MLGs throughout the 4 year experiment. Based on the data, the genetic and pathotype changes detected in the population recovered from the microplots must have happened in the introduced isolate, because no preexisting forms of *P. sojae* were detected at the site. The most likely explanation for the genetic changes in the isolates of *P. sojae* recovered from soil samples collected from the microplots would be: mutation, mitotic recombination, sexual recombination during selfing,

and outcrossing. The new allele ‘D’ detected in locus PS01 may have arisen from a single stepwise mutation, loss of a 6-bp repeat, in allele ‘E’ resulting in a change in size from 425 to 419 bp.

No heterozygosis is predicted in homothallic species of *Phytophthora* that have been established for more than a few generations, since self-fertilization reduces the amount of heterozygosis by one half every generation (23). For one locus PS01, however, our data showed that the proportion of heterozygote individuals tended to increase rather than decrease throughout the study. Although the index of association ($I_A$) suggests asexual reproduction, the high heterozygosity values (*Ho*) we observed in this study suggest processes other than asexual reproduction and selfing were occurring.

Laboratory studies have shown evidence of outcrossing between isolates of *P. sojae* when co-cultured in vitro (58, 61), and we recently reported evidence of outcrossing occurring in the field (Chapter 2). Co-infection of soybean by more than one isolate of *P. sojae* has been demonstrated in greenhouse experiments (35), and more recently within single plants from the field (Chapter 2). Coinfection may result in outcrossing between isolates of *P. sojae* that are in close proximity to each other in disease tissue. Although no visible above ground symptoms of PRR were observed during the four year duration of our trial, root rot due to infection by *P. sojae* was not assessed. We suggest that pathogen shifts may have occurred in root infections and resultant lesions that occurred below ground and were not quantified.

Our research has demonstrated that *P. sojae* has the potential to shift pathotype or evolve new genotypes very quickly. We were unable, however, to demonstrate that pathotype shift was a function of the *Rps* gene used. Thus, overuse of an *Rps* gene is not the

primary reason for changes in pathotype structure of *P. sojae* nor does continuous rotation with high partial resistance cultivars prevents genetic shifts.

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Table 1. Soybean cultivar rotations planted in microplots at the Iowa State University Northern Research Farm from 2007 through 2010.

| Year | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 2007 | Sloan[a] | Sloan | 2834 RR | 2834 RR | Stine 2402 | 2834 RR |
| 2008 | Sloan | 2834 RR[b] | Stine 2402[c] | Williams 79 [d] | Stine 2402 | 2834 RR |
| 2009 | Sloan | Sloan | Stine 2402 | L83-570[d] | Stine 2402 | 2834 RR |
| 2010 | Sloan | 2834 R | 2834 RR | 2834 RR | Stine 2402 | 2834 RR |

[a] cv. Sloan, no *rps* genes and low partial resistance.

[b] cv. 2834RR with *Rps*1k and low partial resistance.

[c] cv. Stine 2402 with no *rps* genes and moderate to high partial resistance.

[d] cv. Williams 79 with *Rps* 1c and cv. L83-570 with *Rps* 3a.

Table 2. Pathotype diversity of isolates of *Phytophthora sojae* recovered from soil samples collected from six soybean cultivar rotation treatments over four years (2007-2010).

| Rotation Treatment | No. isolates of *P. sojae* recovered | No. pathotypes of *P. sojae* detected | No. of race 1[a] isolates recovered | No. non-race 1[b] isolates recovered |
|---|---|---|---|---|
| 1 | 13 | 6 | 5 | 8 |
| 2 | 34 | 7 | 13 | 21 |
| 3 | 30 | 7 | 19 | 11 |
| 4 | 7 | 2 | 3 | 4 |
| 5 | 30 | 8 | 14 | 16 |
| 6 | 7 | 3 | 5 | 2 |
| Total | 121 | 14 | 59 | 62 |

[a] race 1 = virulent formula ( 7), identical race/pathotype as isolate PR1 use to inoculate the experiment.

[b] non-race 1 include virulence formulas (1d), (1d,7), (1a,7), (2,7), (3b,7), (1c,7), (1d,2,7), (1a,1c,7), (1k,2,7), (1a,1c,2,7), (1a,1c,1d,7), and (1d,2,3b,7).

Table 3. Loci, primers, and alleles based on approximate band sizes as determined by GeneMapper analysis for eight microsatellite loci on 121 isolates of *Phytophthora sojae* recovered from soil collected from a soybean cultivar-rotation study (2007 to 2010)

| Locus | Sequence motif in isolate P6497[a] | Primer[b] | Label on forward primer | Allele size (bp) and designated letter |
|---|---|---|---|---|
| PS01 | $(GACACT)_{49}$ | PS01-F<br>PS01-R | 6 FAM | 419(D),**425**[c](E),**431**(F) |
| PS05 | $(TCAG)_{34}$ | PS05-F<br>PS05-R | HEX | **262**(A),339(D),343(E) |
| PS10 | $(CAAAC)_{27}$ | PS10-F<br>PS10-R | HEX | 191(B),**221**(E),226(F) |
| PS12 | $(GCTGTT)_{23}$ | PS12-F<br>PS12-R | HEX | **251**(A),257(B),310(E) |
| PS16 | $(ATTAT)_{20}$ | PS16-F<br>PS16-R | 6 FAM | 405(B),**470**(D),475(E) |
| PS24 | $(CT)_{16}+(CT)_{3}$ | PS24-R<br>PS24-F | 6 FAM | **252**(B) |
| PS29 | $(TAC)_{15}$ | PS29-R<br>PS29-F | HEX | **249**(A),270(B) |
| PS33 | $(AT)_{15}$ | PS33-R<br>PS33-F | 6 FAM | **250**(A) |

[a] '+' indicates that two simple repeats were separated by other bases.

[b] primer sequence detailed in Dorrance and Grundwald (2009).

[c] alleles in bold are the alleles present in PR1, isolate used to inoculate the experiment. Genotype PR1: 425/431, 262/262, 221/221, 251/251, 470/470, 252/252, 249/249, 250/250 for SSR PS01, PS05, PS10, PS12, PS16, PS24, PS29, and PS33, respectively.

Table 4. Genetic diversity statistics of 121 isolates of *Phytophthora sojae* recovered from soil samples collected from a soybean cultivar-rotation study done over four years (2007 to 2010)

| ***Treatment*** | ***1*** | ***2*** | ***3*** | ***4*** | ***5*** | ***6*** | ***Total*** |
|---|---|---|---|---|---|---|---|
| No. isolate | 13 | 34 | 30 | 7 | 30 | 7 | 121 |
| No. genotypes | 4 | 8 | 8 | 5 | 10 | 4 | 21 |
| *Ho* [a] | 0.038 | 0.039 | 0.069 | 0.048 | 0.052 | 0.048 | 0.037 |
| *UHe* [b] | 0.312 | 0.207 | 0.250 | 0.469 | 0.452 | 0.293 | 0.248 |
| *F* [c] | 0.92 | 0.81 | 0.83 | 0.80 | 0.89 | 0.88 | 0.86 |
| $I_A$ [d] | 3.10 | 0.97 | 2.14 | 1.97 | 3.17 | 3.32 | 2.46 |
| *P* [e] | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |

[a] Observed heterozygosity averaged across loci.

