11-4-2017

Pathogenicity and Reproduction of Isolates of Reniform Nematode, Rotylenchulus Reniformis, From Louisiana on Soybean and Utility of Single Nucleotide Polymorphisms to Evaluate Genetic Variability

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PATHOGENICITY AND REPRODUCTION OF ISOLATES OF RENIFORM NEMATODE, *ROTYLENCHULUS RENIFORMIS,* FROM LOUISIANA ON SOYBEAN AND UTILITY OF SINGLE NUCLEOTIDE POLYMORPHISMS TO EVALUATE GENETIC VARIABILITY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by
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M.Sc., Louisiana State University, 2013
December 2017

I would like to dedicate this dissertation to my beloved parents and my loving wife Pramuditha and daughter Senaya.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my major professor Dr. Charles Overstreet. I will be forever in debt for all his advice, encouragement and friendship throughout my program. I thank you for showing me the path for a better future and making our work together a wonderful experience that I will always remember. Without your guidance and constant motivation this degree would not have been possible.

I am especially thankful to Dr. Edward C. McGawley for being a wonderful mentor and a great teacher. I learned a great deal from you and really appreciate your advice, support, and constructive criticism throughout.

I wish to express my sincere gratitude to Dr. Sally Stetina and Dr. Jeff Ray at United States Department of Agriculture, for their support and guidance throughout and teaching me novel techniques in nematology as well as been good mentors.

I would like to thank Dr. Rodrigo A. Valverde, Department of Plant Pathology and Crop Physiology and Dr. Vincent L. Wilson Collage of the Cost and Environment at LSU for helping me to make this work a success.

I thank all the friends I met at Dr. Overstreet's laboratory, Felipe Godoy, Churamani Khanal, Benjamin McInnes and Deborah Xavier-Mis for their friendship and support. All my collogues faculty and staff in the department of Plant Pathology and Crop Physiology for providing me a pleasant and cheerful environment to work in.

I thank Dr. David Blouin of the Department of Experimental Statistics at LSU for review and approval of experimental designs and data analysis employed in this research.

All the greenhouse personals including, Ms. Oster Clodett, Mr. Bill Salzer, Stephen Salzer and Josh Granger deserves a special thank for their support and for been really good friends throughout my time at LSU.

I am fortunate to have a wonderful family that supported me throughout my life. Their love and encouragement have been pillars of my strength. I do not have enough words to thank my beloved wife Pramuditha and my lovely daughter Senaya; for their endless love and care throughout difficult times. Nothing is possible without you two!

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ABSTRACT

Experiments were conducted to evaluate soybean, *Glycine max* (L.) Merr., responses to indigenous isolates of the reniform nematode (*Rotylenchulus reniformis*) in Louisiana and to understand the genetic variability of these native isolates. Microplot and greenhouse experiments were conducted to evaluate the comparative reproduction and pathogenicity of single egg-mass populations of *R. reniformis* isolated from West Carroll (WC), Rapides, Tensas and Morehouse (MOR) parishes of Louisiana. Data from full-season microplot trials, displayed significant differences in reproduction and pathogenicity of the nematode with the commercial soybean cultivars REV 56R63, Pioneer P54T94R, and Dyna-Gro 39RY57. Significantly low population density was observed in the isolate from the MOR parish compared to that of the least reproducing WC isolate. The MOR isolate was also the most pathogenic and resulted in significantly less soybean plant and pod weights compared to the control. In 60 day greenhouse trials, susceptible cultivar Progeny P4930LL and the resistant germplasm lines PI 90763 and PI 548316 were added together with the same cultivars used in the microplot trials. Similar to the microplot trials, the MOR isolate had the least level of reproduction compared to that of WC and presented the greatest level of pathogenicity. In both microplot and greenhouse trials, the soybean cultivar REV 56R63 had a significant reduction in reniform numbers compared to cultivars Pioneer P54T94R and Dyna-Gro 39RY57. The second set of experiments were conducted to understand the amount of genetic variability present in the 13 reniform nematode isolates from Louisiana, Mississippi, Arkansas, South Carolina and Georgia with the use of Single Nucleotide Polymorphism (SNP) analysis. Thirty one chosen SNPs were tested against the reniform nematode isolates using kompetitive allele-specific PCR genotyping assay. Out of the 31 SNPs tested, 26 SNPs amplified genomic DNA of the reniform nematode isolates. Four

SNPs out of all tested were able to distinguish genetic differences between and among tested geographic isolates of reniform nematode from Louisiana, Mississippi, and Arkansas. Even with limited numbers of samples, a genetic variability was observed with 3 SNPs between South Carolina, and Georgia isolates. The results obtained in this study might be extremely useful in resistance breeding programs as well as providing soybean cultivar recommendations for growers in different geographical locations.

CHAPTER 1. INTRODUCTION

1.1 Soybeans

Soybean, *Glycine max* (L.) Merr., is a major crop in the United States. It was first introduced from China to the U.S. in 1765 (Hartman *et al*., 1999). In 2014, soybeans were planted on 117 million hectares worldwide with a production of 275 million metric tons (FAOSTAT, 2017). According to Anonymous, 2017a, there were about 34.8 million hectares planted to soybean for the year 2016 in the U.S. The top soybean producing states are Illinois, Iowa, Minnesota, North Dakota and Indiana (Anonymous, 2017a; b). To date, the United States is the largest soybean producer in the world producing about 32% of the world's soybean supply, followed by Brazil and Argentina, which produces 28% and 21% of world's soybeans respectively (Anonymous, 2017b). Soybean seeds are rich in oil (20%) and protein (40%). Processed soybeans are the world's largest source of animal protein feed and the second largest source of vegetable oil (Anonymous, 2017c). Soybean oil is mainly used for human consumption, but also used for production of adhesives, coatings and printing inks, lubricants, plastics and specialty products (Anonymous, 2017d). In 2015, the United States alone had a revenue of 34.5 billion U.S. dollars in the industry (Anonymous, 2017e). In the year 2016 about 8 million hectares of land in the southern United States were devoted to soybeans and were produced about 24.3 million metric tons of beans. Out of the 16 southern states, Louisiana was able to produce an average of 3261.68 kg/ha of soybeans in 2016 (Allen *et al.,* 2017).

1.2 Soybean growth and development

Optimal soil temperature for the germination of the soybean seeds falls between 12.7 ºC and 15.5 ºC. Plants need at least a soil temperature of 20 ºC and 635 mm of water during the five month growing season for the best performance and yield. Soybeans are classified into maturity

groups based on the days from emergence to maturity. These plants need a specific length of dark period for flowering to occur (Hartwig, 1973). Based on this response to photoperiod, they are classified into thirteen maturity groups. These 13 maturity groups range from 000 to X. The cultivars needing the shortest dark period are classified in group 000 and are adapted to grow in higher latitudes. Groups IX and X are primarily grown in subtropical to tropical areas. Soybeans can be divided into two categories according to their growth pattern. The two types consists either determinate or indeterminate growth. Varieties grown in the North are known as indeterminate. They continue main stem growth indefinitely after first flowering and include the maturity groups from 0 up to 4.9. Whereas determinate soybeans which predominantly are grown in the Southern U.S., terminate main stem growth shortly after first flowering and maturity groups 5 to 8 are included in this category. (Paderson, 2004; Anonymous, 2017f). Soybean plant's life cycle is divided into two categories which includes the vegetative and the reproductive period. Vegetative period occurs from the emergence until first flowering, whereas the reproductive period extends from first flowering until maturity. Vegetative period begin from V1 stage and lasts until the beginning of the reproductive period. Reproductive period are designated using the letter "R". The classification of the Reproductive period ranges from R1 to R7, from first flowering to the end of seed filling respectively (Fehr *et al*., 1971).