[b] Unbiased expected heterozygosity averaged across loci; $UHe = (2n/2n-1)(1-\sum p^2_i)$ where $p$ is the frequency of the $i^{th}$ allele.

[c] Wright's fixation index; $F$ close to zero are expected under random mating while values close to one indicate inbreeding.

[d] Index of association = $V_o/(V_e-1)$, where $V_o$ is observed and $V_e$ the expecetd variance of K , and K is equal to the no. of loci at which 2 individuals differ.

[e] Probability of $I_A$ based on 1000 randomizations; null hypothesis = $I_A$ does not differ from purely sexually outcrossing population.

Table 5. Estimate of differentiation (theta, $\Theta_{PT}$) based on eight microsatellite loci among 121 isolates of *Phytophthora sojae* recovered from soil collected from six soybean cultivar-rotation treatments in a four year rotation study (2007 to 2010).

| *Treatment* | ***1*** | ***2*** | ***3*** | ***4*** | ***5*** | ***6*** |
|---|---|---|---|---|---|---|
| 1 | | | | | | |
| 2 | 0.005 | | | | | |
| 3 | 0.000[a] | 0.000[a] | | | | |
| 4 | 0.217 | 0.380* | 0.271* | | | |
| 5 | 0.029 | 0.120* | 0.076* | 0.077 | | |
| 6 | 0.000[a] | 0.000[a] | 0.000[a] | 0.180 | 0.000[a] | |

*Significant at probability P≤0.05, value based on 999 permutations.

[a] $\Theta_{PT}$ were converted to zero due to negative values.

Table 6. Genetic diversity statistics of 121 isolates of *Phytophthora sojae* recovered from soil of six soybean cultivar-rotation treatments within each of the eight microsatellite loci assessed.

| *Loci* | ***PS01*** | ***PS05*** | ***PS10*** | ***PS12*** | ***PS16*** | ***PS24*** | ***PS29*** | ***PS33*** |
|---|---|---|---|---|---|---|---|---|
| *Ho*[a] | 0.267 | 0.000 | 0.010 | 0.011 | 0.005 | 0.000 | 0.000 | 0.000 |
| *UHe*[b] | 0.440 | 0.274 | 0.301 | 0.305 | 0.314 | 0.000 | 0.349 | 0.000 |
| *F*[c] | 0.326 | 1.000 | 0.940 | 0.961 | 0.964 | -[d] | 1.000 | -[d] |

[a] Observed heterozygozity averaged across treatments.

[b] Unbiased expected heterozygosity averaged across treatments; $UHe = (2n/2n-1)(1-\sum p^2_i)$ where $p$ is the frequency of the $i^{th}$ allele.

[c] Wright's fixation index where $F = (He - Ho)/He$.

[d] No $F$ can be calculated for monomorphic loci.

Table 7. New alleles (different from alleles in the isolate used to inoculate experiment) detected using eight SSRs on 121 isolates of *Phytophthora sojae* throughout four-year soybean cultivar-rotation experiment (2007-2010).

| SSR | Allele in PR1[a] | New allele | Recovered from treatment | No. of isolates | Years recovered |
|---|---|---|---|---|---|
| PS01 | 425/431 | 419 | 3 | 2 | 3 |
| | | | 5 | 1 | 3 |
| | | 339 | 5 | 10 | 2,3,4 |
| | | | 6 | 1 | 3 |
| PS05 | 263 | 339 | 5 | 10 | 3 |
| | | | 6 | 1 | 3 |
| | | 343 | 1 | 2 | 3,4 |
| | | | 3 | 2 | 3 |
| | | | 4 | 3 | 3,4 |
| PS10 | 221 | 191 | 1 | 2 | 3,4 |
| | | | 3 | 2 | 3 |
| | | | 4 | 3 | 3,4 |
| | | | 5 | 10 | 2,3,4 |
| | | 226 | 2 | 2 | 3 |
| PS12 | 251 | 257 | 3 | 1 | 3 |
| | | 310 | 1 | 2 | 3,4 |
| | | | 3 | 5 | 3 |
| | | | 4 | 5 | 3,4 |
| | | | 5 | 10 | 2,3,4 |
| | | | 6 | 1 | 3 |
| PS16 | 470 | 405 | 1 | 2 | 3,4 |
| | | | 2 | 2 | 3 |
| | | | 3 | 4 | 3 |
| | | | 4 | 3 | 3,4 |
| | | | 5 | 9 | 2,3,4 |
| | | | 6 | 1 | 3 |
| | | 475 | 2 | 1 | 3 |
| PS24 | 252 | none | - | - | - |
| PS29 | 249 | 270 | 1 | 2 | 3,4 |
| | | | 2 | 8 | 2,3,4 |
| | | | 3 | 4 | 3 |
| | | | 4 | 4 | 3,4 |
| | | | 5 | 9 | 2,3,4 |
| | | | 6 | 1 | 3 |
| PS33 | 250 | none | - | - | - |

[a] isolate used to inoculate experiment. Genotype PR1: 425/431, 262/262, 221/221, 251/251, 470/470, 252/252, 249/249, 250/250 for SSR PS01, PS05, PS10, PS12, PS16, PS24, PS29, and PS33, respectively.

Figure legend

Fig. 1. Number of isolates and pathotypic diversity of 121 isolates of *P. sojae* recovered from soil from six soybean cultivar rotation treatments in a four year study (2007-2010).

[a] identical race/pathotype as isolate PR1 used to inoculate the experiment.

Fig. 2. Number of pathotypes of *Phytophthora sojae* recovered from soil collected from each soybean cultivar-rotation treatment, by sampling time and year in a four year study (2007-2010).

[a] race 1 = virulent formula ( 7), identical race/pathotype as isolate PR1 used to inoculate the experiment.

[b] others include virulence formulas; (1d), (1d,7), (1a,7), (2,7), (3b,7), (1c,7), (1d,2,7), (1a,1c,7), (1k,2,7), (1a,1c,2,7), (1a,1c,1d,7), and (1d,2,3b,7).

Fig. 3. Percent of isolates of *Phytophthora sojae* (N=121) recovered from soil samples collected from a soybean cultivar rotation study that were compatible on soybean *Rps* resistant genes in a four year soybean cultivar-rotation study (2007-2010). The site was *P. sojae* free, and was inoculated on year one with *P. sojae* race 1, which is compatible on *Rps* 7.

Fig. 4. Number of multilocus genotypes and their diversity based on eight microsatellite loci of 121 isolates of *Phytophthora sojae* recovered from soil samples collected from rotation treatments in a four year (2007 to 2010) soybean cultivar-rotation study .

Multilocus genotypes (MGLs) based on 6 polymorphic microsatellites with primer pairs: PS01, PS05, PS10, PS12, PS16, PS29 (excluded PS24 and PS33 due to monomorphism).

[a] Stoddart and Taylor's genotypic diversity index (*G*) with rarefaction; minimum value of 1.0 (one MGL in the population) and maximum value of 7.0 (all individual with different MGLs).

[b] identical MLG as isolate PR1 used to inoculate the experiment.

Fig. 5. Dendrogram of six populations of *Phytophthora sojae* recovered from soil samples collected from six soybean cultivar-rotation treatments in a four year study (2007 to 2010). The dendrogram was generated by UPGMA (unweighted pair group method) based on the allele frequency of eight microsatellite loci in *P. sojae*. Bootstrap values are shown along the branches. Populations are designated by treatment number. Bar indicates Nei's genetic distance.

Fig. 6. Observed and expected frequencies for monogenic segregation of alleles within the locus PS01 microsatellite marker of *Phytophthora sojae* ( $\chi^2_{1:2:1} = 52.6$, $P = 0.0000$)

Allele E= 425 bp having 50 six bp repeats.

Allele F= 431 bp having 51 six bp repeats.

Fig. 7. Observed number of isolates of *Phytophthora sojae* with different alleles within locus PS01 microsatellite marker in year three of a soybean cultivar-rotation study conducted over four years (2007 to 2010).

Allele E= 425 bp having 50 six bp repeats.

Allele F= 431 bp having 51 six bp repeats.

Allele D=419 bp having 49 six bp repeats.

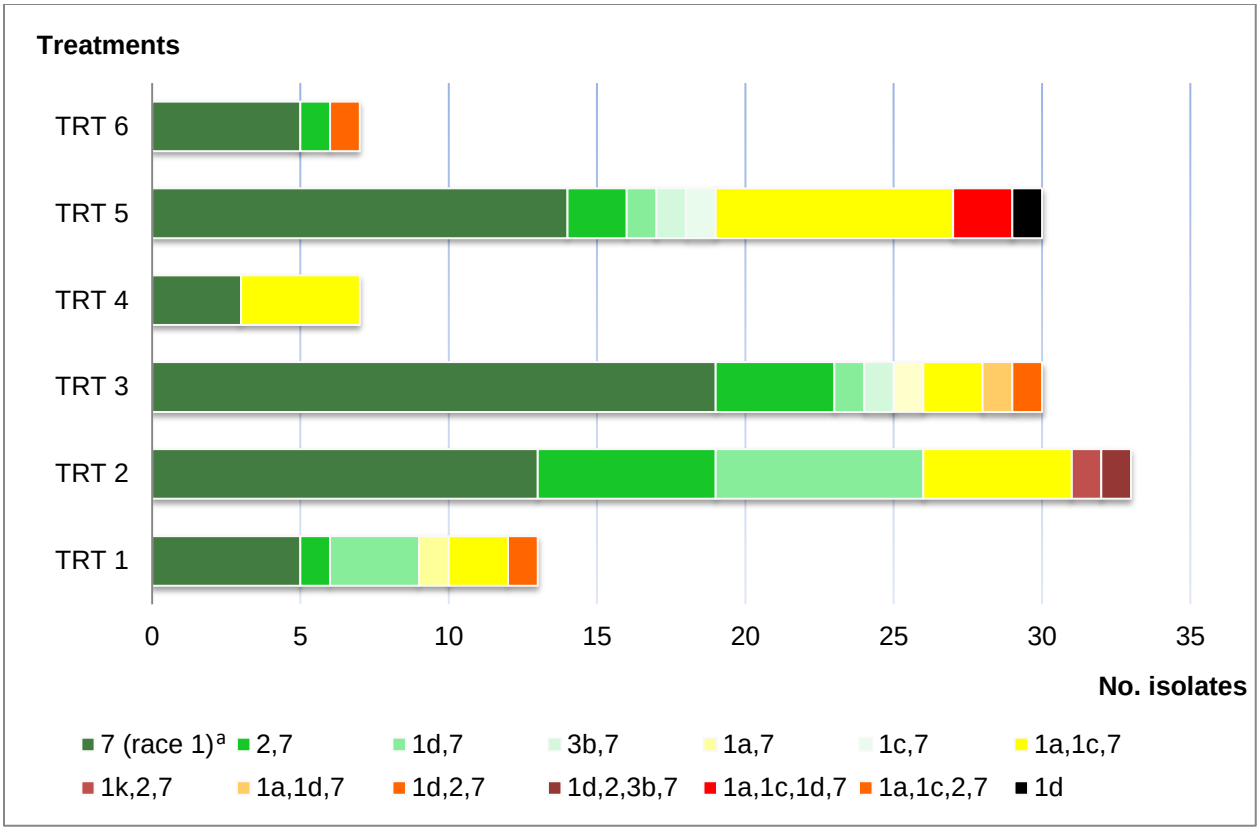

Treatments
TRT 6
TRT 5
TRT 4
TRT 3
TRT 2
TRT 1
0
5
10
15
20
25
30
35
No. isolates
7 (race 1)[a]
2,7
1d,7
3b,7
1a,7
1c,7
1a,1c,7
1k,2,7
1a,1d,7
1d,2,7
1d,2,3b,7
1a,1c,1d,7
1a,1c,2,7
1d

Fig. 1

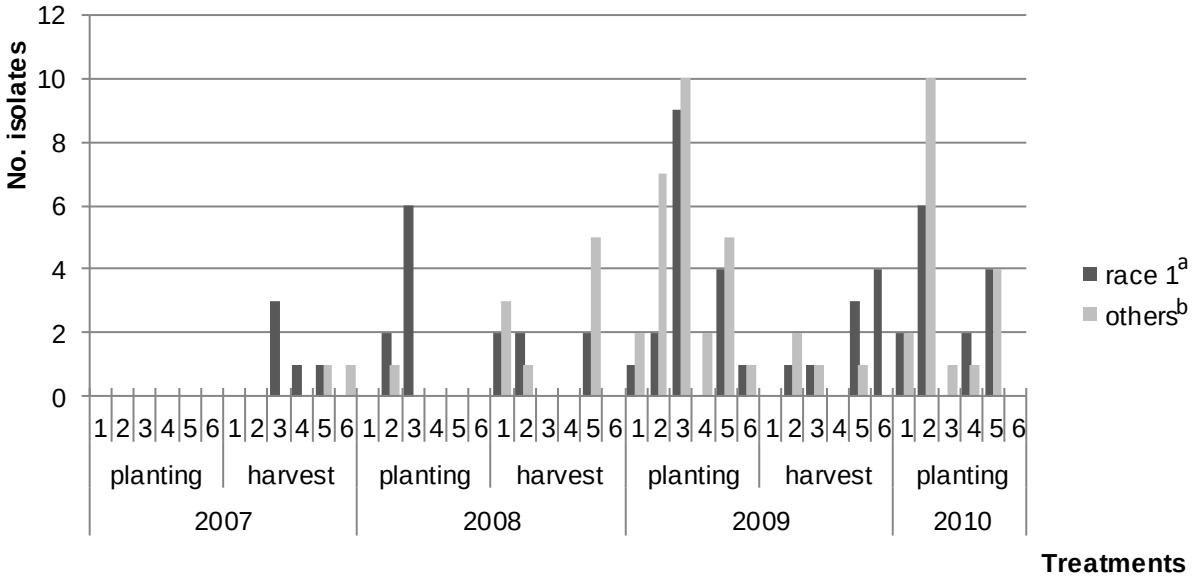

12
10
8
6
4
2
0
No. isolates
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
planting
harvest
planting
harvest
planting
harvest
planting
2007
2008
2009
2010
Treatments
race 1a
othersb