1.3 Nematode damage to soybeans

There are a number of diseases damaging to soybeans. According to the annual report of the Southern Soybean Disease Workers, in the southern U.S. only, around 2.43 million metric tons were lost due to diseases in soybeans (Allen *et al.,* 2017). Out of these disease causing pathogens in soybeans, nematodes play a major role. Several nematode species are known to damaging to soybeans in the United States. These include soybean cyst (*Heterodera glycines*),

root-knot (*Meloidogyne spp.*), lesion (*Pratylenchus scribneri*), sting (*Belonolaimus longicaudatus*), reniform (*Rotylenchulus reniformis*), and Columbia lance (*Hoplolaimus Columbus*) nematodes (Padgett, 2011). These nematodes cause a large economic impact to the industry and cause significant reductions in yields. According to the literature, the largest soybean yield losses from 2006 to 2009 in the U.S. soybean production states were due to the damage caused by the soybean cyst nematode (SCN) (Koenning and Wrather, 2010). The loss in 2016, in the southern soybean producing region due to the SCN was around 0.5 million metric tons. This represents 21% out of the total losses due to all diseases combined. This trend has been observed to be consistent across the years. Therefore, these data provide valuable information about the relative importance of nematodes as a pest of soybeans.

1.4 Reniform nematode

In the southern United States, reniform nematode has become the more prominent nematode species damaging to both soybeans and cotton. Reniform nematode belongs to the genus *Rotylenchulus,* which includes 11 recognized species. Out of the nine, *Rotylenchulus reniformis*, or the reniform nematode, is by far the most damaging and causes the largest economic losses to a variety of economically important crops (Robinson *et al*., 1997). It was first described as a plant parasitic nematode in Hawaii by Linford and Oliveira in 1940. A reniform nematode problem was first reported in the United States by Smith and Taylor in 1941. This nematode is considered a tropical/subtropical pest and has a wide host range (Koenning *et al*., 2004; Robinson *et al*., 1997). Reniform nematode can survive in soil for long periods without the presence of the host. This is due to its ability to enter an anhydrobiotic state. This state of reduced metabolism provides higher survivability for the nematode. Additionally, high reproductive rate and ability to migrate deep in soil allows the nematode to survive and

repopulate the "cultivation" layer of soil when conditions are unfavorable (Koenning *et al*., 2004). During the past 15 -20 years, reniform nematode has become the dominant nematode species in a number of states, including Louisiana, together with a large decline in soybean cyst nematode problems (Gazaway, 2005; Overstreet and McGawley, 1996; 1998; 2000; Overstreet, 2006; 2015). At present, the nematode has a wide distribution in the cotton producing area of the southern U.S. (Bagwell *et al*., 2006). Due to the shift in commodity prices, farmers are now switching from cotton to more profitable crops such as soybean and corn. Problems arise when these cotton lands previously infested with the reniform nematode are now being used for these new crops. Due to this recent switch, very little research has been conducted to understand the effects of this nematode on the damage to soybeans. Symptoms caused by *Rotylenchulus reniformis* are similar to that of other nematodes. That is, plants become stunted, develop poorly with low yields, and lack of vigor (Overstreet and Wolcott, 2007). Detection of reniform nematode damage is very difficult to diagnose because they do not produce distinctive galling symptoms like root-knot nematode (Overstreet and Wolcott, 2007). Over the past several decades, reniform nematode has become much more widely distributed and losses have increased dramatically in most of southern states including Louisiana. A survey during 1994-1995 showed that reniform nematodes have spread widely through the state and estimated acreage infected was about 510,000 (Overstreet and McGawley, 1996). In the year 2016, reniform nematode alone caused about 92,000 metric tons lost in southern soybean production. Out of the 16 southern soybean producing states, Mississippi, Louisiana, South Carolina, Alabama and Georgia had considerably higher damage due to the reniform nematode (Allen *et al.,* 2017).

1.5 Reniform nematode management

There are a number of different techniques that have been employed for the control of reniform nematodes. Some common strategies include the use of resistant cultivars, crop rotation, biological control, nematicide application, and the use of precision agriculture (Koenning *et al*., 2004). Out of the several available management practices, use of resistant cultivars, crop rotation and use of nematicide are frequently employed practices in Louisiana (Overstreet *et al*., 2014).

Crop rotation is one of the main management practices used for the control of the reniform nematode. For this, rotation is done with a resistant or a poor host crop such as corn, milo, resistant soybean, peanut and sugarcane (Overstreet, 2015). According to literature, a two year rotation with a non-host crop can significantly reduce high populations of reniform nematodes to a more manageable levels (Overstreet *et al*., 2014). Crop rotation is a preferable management practice within Louisiana producers due to the favorable pricing of grain crops.

The use of nematicide goes as far back as the 1800's. During the time period chemical known as carbon bisulphide was tested as a nematicide against both sugerbeet nematode (*Heterodera schachtii*) and root-knot nematode (Taylor, 2003). With the use of modern technology, nematicides that are more efficient are now available in the market. There are number of nematicide listed for the use in Louisiana against a variety of nematode species on soybean. Most of the modern nematicides are available as seed treatments. Common seed treatment nematicides available in Louisiana are Poncho VoTivo, ILeVO, and Avicta Complete Bean. Telone II, is a widely used recommended nematicide that's been used as a preplant nematicide for heavy nematode infestation (Hollier *et al*, 2017).

The use of resistant cultivars is the best option as it is more economical and environmentally friendly (Stetina *et al.*, 2014; Robbins *et al.*, 2014; Overstreet, 2015). In the USA, soybeans have demonstrated resistance against reniform nematode, and some resistent cultivars have been developed. This resistance is mainly transferred from soybean varieties such as Peking, PI 437654 and PI 90763 which were resistant to soybean cyst nematode (*Heterodera glucines*) (Robbins *et al.*, 1994a,b; 2014; Davis *et al*., 1996; Robbins and Rakes, 1996). The mechanism of resistance was studied by Rebois *et al*. (1975) while observing the physiological changes that occur during syncytium development. According to Rebois *et al*., 1975 susceptible plants under go two phases which involve partial cell wall lysis and separation followed by an anabolic phase which involves organelle proliferation and secondary wall deposition. In the resistant plants, the first step is increased leading to accelerated cell lysis. There are about six resistant soybean varieties recommended for Louisiana for the year 2015. The recommended varieties Armor A4450, Asgrow AG5535 GENRR2Y, Delta Grow DG4940RR, Delta Grow DG5230 GENRR2Y, Dyna–Grow S52RY75, and MPG 5214NRR, have shown some level of resistance in trials conducted in both Arkansas and Louisisna. With our preliminary studies, soybean cultivars DEL 4940 and Univ. Missouri S11-20356 have showed some level of resistant against two reniform isolates in Louisiana under greenhouse and field conditions.