Fig. 2

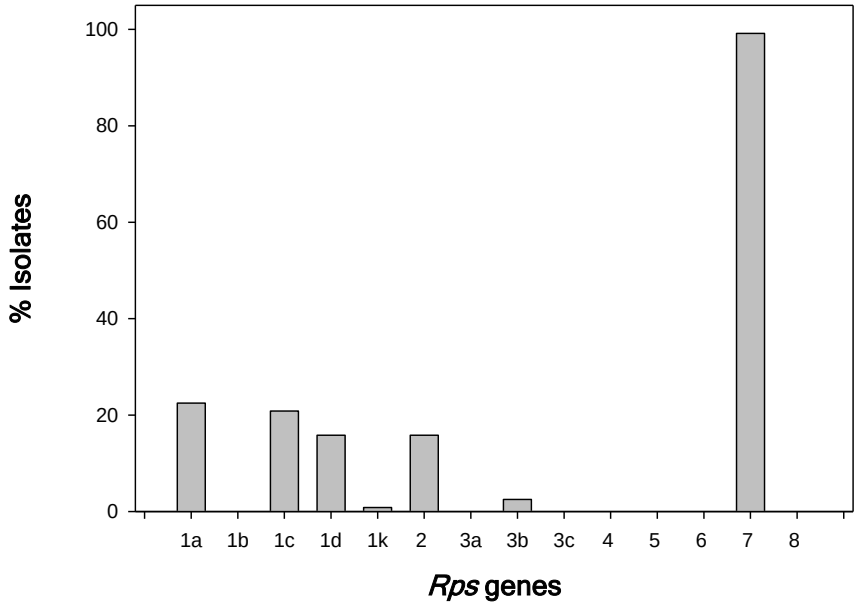
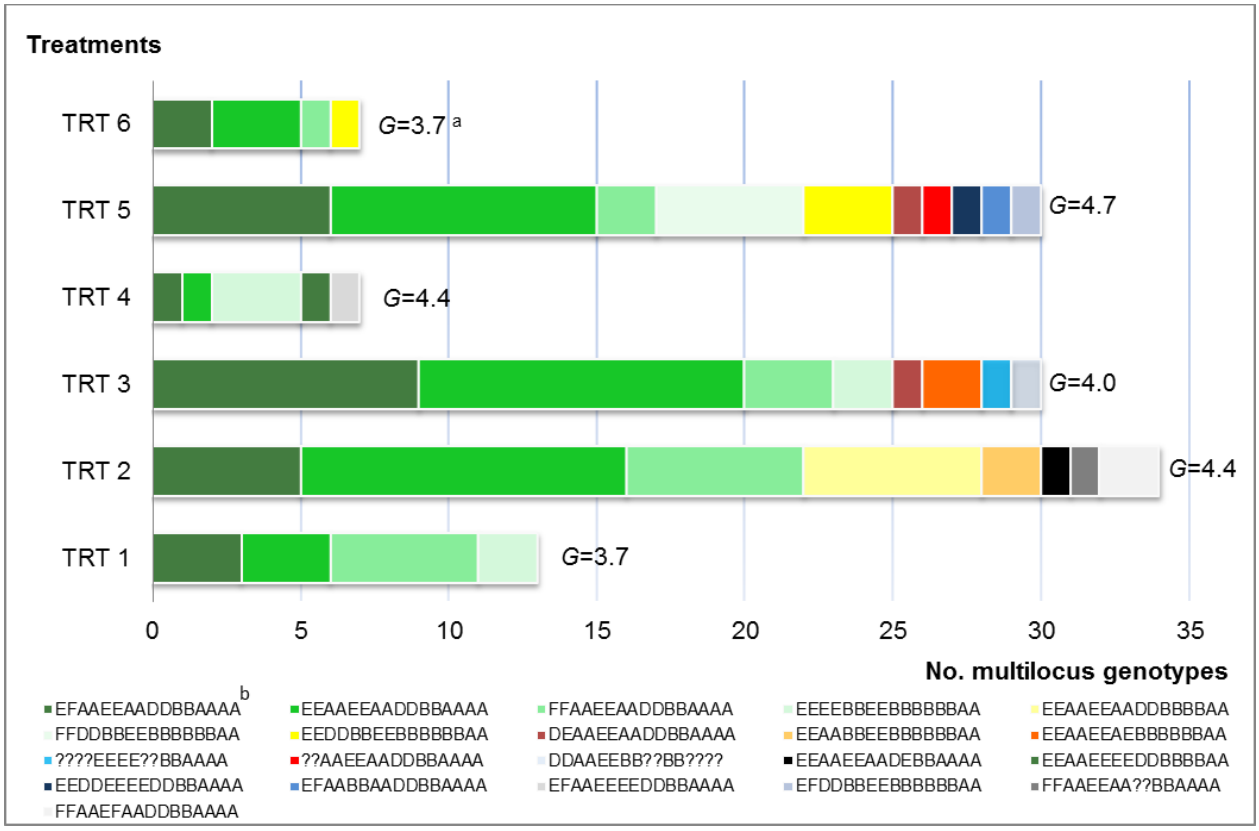

100
80
60
40
20
0
% Isolates
1a
1b
1c
1d
1k
2
3a
3b
3c
4
5
6
7
8
Rps genes

Fig. 3


Treatments
TRT 6
TRT 5
TRT 4
TRT 3
TRT 2
TRT 1
G=3.7 a
G=4.7
G=4.4
G=4.0
G=4.4
G=3.7
0
5
10
15
20
25
30
35
No. multilocus genotypes
b
EFAAEEAADDBBAAAA
EEAAEEAADDBBAAAA
FFAAEEAADDBBAAAA
EEEEBBEEBBBBBBAA
EEAAEEAADDBBBBAA
FFDDBBEEBBBBBBAA
EEDDBBEEBBBBBBAA
DEAAEEAADDBBAAAA
EEAABBEEBBBBBBAA
EEAAEEAEBBBBBBAA
????EEEE??BBAAAA
??AAEEAADDBBAAAA
DDAAEEBB??BB????
EEAAEEAADEBBAAAA
EEAAEEEEDDBBBBAA
EEDDEEEEDDBBAAAA
EFAABBAADDBBAAAA
EFAAEEEEDDBBAAAA
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FFAAEEAA??BBAAAA
FFAAEFAADDBBAAAA