1.6 Diversity among *Rotylenchulus reniformis* species

In the literature, there have been reports showing differences in reproduction and pathogenicity within the species of *Rotylenchulus reniformis*. This difference is mainly due to the geography of the isolates. McGawley *et al.* (2010, 2011) have demonstrated the variability in pathogenicity and reproduction using six reniform nematode isolates on both cotton and soybean. According to their results, the different isolates had significantly affected both nematode counts

and plant measurements in their 120 day microplots experiments. Until recently, very little was known about the genetic variability within reniform nematode populations. This variability is a major factor that affects the use and the durability of using crop resistance as a management tool. In a recent study on cotton, using four different reniform isolates from Texas, Louisiana, Mississippi and Georgia revealed a strong genetic variability within the populations, and also found pathogenetic difference (Arias *et al.,* 2009). This study additionally revealed that the samples tested within the state of Mississippi also showed genetic variability in reniform populations. Similar results were observed in the study conducted by Leach *et al* (2012a) using isolates from southern United States, Colombia and Japan. A recent study found that even the crop rotation can have an effect on the genetics of the population by expressing variability with different rotation schemas (Leach *et al.*, 2012b). This genetic diversity is very important for management practices because the varieties considered as resistant in one location might not hold the resistance in another geographical location. Therefore, the knowledge of the genetic variability of the populations is critical to give proper recommendations for selecting suitable soybean varieties.

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CHAPTER 2. PATHOGENICITY AND REPRODUCTION OF ISOLATES OF RENIFORM NEMATODE, *ROTYLENCHULUS RENIFORMIS,* FROM LOUISIANA ON SOYBEAN

2.1 Introduction

Soybean is a major crop that has an enormous impact on the economy of the United States. There were about 83 million ha of soybean planted in 2016 throughout the country (Anonymous, 2017). In 2016, about 8 million ha in the southern United States were devoted to soybean and produced about 24.3 million metric tons of soybeans with yield in Louisiana at 1.7 million metric tons (Allen *et al*., 2017).

In the United States, several nematode species including *Rotylenchulus reniformis* are known to damage soybeans (Noel and Schroeder, 2015). Even though the soybean cyst nematode (SCN) is more prevalent in soybeans in the mid-west, the reniform nematode is more widespread and damaging to soybean in the South (McGawley and Overstreet, 2015). Reniform nematode belongs to the genus *Rotylenchulus,* which includes 11 recognized species (Robinson *et al*., 1997; Berg *et al*., 2016). Of these, *R. reniformis* causes the greatest economic loss (Robinson *et al.,* 1997). *Rotylenchulus reniformis* was identified in Hawaii in 1940 (Linford and Oliveira), and reported in Louisiana, USA in 1941 (Smith and Taylor). Over the past 2 decades, this nematode has become the dominant nematode species in several southern states, including Louisiana (Gazaway, 2005; Overstreet and McGawley, 1998; 2000; Overstreet, 2006; 2015).

Currently, *R. reniformis* is distributed throughout the 16 cotton producing states of south-east and mid-south of the U.S. (Bagwell *et al.,* 2006). In this region, many producers have recently switched their cropping preference from cotton to the more profitable soybean. This change in cropping preference had produced immediate challenges to soybean growers due to the widespread occurrence of *R. reniformis* and the host-suitability of many soybean cultivars. In this region in 2016, reniform nematode caused losses in soybean yield estimated at 92,000 metric

tons (Allen *et al.,* 2017). Mississippi and Louisiana report the greatest yield losses and plant damage to this nematode (Allen *et al*., 2017).

Management strategies for reniform nematode include resistant cultivars, crop rotation, biological control, nematicide application, and precision agriculture (Koenning *et al.,* 2004). Resistant cultivars are the most desirable but least frequently used management option. This is due to lack of more profitable traits than those available in some cultivars classified as susceptible (Stetina *et al.,* 2014; Robbins *et al.,* 2015; Overstreet, 2015).

There are reports describing differences in reproduction and pathogenicity among geographic isolates of *R. reniformis* on both cotton and soybean (McGawley *et al*., 2010; 2011; Xavier *et al*., 2014; Bhandari *et al*., 2015; Arias *et al*., 2009). Moreover, the study by McGawley *et al* in 2011 showed that the nematode was actually more damaging to soybean than to cotton. Isolates of the nematode from Louisiana and Mississippi had significantly greater rates of reproduction and were more virulent than the isolates from Alabama, Arkansas, Hawaii, and Texas. Stetina *et al.* (2014) speculated that the geographic origin of isolates of the nematode may have an impact on resistance to *R. reniformis* in soybean.

Variability in the reproduction and pathogenicity among reniform nematode populations has a major impact on management options including breeding, cultivar selection and nematicide and rotation recommendations. For example, soybean cultivar recommendations for Louisiana are made on the basis of reproduction data for isolates of the nematode present in Arkansas (Robbins *et al.,* 2015). To date, no studies have been conducted to evaluate reproductive and pathogenic variation in indigenous isolates of *R. reniformis* on cultivars of soybean produced in Louisiana. A better understanding of *R. reniformis* within Louisiana will enhance nematode management recommendations and assist plant breeders and seed companies in producing or

selecting cultivars with resistance. To date, cultivars with resistance to the reniform nematode have primarily been derived almost exclusively from germplasm sources containing resistance to the SCN. Therefore, the objectives of this work were to evaluate the host status and susceptibility of soybean cultivars popular in Louisiana and germplasm lines of PI 90763 and PI 548316 hereafter referred to as PI90, and PI54, respectively, which have known resistance to SCN and reniform nematode, to isolates of *R. reniformis* present in Louisiana.

2.2 Materials and methods

2.2.1 General procedures

Isolates of reniform nematode were collected from Rapides (RAP), Tensas (TEN), Morehouse (MOR) and West Carroll (WC) parishes, confirmed morphologically as *R. reniformis* and used to establish single egg mass (SEM) cultures. These cultures were maintained under greenhouse conditions on tomato (*Solanum lycopersicum* L. cultivar Rutgers PS, Seedway; Hall, New York 14463) and employed in greenhouse and microplot experiments with the soybean cultivars REV 56R63, Pioneer P54T94R, Progeny P4930LL and Dyna-Gro 39RY57 which will be abbreviated as RV56, Pp54, Pr49, and DG39, respectively hereafter. Details of greenhouse and microplot experiments are presented below under the appropriate subheadings.

Pots for all experiments as well as a soil mixture consisting of one part sand and three parts commerce silt loam soil (fine-silty, mixed, superactive, nonacid, thermic Fluvaquentic endoaquepts) utilized in all experiments was heat sterilized for 5 hrs. at 135°C prior to use. In each test, two soybean seeds were planted to a depth of 2.5 cm and thinned to one per pot after germination. Soil was infested by pipetting aqueous suspensions of vermiform individuals of *R. reniformis* into three depressions arranged into a triangular pattern, 0.5-cm diam. × 5- to 7.5-cm deep, surrounding a 10-day-old seedling. Inoculum for all tests contained a mixture of juveniles, pre-adult females, and males at a level, irrespective of pot size, of 6 per gram of soil. Therefore,

inoculum density was 5,500 per pot in greenhouse tests and 50,000 per pot in microplot tests. Half of the inoculum was added to soil in microplots at 10 days after planting and the remainder at 21 days.

In all cases, nematode population density was estimated by extracting a 250g subsample of soil from each pot using a semi-automatic elutriator (Byrd *et al*., 1976) and the centrifugal/sugar flotation technique (Jenkins, 1964). Vermiform life-stages were enumerated using a dissecting microscope at 40X. All experiments were repeated once. Standard fertilization, weeding and insect management practices were employed in all trials.