Fig. 4

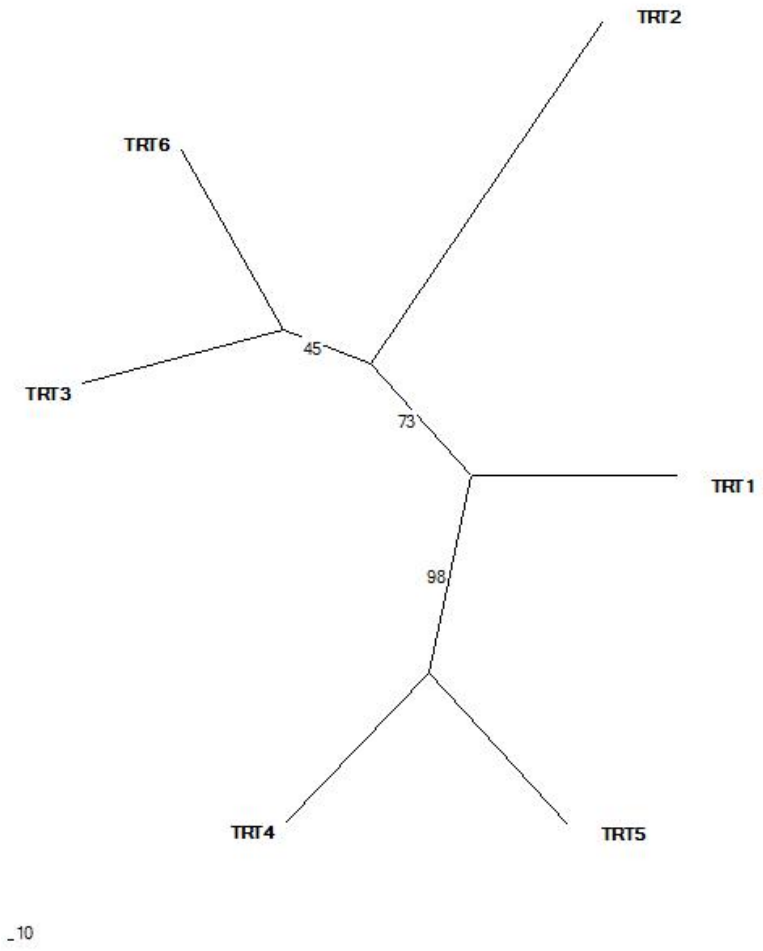


Fig. 5

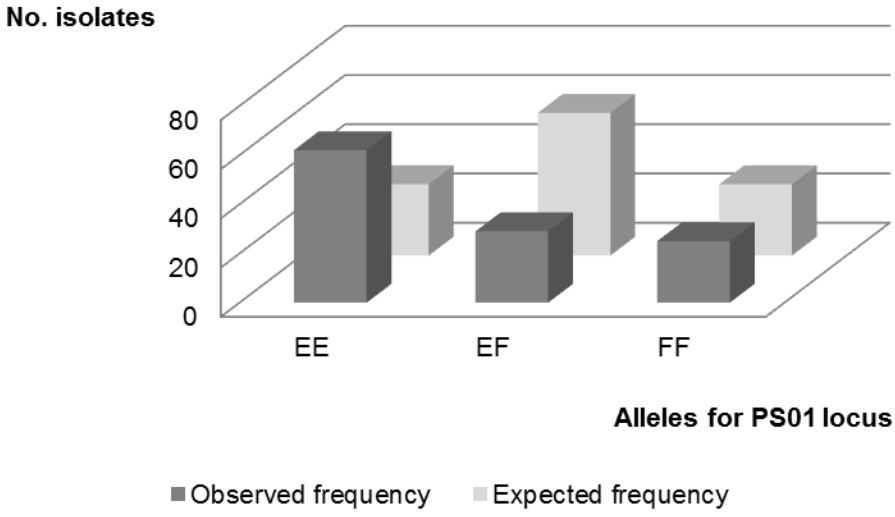
No. isolates
80
60
40
20
0
EE
EF
FF
Alleles for PS01 locus
Observed frequency
Expected frequency

Fig. 6

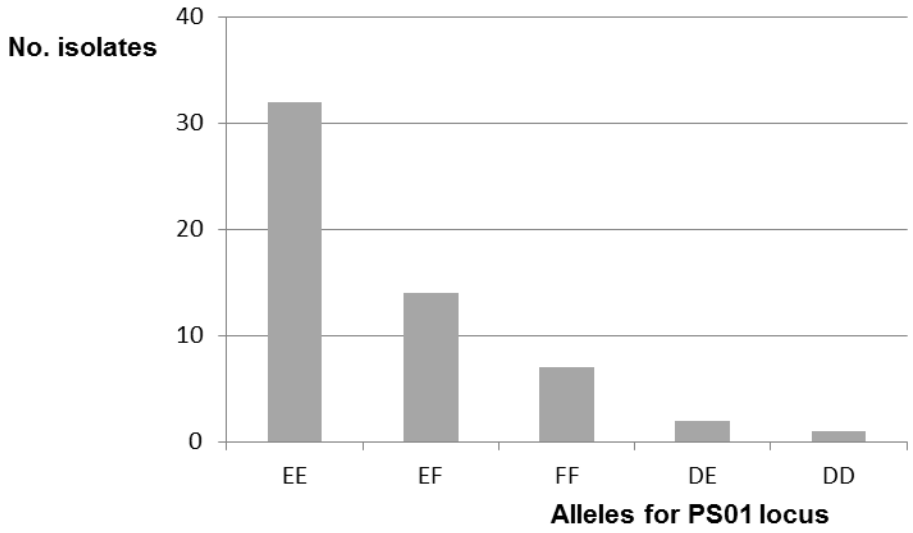


Fig. 7

# CHAPTER 4. A MODIFIED METHOD TO SCREEN FOR PARTIAL RESISTANCE TO *Phytophthora sojae* IN SOYBEAN

A paper to be submitted to *Crop Science*

S. Stewart, and A. E. Robertson

Department Plant Pathology, Iowa State University, Ames, IA 50011

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Corresponding author: A.E. Robertson, alisonr@iastate.edu

## ABSTRACT

Stewart, S., Robertson, A.E. 2011. A modified method to screen for partial resistance to *Phytophthora sojae* in soybean.