2.2.2 Analysis of data

Each experiment employed a factorial treatment structure and was established as randomized block design with five replications. Data obtained from all studies were analyzed using SAS JMP version 12.0 (SAS Institute, Cary, NC) analysis of variance (ANOVA) and Fisher's LSD mean separation technique ($P \leq 0.05$). Analysis was conducted using the "Fit Model" module of SAS JMP, version 12.0. Analysis of variance was conducted using test as a fixed effect and there were no significant test by treatment interaction in any of the tests described herein. Therefore data from all like trials was combined for analysis.

2.2.3 Greenhouse experiments with 4 reniform isolates, 4 soybean cultivars and 2 germplasm lines

This study involved six soybean genotypes: four cultivars of soybeans widely planted in Louisiana and one resistant PI90 and one moderately resistant PI54 germplasm line. Terra cotta pots having a top diameter of 15cm and containing 1.6 kg of soil mixture were used. A total of 150 pots were established to evaluate the 6 genotypes, 4 isolates of reniform nematode, a non-inoculated control for each cultivar and 5 replications. The experiments were terminated after 60 days and nematode life stages in soil were quantified as described above. Eggs were extracted

from entire root system. Root samples were agitated in 0.6% NaOCl for 10 min to dislodge eggs from egg masses (Hussey and Barker, 1973). Eggs of reniform nematode were stained using the red-food coloring technique (Thies *et al*., 2002) and numbers present on the whole root system were enumerated at 40X magnification using a dissecting microscope. Fresh shoot and root materials were dried at 30-35°C for two weeks and weighed. Average greenhouse temperature was maintained at 80-85°C. Supplemental lighting was added above the experimental area to give a 16 hrs light period.

2.2.4 Microplot experiments with 4 reniform isolates and 3 soybean cultivars

Terra cotta pots having top diameters of 35.6 cm were used as microplots. Microplots were placed in depressions in soil so that only the rim was exposed. Each microplot was filled with 13.6 kg of soil mixture. The entire microplot area was bounded by an aluminum Quonset hut skeletal frame open at both ends. The skeletal frame was covered with polyethylene (6 mm) film and one layer of 20% reflective foilcloth to protect plants from excessive rainfall and to maintain near-ambient air and soil temperatures. A total of 75 microplots were established to evaluate 3 cultivars RV56, Pp54, and DG39, 4 isolates of the nematode, a non-inoculated control for each cultivar and 5 replications. Establishment of plants, inoculation with nematodes, and processing of plant and nematode materials after 125 days were as described above. Additional plant data collected included: numbers of pods per plant, pod weight per plant, weight of 100 seeds, total seed weight per plant and plant dry weight. All plant materials were dried at 30-35°C for two weeks before measuring the weights.

2.3 Results

2.3.1 Greenhouse experiments with 4 reniform isolates, 4 soybean cultivars and 2 germplasm lines

Data from greenhouse experiments are summarized as Table 2.1. Across genotypes of soybean and isolates of the nematode, there were significant main and interactive effects that impacted both nematode and plant parameters. Significant soybean main effects influenced both vermiform nematode stages in soil and eggs per root system as well as final dry root weight. Main effects of reniform isolate as well as interactive effects of soybean and isolate significantly influenced only the nematode.

Individual treatment means across the 6 soybean genotypes and geographic parish of origin of each of the 4 isolates of *R. reniformis* are presented as Figure 2.1. Soil populations of the WC isolate of the nematode recovered from RV56, which averaged 40.9 thousand per 500cm^3 of soil, were significantly greater than the 17.7 and 15.0 thousand recovered from this genotype with the TEN and MOR isolates, respectively. Similarly, soil populations of the isolate from WC recovered from Pp54, 111.2 thousand, were significantly greater than the 87.9, 75.7 and 56.0 thousand for the RAP, TEN and MOR isolates, respectively. Of the 4 isolates, reproduction by the ones from RAP and TEN parishes on DG39 was very similar, averaging 76.3 and 75.8 thousand per 500cm3 of soil, and were significantly greater than the 50.5 and 48.2 averages for the isolates from WC and MOR parishes. Reproduction by all 4 isolates of the nematode was similar and not significantly different on Pr49, averaging respectively 36.4, 39.9, 42.3 and 29.3 thousand per 500 cm3 of soil for WC, RAP, TEN and MOR parishes.

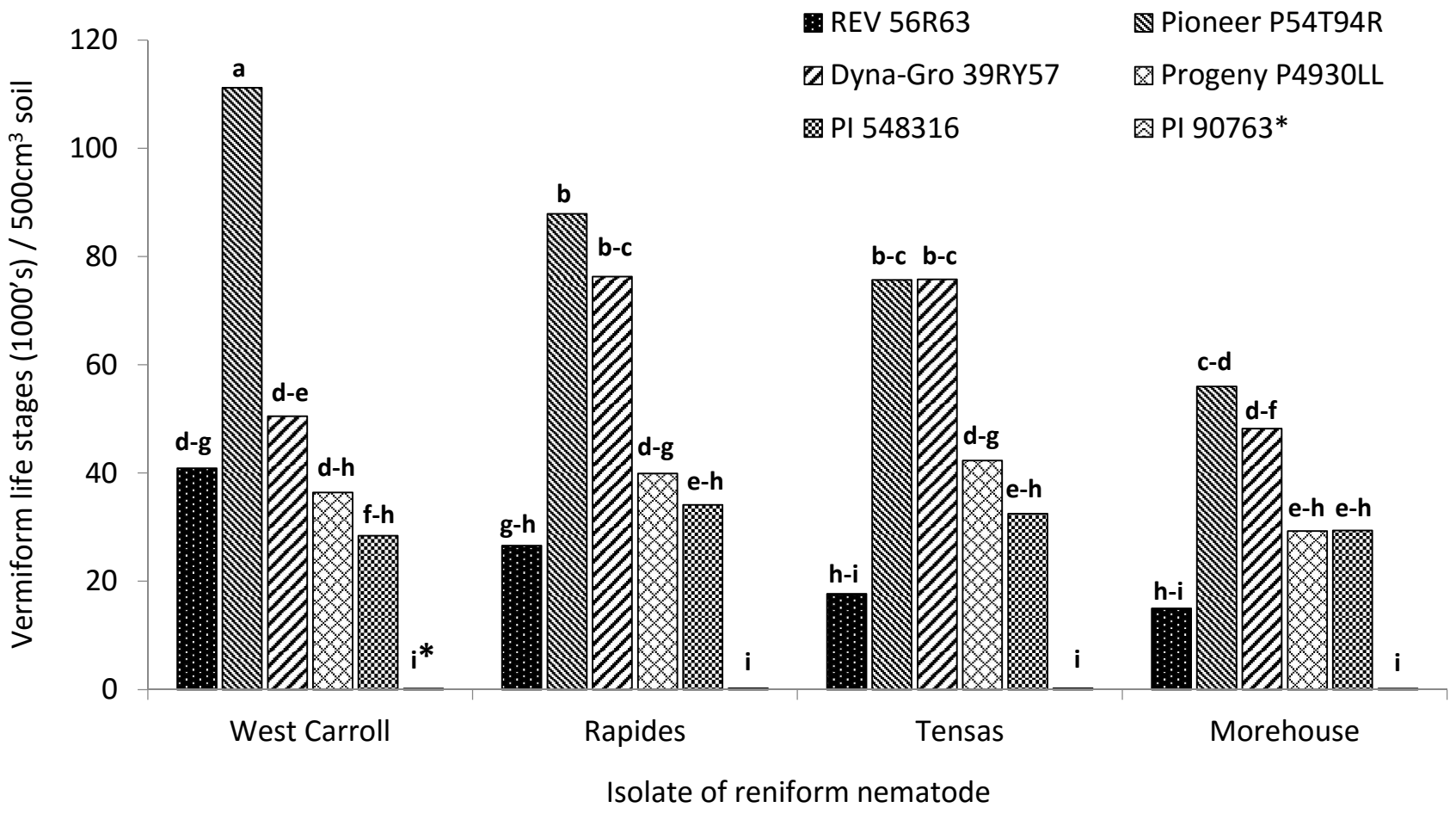

Figure. 2.1. Vermiform life stages of *Rotylenchulus reniformis* per 500cm3 of soil, after 60 days in a greenhouse environment from soybean genotypes REV 56R63 (RV56), Pioneer P54T94R (Pp54), Dyna-Gro 39RY57 (DG39), Progeny P4930LL (Pr49), PI 90763 (PI90) and PI 548316 (PI54). Data are means of 10 replications averaged over two trials. *indicates the mean value (West Carroll; 200, Rapides; 240, Tensas; 250, and Morehouse; 160) for vermiform life stages per 500cm3 soil of *R. reniformis* with the germplasm line PI 90763. Bars with common letters are not significantly different based on Fisher's LSD test ($P \leq 0.05$)

Table 2.1. Main and interaction effects (*P* values) of four isolates of *Rotylenchulus reniformis* endemic in Louisiana on six genotypes of soybean in a greenhouse environment[x].

Source	DF	Vermiform life stages	Eggs per root system	Shoot weight	Root weight
Soybean (S)[y]	5	<0.0001**	<0.0001**	0.260	<0.0001**
Isolate (I)[z]	4	<0.0001**	<0.0001**	0.930	0.999
S × I	20	<0.0001**	<0.0001**	1.000	0.973

[x]Data were combined over two 60-day trials and are means of ten replications. Plant material was dried at 30-35 °C. Data were analyzed as a 6 × 5 factorial with ANOVA ($P \leq 0.05$); ** indicates *P* values significant at the 0.01% level.
[y]Soybean were the cultivars REV 56R63, Pioneer P54T94R, Dyna-Gro 39RY57, and Progeny P4930LL, and the germplasm lines PI 90763 and PI 548316.
[z]Isolates were derived from single egg mass from roots of soybean from West Carroll, Rapides, Morehouse and Tensas parishes.

Also with PI54, reproduction by the 4 isolates was similar and not significantly different, with population density values of 28.4 thousand for MOR, 34.1 thousand for RAP, 32.5 thousand for TEN and 29.4 thousand for the MOR isolate. Lastly, population levels of the nematode in soil for each of the isolates on PI90 actually fell below the initial infestation level averaging about 0.2 thousand per root system for each of the 4 isolates of the nematode.

The overall pattern of Figure 2.2 mirrors closely that of Figure 2.1 for soil stages of the nematode and visualizes the production of eggs by females of the 4 isolates of *R. reniformis* on the 6 soybean genotypes. Data are expressed as thousands of eggs per isolate extracted from the entire root system of each genotype. From RV56, 4.5, 4.7, 4.0 and 2.7 thousand eggs per plant, with no significant differences among the 4 isolates, were recovered for the WC, RAP, TEN and MOR isolates. As with juveniles from the WC isolate in soil for Pp54, the 30 thousand eggs per plant from this genotype was significantly greater than the numbers recovered from roots of the other 3 isolates.

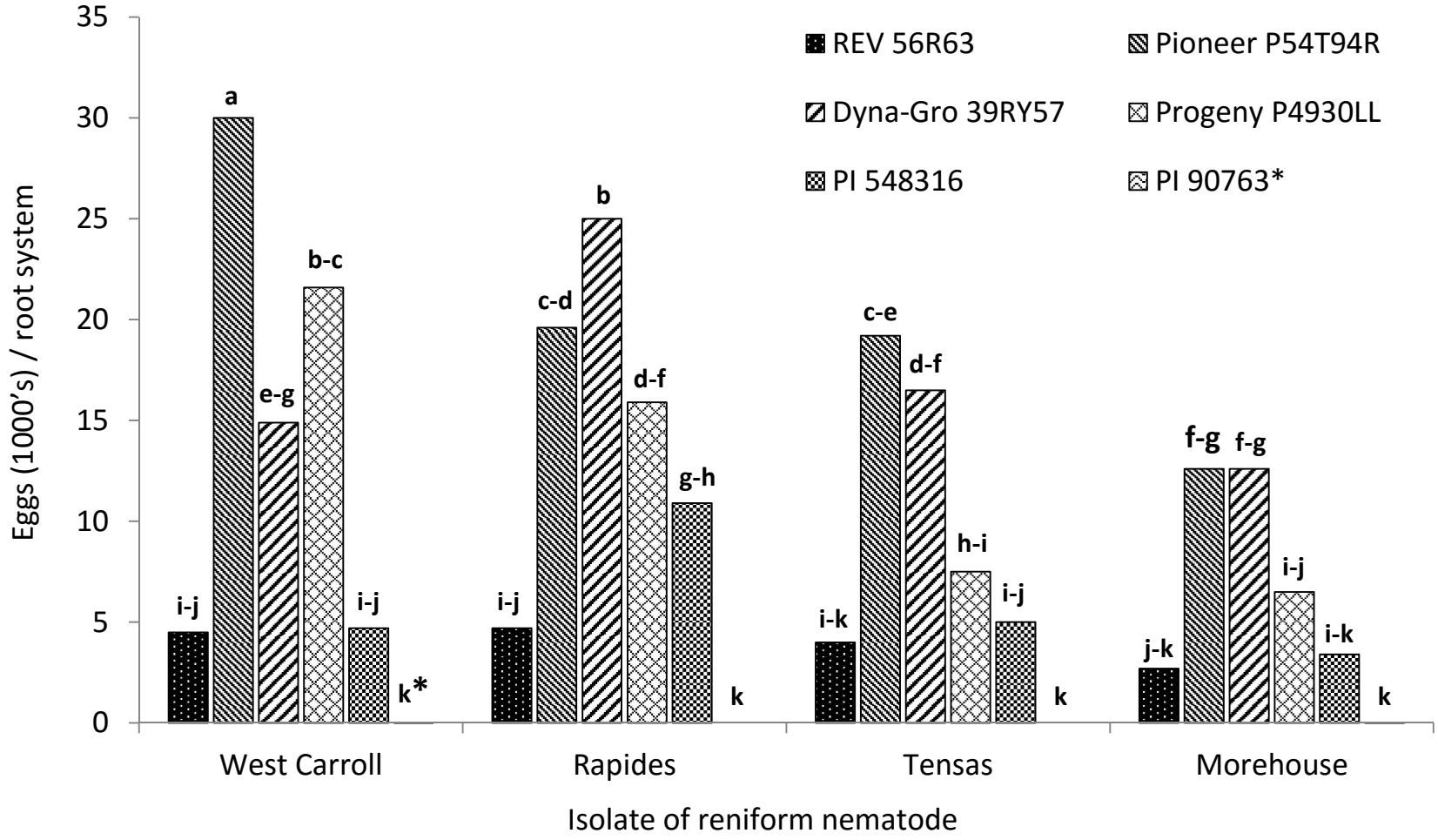

Figure. 2.2. Egg stages of *Rotylenchulus reniformis* from whole root systems of soybean genotypes REV 56R63 (RV56), Pioneer P54T94R (Pp54), Dyna-Gro 39RY57 (DG39), Progeny P4930LL (Pr49), PI 90763 (PI90) and PI 548316 (PI54) after 60 days in a greenhouse environment. Data are means of 10 replications averaged over two trials. *indicates the mean value (West Carroll; 2, Rapides; 0, Tensas; 0, and Morehouse; 4) for eggs per root system for *R. reniformis* with the germplasm line PI 90763. Bars with common letters are not significantly different based on Fisher's LSD test ($P \leq 0.05$).