A modification in the standard layer test used to screen for partial resistance to *Phytophthora sojae* in soybean is proposed. The modification uses rice infested with *P. sojae* instead of the agar layer, and evaluation is done by dry root weight instead of the customary 1-10 visual scale used. For the variables evaluated (partial resistance, dry root weight), the rice method did not differ statistically from the layer test. Furthermore, the rice method has several advantages: it is cheaper, set up is less time consuming, and it allows the use of more than one pathotype in a single test. Screening for partial resistance using a mixture of several pathotypes ensures compatible interactions between the isolates chosen and all known *Rps* genes, thus ensuring that no *Rps* gene masks the partial resistance trait while screening. Since dry root weight is a continuous and objective variable, it ensures precision and accuracy measuring a quantitative disease trait. Dry root rate is also non-rater dependent and requires minimal training.

Phytophthora root and stem rot (PRR) caused by *Phytophthora sojae* is an economically important disease of soybean in Iowa and worldwide (3, 14, 35, 36). The most effective way of managing the disease has been through the use of single gene/race specific host resistance, which is based on a gene-for-gene interaction between an avirulence gene in *P. sojae* and an *Rps* resistance gene in soybean (12). The pathogen, however, has the ability to rapidly adapt and overcome this type of resistance, resulting in populations that are able to infect soybean plants with *Rps* genes (21).

Partial resistance (PR), which has also been called field-, rate reducing-, horizontal-, and quantitative resistance or tolerance, is an alternative to race specific resistance (2, 33). Partial resistance is effective against all physiological races of the pathogen and is expressed as a reduced level of root rot. This type of resistance is polygenic, and limits the lesion growth rate of the pathogen within the host tissue allowing the host to better tolerate the infection when compared to a fully susceptible host (6, 11, 32). Thus, tolerance should be more stable than single-gene resistance because of the lack of selection pressure imposed on the pathogen (27). Buzzel and Anderson (1982) proposed combining partial resistance with specific *Rps* genes to provide long-term management of PRR and avoid the boom-and-bust cycle of single gene deployment.

More than 55 described races of *P. sojae* have been identified on the basis of compatible (susceptible) and incompatible (resistant) reactions. Race designation is obtained after inoculating on a set of differentials comprised of seven to 14 soybean lines that each have one *Rps* gene to *P. sojae* (5, 6, 11). Since new virulence gene combinations or pathotypes continue to emerge in the pathogen (1, 7, 15-17, 24, 26, 37), the use of a race classification system has been discouraged (38). Pathotypes or virulence formulas,

indicating the *Rps* gene in soybean that the isolate is able to infect, are now used to describe the more than 200 pathotypes of this pathogen found worldwide (7).

Potentially there can be 32,768 possible virulence combinations or pathotypes ($2^N$, N=no. *Rps* genes) that could exist in a single population of *P. sojae* since there are 15 known *Rps* genes in soybean that confer resistance to PRR (9, 13, 29). In previous reports, 54 and 56 pathotypes were detected from two intensively sampled commercial soybean fields in Ohio (7), while 11 and 18 pathotypes were recovered from two commercial soybean fields in Iowa (23). Interestingly, as many as four pathotypes were detected in some sub-samples of soil, indicating that a single soybean plant may be physically and spatially subjected to infection by more than one pathotype (23). Consequently, the complexity within the population of *P. sojae* in a single field makes it almost impossible to choose a soybean cultivar with the correct *Rps* gene or genes required to resist infection by the pathogen in a particular field. Thus, PR could be an effective management tool against all physiological races of *P. sojae* especially under high disease pressure.

Traditionally, breeding efforts have focused on incorporating major *Rps* gene resistance into soybean lines, it is easier to recognize and is more easily introgressed in a good agronomic and high yielding soybean type. Few cultivars with high levels of PR are currently available (6, 28), and the challenge faced by soybean breeders and pathologists is to find easy, feasible and effective ways to incorporate partial resistance into superior soybean cultivars.

Since the early 1980s, researchers have evaluated numerous methods to screen for PR (18, 19, 25, 30, 31, 34). Some direct methods include inoculating wounded cotyledons or roots, while others inoculated non-wounded aeroponic grown plants (19, 31, 34). Indirect

methods have also been explored, such as quantifying preformed epidermal suberin (22). The layer test has been the most widely accepted and has become the standardized method used to screen soybeans for partial resistance to *P. sojae* in the greenhouse (6, 11, 15, 25, 30). In the layer test, an agar culture of the pathogen is placed at a certain distance below seeds at planting time. Seedling roots grow through the *P. sojae*-inoculated agar layer at approximately the same time that the seedling's unifoliate leaves expand, thus infection of the roots coincide with soybean stage (VC) at which PR becomes active in the plant (7). Although this method is widely used in many soybean breeding programs to screen for PR, it is cumbersome because it requires handling a high number of agar plates. Furthermore, evaluation of PR using this method is based on a visual rating scale (1 to 10) which requires training and can be subjective. Moreover, the use of more than one isolate in a single test is limited by the number of agar layers the roots are capable of penetrating through.

Our goal was to develop an easier and more objective method than the layer test, that could be used by soybean breeders to screen for PR. Compared to the standard layer test, the rice screen tests provides an assessment of PR that it is more precise, non-rater dependent, quantitative and accurate.

## MATERIALS AND METHODS

***Phytophthora sojae* isolates and pathotype characterization**. Isolates of *P. sojae* recovered from PRR-diseased plants were selected based on their pathotype and on their interaction with selected soybean cultivars. Isolates were purified and mono-zoospored (25), and pathotypes of *P. sojae* were determined with the hypocotyl inoculation technique using a 15 differential set (4, 5, 25). Standard soybean cultivars or lines, each with a specific *Rps*

gene, were grown in trays in the greenhouse. The cultivars or lines used were: L88-8470 (*Rps*1a, Muckden source), L77-1863 (*Rps*1b, Hanga source), Williams 79(*Rps*1c), L99-3312 or PI 103091 (*Rps*1d), Williams 82 (*Rps*1k), L82-1449 (*Rps*2, CNS source), L83-570 (*Rps*3a), L91-8347 (*Rps*3b), L92-7857 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), Altona or L89-1581 (*Rps*6), L93-3258 or Harosoy (*Rps*7), PI 399073 (*Rps*8) and Williams (susceptible).

Soybean seeds were grown in potting mix (equal volume of soil, sand and vermiculite) in trays in the greenhouse. Ten 7-day-old seedlings of each differential were inoculated by making a slit in the hypocotyl with a syringe filled with a slurry of a 7-10 day old culture of each isolate, and placing approximately 0.2 to 0.4 ml of slurry into the slit (10, 25). A plastic covering was placed over the tray for 12-16 hours to prevent inoculum from drying. Seven to 10 days after inoculation, PRR incidence was evaluated. Plants which died or developed distinct symptoms of PRR were classified as susceptible, while resistant plants developed a hypersensitive reaction (slight necrotic lesion around the wound). The differential was considered susceptible when at least 7 out of 10 seedlings showed disease symptoms. The test was repeated at least twice for each isolate. Two isolates were selected for use in the rice screen test, isolate 1023-1c compatible on soybean resistant gene *Rps* 7 (race 1) and isolate 1019-1.11c compatible on *Rps* 1a, 1b, 1c, 1k and 7, and used in a 1:1 mixture to screen cultivars.