Root systems of DG39 yielded a significantly greater number of eggs, 14.9 thousand, with the RAP isolate with the other 3 isolates; 16.5 for TEN, 14.9 for WC and 12.6 for MOR. With Pr49 there was almost significantly declining stair-step effect in egg numbers per root system across the 4 isolates of the nematode: eggs densities averaging 21.6 thousand for the WC isolate, 15.9 for RAP, 7.5 for TEN and 6.5 for MOR. From roots of PI54 the number of eggs of the RAP isolate recovered averaged 10.9 thousand and was significantly greater than the 4.7 thousand for the WC isolate and the 5.0 and 3.4 for the TEN and MOR isolates, respectively. Very few to no eggs of the 4 nematode isolates were recovered from PI90.

2.3.2 Microplot experiments with 4 reniform isolates and 3 soybean cultivars

In the microplot environment, there were significant main effects of cultivar and isolate but no cultivar by isolate interactions (Table 2.2).

Table 2.2 Main and interaction effects (*P* values) of four isolates of *Rotylenchulus reniformis* endemic in Louisiana on three soybean cultivars in a microplot environment[x].

Source	DF	Vermiform life stages	Number of pods	Pod weight	100 seed weight	Seed weight per plant	Plant weight
Cultivar (C)[y]	2	0.001**	0.255	0.908	<0.0001**	0.062	0.672
Isolate (I)[z]	4	<0.0001**	0.141	0.0003**	0.940	0.956	0.035**
C × I	8	0.069	0.474	0.226	0.323	0.167	0.436

[x]Data were combined over two full season trials and are means of ten replications. Plant material was dried at 30-35 °C. Data were analyzed as a 3 × 5 factorial with ANOVA ($P \leq 0.05$); ** indicate *P* values significant at the 0.01% level.
[y]Cultivars were REV 56R63, Pioneer P54T94R, and Dyna-Gro 39RY57 that were recommended for use in Louisiana in 2015.
[z]Isolates were derived from single egg masses from roots of soybean from West Carroll, Rapides, Morehouse and Tensas parishes.

The influence of cultivar significantly impacted reniform nematode juvenile stages in soil and hundred seed weight. The influence of isolate was significant for life stages of reniform nematode in soil and weights of soybean pods and plants. In the microplot environment, there were significant main effects of cultivar and isolate but no cultivar by isolate interactions. The

influence of cultivar significantly impacted reniform juvenile stages in soil and hundred seed weight (Table 2.3). The influence of isolate was significant for life stages of reniform nematode in soil and weights of soybean pods and plants (Table 2.4). Across the 4 isolates of *R. reniformis,* soil populations from RV56 were significantly lower in number, averaging 61.5 thousand per 500cm3 of soil, than those recovered from soil with the cultivars Pp54 or DG39 that averaged 111.6 and 103.7 thousand vermiform life stages, respectively (Table 2.3).

Table 2.3. Main effect of three cultivars of soybean on vermiform life stages and seed weight across four isolates of *Rotylenchulus reniformis* in a full season microplot environment[x].

Cultivars[y]	Vermiform life stages per 500cm^3 of soil (1000's)[z]	100 seed weight (g)
REV 56R63	61.5 b	12.4 b
Pioneer P54T94R	111.6 a	11.4 c
Dyna-Gro 39RY57	103.7 a	15.2 a

[x]Data were combined over two full season trials and are means of ten replications. Seeds were dried at 30-35 °C for two weeks.
[y]Cultivars were recommended for use in Louisiana in 2015.
[z]Data were analyzed with ANOVA and Fisher's LSD test ($P \leq 0.05$). Within columns, means followed by a common letter are not significantly different.

Seed weights averaged 15.2 g for DG39, significantly less, 12.4 g, for RV56 and even less, 11.4 grams for Pp54. The lowest soil population levels of the nematode, 76.3 thousand, were from the MOR isolate (Table 2.4.) Populations of the other 3 isolates were significantly greater, averaging 143.3 for WC, 125.0 for RAP and 117.0 for TEN. Reproductive values reflected these population densities in soil. However, while exhibiting the lowest level of reproduction of the 4 isolates, the MOR isolate was the most damaging. Weights for pods and were reduced significantly in comparison to those of both non-inoculated controls and other isolates. Weights of plants were reduced significantly by isolates from RAP and MOR, which averaged 114.2 and 99.6g, respectively compared to the non-inoculated control.

Table 2.4. Main effect of isolate of *Rotylenchulus reniformis* on vermiform life stages, pods weight, and plants weight across three cultivars of soybean in a full season microplot environment[w].

Isolate[x]	Vermiform life stages per 500 cm3 of soil (1000's)[y]	Reproductive value[z]	Pod Weight (g)	Plant Weight (g)
Control	0 c	0	110.9 a	141.9 a
WC	143.3 a	77.9	99.4 ab	127.2 ab
RAP	125.0 a	67.9	88.7 b	114.2 bc
MOR	76.3 b	41.5	61.5 c	99.6 c
TEN	117.0 a	63.6	89.3 b	115.0 abc

[w]Data were combined over two full season trials and are means of ten replications. Plant material was dried at 30-35 °C. Cultivars of soybean were REV 56R63, Pioneer P54T94R, and Dyna-Gro 39RY57.
[x]Reniform nematode isolates were each derived from single egg masses isolated from roots of soybean from West Carroll (WC), Rapides (RAP), Morehouse (MOR) and Tensas (TEN) parishes.
[y]Data were analyzed with ANOVA and Fisher's LSD test ($P \leq 0.05$). Within columns, means followed by a common letter are not significantly different.
[z]Reproductive values were calculated by dividing the estimated numbers of vermiform stages per microplot (13.6 kg of soil) by the infestation level of 50,000 vermiform life stages.

2.4 Discussion

The nematological literature documents variability in the pathogenicity and reproduction within species of many plant parasitic nematodes. Variation in soybean cyst nematode (SCN) populations was described as far back as the 1970's (Golden *et al.*, 1970). Since then, many studies have confirmed the existence of variability in populations of SCN (Niblack *et al.*, 2002; Colgrove *et al*., 2002). Similarly, variability has been described in major root-knot nematode species. The host differential assay of Hartman and Sasser, 1985, which differentiated 4 major races of root-knot nematode *M. incognita*, is currently valid and used. The literature also describes variation in virulence within populations of *Meloidogyne incognita* on different crop species (Cevantes-Flores et al., 2002; Anwar and McKenry, 2007). Anwar and McKenry (2007) found that virulent populations of *Meloidogyne incognita* were associated with physiological changes both in the plant and in the nematode with the development of larger galls and giant cell