**Inoculum preparation.** *P. sojae*-rice infested inoculum was prepared by autoclaving batches of 50 grams of common rice in 36 ml of distilled water in 250 ml Erlenmeyer flasks twice within a 24 h period. Cooled rice grains were loosened under aseptic conditions in between each autoclaving. Three Erlenmeyer flasks each were inoculated with 10 pieces (0.5

$cm^2$) of 4-6 day old mycelium of a *P. sojae* isolate 1023-1c or 1019-1.11c grown on diluted V8 media (40 ml V-8 juice, 0.6 g $CaCO_3$, 0.2 g Bacto yeast extract, 1 g sucrose, 0.01 g cholesterol, 20 g Bacto agar, 1 l distilled water). Inoculated flasks were kept at room temperature (25 $^0$C) for 10-14 days, and shaken once per day to prevent clumping. Immediately prior to use, inoculated rice grains were removed from flasks, loosened and equal volumes of infested rice belonging to the each isolate were mixed together thoroughly. Ten $cm^3$ of this mixture was used to inoculate each 32-oz cup. The amount of inoculated rice used in the screening test was established in previous experiments, where no significant differences in root rot were found using 5, 10 or 15 $cm^3$ of inoculated rice per cup (data not shown). Inoculum for the standard layer test consisted of the same two isolates each grown for 10 days on diluted V8 juice agar plates. One plate of each isolate was placed on top of the other to inoculate each 32-oz cup.

**Soybean cultivars.** Six cultivars with different sources of resistance to *P. sojae* were used to compare the standard layer test to the rice method: susceptible cv. Sloan with no *rps* genes and low partial resistance (8, 10), cv. Stine 2402 with no *rps* genes and moderate to high partial resistance (10), cv. 2834RR with *Rps*1k and low partial resistance (10), and three lines provided by Syngenta with high, moderate and low PR as described by the company (Table 1). All varieties lacked race-specific resistance to at least one of the isolates used in the mixture; this was proven earlier by hypocotyl injection test of the isolates onto the six cultivars (data not shown).

**Partial resistance screening.** Styrofoam cups (32-oz) with three holes punctured in the base were 1/3-filled with A4 coarse vermiculite, 5 cm depth. The inoculum (infested rice or agar) was placed on top of this first layer of vermiculite, and the cups were filled up to 2/3

level with vermiculite, approximately 13 cm from the bottom of the cup. Fifteen seeds of the soybean variety to be tested was placed in a pile at the center of each cup, covered with vermiculite and watered until runoff. Thereafter, cups were watered once a day and kept in greenhouse at 20 ± 5 º C with a 12 hour supplemented light cycle.

**Experimental design, assessment and analysis.** A complete randomized design with two factors, 6 varieties (Table 1) by 3 treatments was used, with 5 replications. Treatments were: i) 10 $cc^3$ of a 1:1 volume mixture of *P. sojae* isolates 1023-1c and 1019-1.11 inoculated rice, ii) a double agar layer where each layer corresponded to a fully grown culture of *P. sojae* isolates 1023-1c and 1019-1.11, iii) and a non-inoculated control. Thus, a total of 90 32-oz foam cups were arranged randomly on the greenhouse bench. The experiment was repeated twice. Four weeks after planting, the number of surviving plants in each cup was counted. Plants were removed from the cup, and roots gently washed to remove all traces of vermiculite. Partial resistance was evaluated using a visual 1-10 scale, where 1 is no root rot and 10 is all seedlings killed before emergence (25). Roots from individual cups were placed in a paper bag and oven-dried at 50° C for 24 h. Total dry root weight (tDrw) for each cup was weighed, and individual dry root weight (Drw) for each root was obtained by dividing tDrw by the number of surviving plants present in each cup. Corrected root weight (cDRW) was calculated as root weight of a cultivar relative to the root weight of its non-inoculated control.

PROC GLM procedure using software SAS (SAS Institute, Cary, NC) was used and contrast between factors were estimated. Correlations were computed with PROC CORR using SAS (SAS Institute, Cary, NC).

## RESULTS

No significant interactions were observed between experiment x treatment x cultivar for any of the variables evaluated, thus both experiments were pooled for the analysis. The second experiment had significantly higher disease level as measured by all variables ($P < 0.0001$). Single degree freedom comparisons of non-inoculated control vs. inoculated treatments using contrast statements revealed highly significant differences ($P < 0.0001$) for PR rating, Drw and cDrw (Fig. 1). When contrast were used to compare the standard layer test vs. the rice method, no significant differences were revealed for PR rating, Drw, and cDrw ($P$=0.193, $P$=0.489 and P=0.149, respectively) (Fig. 1). There was an overall reduction of 68.2% in Drw and 64.3% in cDrw, when inoculated roots were compared to non-inoculated roots.

The level of partial resistance differed among cultivars (Table 1). Cultivars ranked as expected for PR based on pubished information (10) and on information supplied by company. Thus, cultivars reported to have moderate to high levels of PR, had the highest level of PR using the rating scale, Drw and cDrw. Of three variables, cDrw was the variable that separated cultivars the best according to their expected PR rankings. Cultivars Stine 2402 and S37-F7 ranked the highest for PR according to the corrected dry root weight (Table 1). When contrast statements were used to separate high, moderate and low partial resistant cultivars, all variables significantly separated low partial resistant from high as well as low partial resistant from moderate, but cDrw was the only variable that significantly separated moderate from high tolerant cultivars.

Partial resistance ratings were negatively and significantly correlated to dry root weight ($r = -0.933$, $P < 0.0001$). The relationship between the variables, however, was

improved when root weight for each cultivar was corrected based on the root weight of its non-inoculated control (cDrw) (r = -0.975, $P < 0.0001$) (Fig. 2).

## DISCUSSION

No differences in assessment results from screening were observed between the standard layer test and the rice method, indicating that screening for PR can be done indistinctly using any of the two methods. The advantages of using the rice method, however, are several: 1) the rice method is more than 10-times cheaper to set up than the layer test, considering the price of rice *vs* the price of agar, V8 juice and Petri plates, 2) hundreds of plates can be substituted by one or two bags of inoculated rice in the lab, 3) the ability to screen for PR using more than one pathotype is feasible by mixing equal volumes of inoculated rice each with the desired pathotype, and 4) because of the above mentioned points, large number of soybean genotypes may be screened simultaneously.

One of the major constraints in screening for PR using a large number of lines or cultivars is that the choice of isolate becomes critical to evaluate the trait (10). The isolate of *P. sojae* chosen has to have a compatible interaction (susceptible response) on all the soybean lines or cultivars to be tested. The presence of a *Rps* gene in the lines will mask the PR, consequently, a hypocotyl test should be done on each line or cultivar, prior to the PR screening to ensure the isolate's compatibility. This step is avoided when using the rice method as long as the mixture of pathotypes to be used ensures that all known compatible interactions with all *Rps* genes are represented.