and improvement in success of juveniles transitioning into reproducing adults, compared to less virulent populations. Nematologists have been documenting the incidence of distinct races of *R. reniformis* nematode outside of North America since the 1970's (Dasgupta and Seshadri, 1971). Their host differential assay employing cowpea, castor and cotton distinguished two races of the nematode. Another study by Nakasono in 1983 was translated and published in English in 2004. His work involved isolates of *R. reniformis* from Japan, Hawaii and Texas and identified polymorphism between populations. Nakasono found three morphologically distinct groups of the nematode based on physiological and ecological characteristics. To date, there is only limited information on the variability in reniform nematode in the southern United States (McGawley and Overstreet, 1995; Aguedelo *et al*., 2005; McGawley *et al*., 2010; McGawley *et al*., 2011). Other research conducted by nematologists in Louisiana has evaluated variability in reproduction and pathogenicity of isolates of the nematode within the state (McGawley and Shankaralingam, 1994; Xavier *et al.*, 2014; and Bhandari *et al*., 2015). In all of these studies, which involved both cotton and soybean, and isolates of the nematode from multiple states or just Louisiana, the isolate of the nematode that caused the most damage was the one that reached the highest population level. Data reported herein is in contrast to that since the reniform nematode isolate from MOR parish is the one that reproduced least yet caused statistically the greatest reduction in weight of pods and numerically the greatest reduction in weight of plants. Somewhat similar observation was made by both Noe (1992) and Baimey *et al*. (2009) with three *Scutellonema bradys* isolates on seven yam cultivars have reported that the isolate form Toui in the northern Guinea savannah, having the lowest level of reproduction compared to other two tested isolates, was able to cause the greatest yield reductions. The research conducted by Noe (1992) had used cultivars of peanut, soybean, tomato, tobacco and peppers with 9 *Meloidogyne arenaria* race 1

populations from Georgia, and one each from Alabama, Florida and North Carolina. In his research Noe has reported that top dry weight of the peanut cultivar "Florunner" and the pepper cultivar "Carolina Cayenne" were significantly reduced by an isolate from Georgia having significantly lower reproduction level.

Parallel research conducted by a fellow nematology student here at LSU, Mr. Curamani. Khanal, employs the same populations of reniform nematode discussed in this current research, but uses cotton as the host plant. Data from his research also shows differences in reproduction and pathology of the nematode on soybean. A major difference in results from these two parallel lines of research involve the level of reproduction of MOR isolate on two different hosts. Across cotton genotypes, the MOR isolate exhibited the greatest level of reproduction and caused the greatest level of damage. Conversely, with soybean, the MOR isolate exhibited the lowest level of reproduction, but caused the greatest amount of damage.

Across all soybean genotypes, respectively, MOR isolate reduced plant dry weight by 29.8% and 54.8% relative to those of the non-inoculated controls. This difference in pathogenicity of MOR isolate on soybean and cotton is possibly a function of host. Averaged across four isolates of *R. reniformis* endemic in Louisiana, the reduction in harvest dry weight of plants relative to non-inoculated control was 19.6% for soybean and 27.5% for cotton. Research by McGawley *et al.* (2010, 2011) with isolates of *R. reniformis* from Alabama, Arkansas, Hawaii, Louisiana, Mississippi, and Texas showed that across isolates representing each of these states a negative impact of *R. reniformis* on plant growth and yield was greater on soybean than cotton. Averaged across the six geographic isolates, the reduction in harvest dry weight of plants relative to non-inoculated control was 27.4% for soybean and 19.7% for cotton. However, data for the Louisiana isolate of *R. reniformis* used in that research, which originated from Avoyelles

parish, showed that the isolate from Louisiana was actually more damaging on cotton than soybean. Data presented herein is in agreement with this previous observation as, across endemic isolates, the reniform nematode was more damaging on cotton than soybean.

This difference in reproduction could be attributing to phenotypic polymorphism or genetic variability within this isolate of reniform nematodes as described by Aguedelo *et al*., 2005. To further clarify this finding, studies should be conducted using molecular techniques and morphometric characterization of reniform isolates from various locations in Louisiana on a range soybean lines. Germplasm lines PI54 and PI90 had moderate resistance and resistance levels respectively, against the tested Louisiana isolates and are similar to that of previously tested Mississippi isolates (Stetina *et al*., 2014). The host status of the commercial cultivars used in the microplot trials were reported by Robbins *et al*. (2012, 2013, 2014, 2015). The cultivar RV56 was reported to have lower reproduction of reniform nematode than more susceptible cultivars by Robbins *et al*. (2015). This research found a similar pattern of reproduction among the different isolates of the nematode. The data from these studies provide enough evidence for the variability in resistance of commercial cultivars tested against native reniform isolates. Therefore, this information will be valuable for growers in selecting soybean cultivars suitable for their locations with the consideration of reniform nematode pressure within their geographical locations.

This research yielded information beneficial to the development of management strategies for nematodes and also provides an impetus for further investigations with *R. reniformis*. Notable conclusions from this research include i) there is significant variation among isolates of *R. reniformis* associated with soybean within Louisiana; ii) reniform isolates showed greater variation in reproduction on moderately and susceptible than on resistant cultivars and

germplasm lines; iii) additional studies are justified with commercial soybean cultivars and additional isolates of the nematode.

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CHAPTER 3. UTILITY OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) ANALYSIS TO ELUCIDATE GENETIC VARIABILITY IN *ROTYLENCHULUS RENIFORMIS*

3.1 Introduction

Plant parasitic nematodes are a major problem in commercial crops such as soybean and cotton in the United States (Stetina and Young, 2006; Robinson, 2007; Arias *et al*., 2009; Leach *et al*). In the southern United States, reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) is considered as a major pest due to its devastating impacts on soybean (Allen *et al*., 2017). In 2016, soybean yield losses due to reniform nematode was estimated to be around 5% of the total soybean yields in the southern United States. Out of the 16 soybean producing states in the south, Louisiana, Alabama, and Mississippi had the greatest yield losses due to *R. reniformis* damage (Allen *et al*., 2017).

Out of several management practices, crop rotation, use of nematicides, and the use of resistant soybean cultivars are widely employed. Crop rotation using non-host crops or resistant soybean cultivars are valid management practices that are currently in use. However, reniform nematode populations can easily resurge to an economic threshold level when a susceptible crop is planted (Robinson *et al*., 2007). Even though the use of nematicides as a management strategy is more effective against reniform nematode, it is not the most preferred method due to its negative impacts on human health and the environment (Agudelo *et al*, 2005). Of the management strategies available for the reniform nematode, the use of resistant cultivars is considered to be more durable and economical. To date, there are several soybean germplasm lines and cultivars that have been reported to be resistant to reniform nematode (Robbins *et al.*, 2012; 2013; 2014; 2015; Stetina *et al.,* 2014). Research has shown that durable host plant resistance is proportionate to the amount of variability present in a pathogen (Niblack *et al*., 2002; Noe, 1992; Riggs *et al*., 1981; Van der Beek *et al*., 1999). Research has also revealed that

moderate to high levels of resistance in soybean cultivars and some cotton breeding lines may not be consistent with different geographical isolates of this pathogen (Yik and Birchfield, 1984; Robinson *et al*., 1997; Robinson *et al*., 2004; Weaver *et al*., 2007). This inconsistency in performance of resistant cultivars/breeding lines could be determined by the existence of physiological and genetic variability in geographic isolates of reniform nematode. Utilizing novel molecular techniques to understand this variability would aid in developing durable reniform nematode resistant cultivars. Over the last few decades researchers have been using molecular techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSR), simple sequence repeat (SSR), and single nucleotide polymorphisms (SNPs) to understand genetic variation and to characterize multiple organisms (Grover and Sharma, 2016).