Partial resistance, like most quantitative disease resistance trait is typically assessed by visual estimation of disease severity. Accuracy and precision in this visual estimate is

critical, and especially impacts identification of disease resistance quantitative trait loci (QTL) (20). Variability between raters using direct percentage and a 0-to-9 ordinary rating scale may result in identification of some QTLs only by a subset of raters, moreover, those more experienced raters will have higher precision using the direct percent (20). This rater-dependent scoring variable is avoided when using dry root weight as a direct measure of PR. An additional advantage is that, no expertise is required in weighting roots.

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**Table 1.** Rating and dry root weight of six cultivars of soybean evaluated for partial resistance to *Phytophthora sojae* in the greenhouse

| *Cultivar, resistance*[w] | *Rating* [x] | *Drw* [y] | *cDrw* [z] |
|---|---|---|---|
| Stine 2402, HPR, no *Rps* | 4.18 a | 0.089 d | 0.72 f |
| S25-J5, MPR | 4.22 a | 0.077 cd | 0.57 de |
| S37-F7, HPR | 4.43 a | 0.071 c | 0.64 ef |
| 2834 RR, LRP, *Rps* 1k | 4.50 a | 0.074 c | 0.57 cd |
| S41-M5, LPR | 5.13 b | 0.054 bc | 0.52 bc |
| Sloan, LPR, no *Rps* | 6.04 c | 0.055 ab | 0.41 a |

Values within a column followed by the same letters are not significantly different according to Tukey test ($P \leq 0.05$).

[w] Level of *P. sojae* partial resistance based on published information and information supplied by company: HPR = high partial resistance, MPR = moderate level, LPR = low level of partial resistance.

[x] Partial resistance rating based on 1-10 scale, where 1 is no root rot and 10 all seedlings killed before emergence (Schmitthenner and Bhat, 1994).

[y] Dry root weight per root in grams.

[z] Corrected dry root weight calculated as root weight of a cultivar relative to the root weight of its non-inoculated control.

Figure Legend

**Fig. 1.** Mean rating and dry root weight of six cultivars screened for partial resistance to *Phytophthora sojae* using the rice method, or layer test in comparison to a non-inoculated control treatment.

Rating scale 1-10, where 1 is no root rot and 10 all seedlings killed before emergence (Schmitthenner and Bhat, 1994).

Non-inoculated control significantly differs from the other two treatments for partial resistance rating, dry root weight (Drw) and corrected dry root weight (cDrw) according to GLM contrast ($P<0.0001$ for all three variables).

The standard layer did not differ significantly from the rice method for PR rating, Drw and cDrw according to GLM contrast ($P= 0.193$, $P=0.489$, and $P= 0.149$ ,respectively)

**Fig. 2.** Relationship between standard partial resistance ratings and corrected root dry weight for six soybean cultivars screened for partial resistance to *Phytophthora. sojae*.

Partial resistance rating based on 1-10 scale; 1 is no root rot and 10 all seedlings killed before emergence (Schmitthenner and Bhat, 1994).

Corrected dry root weight calculated as root weight of a cultivar relative to the root weight of its non-inoculated control.

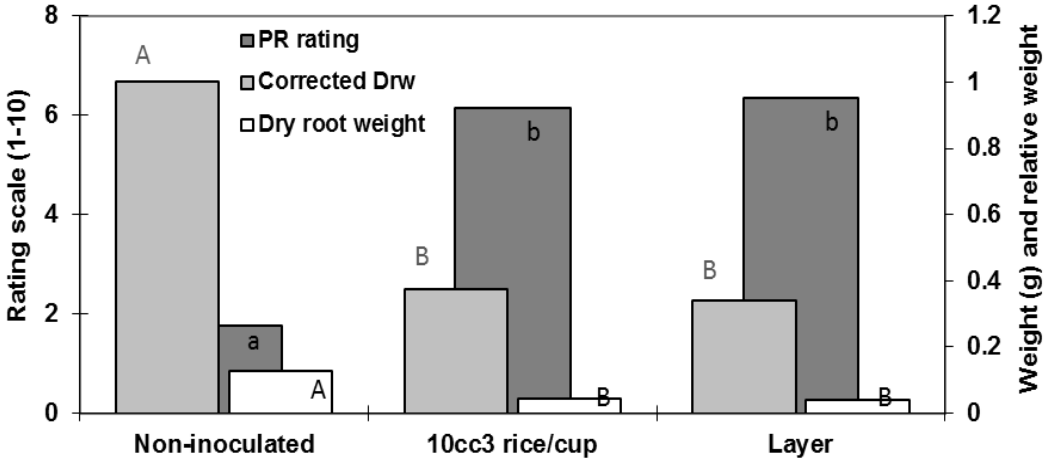
PR rating
Corrected Drw
Dry root weight
Rating scale (1-10)
Weight (g) and relative weight
0
2
4
6
8
0
0.2
0.4
0.6
0.8
1
1.2
A
a
A
B
b
B
B
b
B
Non-inoculated
10cc3 rice/cup
Layer

Fig. 1

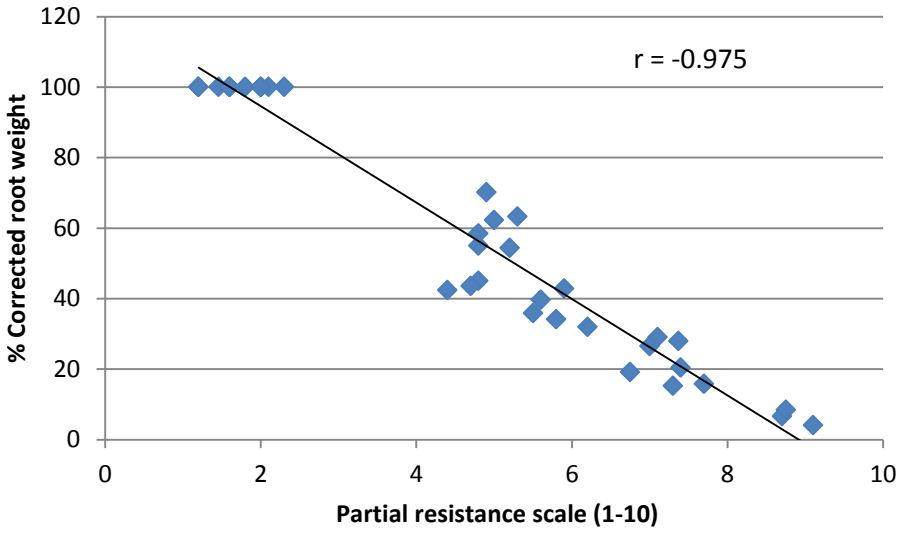
r = -0.975
% Corrected root weight
Partial resistance scale (1-10)
120
100
80
60
40
20
0
0
2
4
6
8
10

Fig. 2