RFLP known as the first generation of molecular markers, are currently not much in use for assaying genetic variability due to complexity, cost effectiveness, and elucidation of lower rate of polymorphism (Gao *et al*., 2016; Yang *et al*., 2017). The second generation of genomic markers, such as microsatellites (SSR), are easy to obtain at lower costs and have a higher polymorphism rate (Gao *et al*., 2016). The few drawback of using SSR markers are that they often considered as tedious in high throughput genotyping protocols and lack an even distribution in the genome (Salem *et al*., 2012). The third generation of markers known as the SNPs, and with the utility of next generation sequencing (NGS) technology has gained popularity in recent years to study genetic variability in organism (Gao *et al*., 2016). Use of SNPs are considered to be a reliable tool for developing a rapid, and high-throughput assay to detect genetic differences between species (Yang *et al*., 2017). SNP analysis has been used in

analyzing variability of many species of fungi, bacteria, virus, nematode, plants, and animals (Yang *et al*., 2017; Gao *et al*., 2016; Linlokken *et al*., 2017; Ojeda *et al*., 2014; Lu *et al*., 2013; Rattei, *et al*., 2007; Morais *et al*., 2006; Faga *et al*., 2001; Figueiredo *et al*., 2013; Samson-Himmelstjerna *et al*., 2007).

Evidence of morphometric, physiological, and genetic variabilities of *R. reniformis* populations have been documented in many parts of the world including Japan (Nakasono, 2004), India (Dasgupta and Seshadri, 1971), Africa (Germani, 1978), Brazil (Rosa *et al*., 2003; Soares *et al*., 2003, 2004), and the United States (Agudelo *et al*., 2005; Tilahun *et al*. 2008, Arias *et al*., 2009, McGawley and Overstreet, 1995; McGawley *et al*., 2010; McGawley *et al*., 2011). There are contradicting results found in the literature regarding the existence of genetic variability among geographic populations of *R. reniformis.* A study conducted by Agudelo *et al*. (2005) using a collection of reniform nematodes samples from ten different states in the United States showed no variation in the first internal transcribed spacer (ITS1) region of the pathogen. Agudelo *et al*. (2005) suggested that microsatellite markers would provide a more reliable alternative to analyze the variability in reniform nematode populations. Tilahun *et al*. (2008) showed contradictory results to Agudelo *et al*. (2005) by finding fairly substantial variation in ITS1 as well as in the 18S regions of the reniform nematode populations from Alabama. Research utilizing microsatellite markers in the literature have shown promising results of detecting genetic variability in geographic isolates of reniform nematode (Arias *et al*., 2009; Leach *et al*., 2012). Use of novel technologies such as the next generation sequencing (NGS) together with SNP analysis would enable analysis of whole genomic DNA of reniform nematode in a more detailed, accurate and reliable way. This approach would be beneficial in determining genetic variability of the nematode *R. reniformis*. To date no published reports are available on

the use of SNP molecular marker analysis to distinguish genetic variability of the reniform nematode. Therefore the main objective of this research was to design and identify SNP molecular markers for the evaluation of genetic variability on endemic populations of reniform nematode.

3.2 Methodology

3.2.1 Reniform nematode isolation and extraction

Geographic populations of *R. reniformis* used in this research are as follows; two from Louisiana, six from Mississippi, three from Arkansas, and one each from South Carolina, and Georgia (Table 3.1).

Table 3.1. Sample ID, origin of samples, population type, and sources of reniform nematode populations used for SNP analysis.

Sample ID	Origin of sample[x]	Isolate[y]	Source[z]
LA1	West Carroll, Louisiana	Single egg mass	Nematode advisory service, LSU
LA2	Tensas, Louisiana	Single egg mass	Nematode advisory service, LSU
MS1	Stoneville, Mississippi	Single egg mass	Salliana Stetina
MS2	Stoneville, Mississippi	Single egg mass	Salliana Stetina
MS3	Stoneville, Mississippi	Single egg mass	Salliana Stetina
MS4	Washington, Mississippi	Single egg mass	Salliana Stetina
MS5	Washington, Mississippi	Single egg mass	Salliana Stetina
MS6	Sunflower, Mississippi	Single egg mass	Salliana Stetina
AR1	Hawkins, Arkansas	Single egg mass	Robert Robbins
AR2	Hawkins, Arkansas	Single egg mass	Robert Robbins
AR3	Kibler, Arkansas	Single egg mass	Robert Robbins
SC1	Clemson, South Carolina	Single egg mass	Paula Agudelo
GA1	Tifton, Georgia	Single egg mass	Richard Davis

[x]Origin of the reniform nematode populations employed in this research
[y]Reniform nematode populations were maintained on tomato under a greenhouse environment
[z]Persons or lab that that provided the initial reniform nematode samples

Reniform nematode populations, with exception of Mississippi, Arkansas, South Carolina and Georgia, were derived from a single egg mass (SEM) before increasing and maintaining SEM populations on tomato (*Solanum lycopersicum* L. cultivar Rutgers PS, Seedway; Hall, New York 14463) in a greenhouse environment. Approximately 300 to 400 gravid reniform nematode females from each population were handpicked with the use of a dissecting microscope and

laboratory utensils from tomato roots and were placed in petri plates containing distilled water before transferred to 2 ml centrifuge tubes.

3.2.2 Extraction of genomic DNA from gravid *R. reniformis*

DNA was extracted from the reniform isolates from Louisiana using a Maxwell 16 (Promega, Madison, WI, USA) automated DNA isolation machine. Five hundred µl of CTAB buffer, 30 µl of Proteinase K (20 mg/µl), 2 µl of RNase A (10 mg/ml, catalog No. EN0531), and 2 µl of lysozyme (500 ng/µl) were added to the 2 ml Eppendorf tubes containing 300 to 400 gravid reniform nematode females. The tubes were vortexed briefly, then gently shaken at 350 rpm while incubating 2 hours at 60 °C. At the end of the incubation process, tubes were vortexed for 5 seconds to mix the solution before processing using the Maxwell 16 FFS Nucleic Acid Extraction System (Catalog No. X9431). At the end of process, the supernatant containing the genomic DNA was collected and transferred to labelled 1.5 ml Eppendorf tubes. A microplate spectrophotometer (Synergy H1Bio-tek, Winooski, VT, USA) was used to quantify the genomic DNA at a UV absorption of 260 nm. After isolating DNA from the SEM reniform nematode sample, DNA was amplified using whole genome amplified as described in Arias *et al.,* 2009. DNA was extracted and amplifications for the reniform isolates from Mississippi, Arkansas, South Carolina and Georgia were conducted following the protocols in Arias *et al.,* 2009 and Arias *et al.,* 2011.

3.2.3 Quantitative increase of genomic DNA using whole genome amplification

Whole genome amplification (WGA) technology was used for the molecular analysis of minute quantities of DNA derived from individual nematodes, thereby enabling the analysis of genetic diversity among and within nematode populations. Genomic DNA for each reniform nematode population was amplified using the WGA process employing GenomePlex® Complete Whole Genome Amplification kits based on the instructions from the manufacturer (Sigma-

Aldrich, St. Louis, MO; Cat. No. WGA2). This step was conducted due to the need of having larger amount of genomic DNA for the multiple SNP analysis. The DNA template for WGA was derived from the genomic DNA isolations that was extracted using the Maxwell 16 FFS Nucleic Acid Extraction System. The whole genome amplification process consisted of fragmentation, library preparation and amplifications steps. For the step of fragmentation, 1 µl of 10X fragmentation buffer and 10 µl of DNA (1 ng/µl) were pipetted in a 200 µl PCR tube. The tube was placed in a PTC-200 thermal cycler (MJ Research, Waltham, MA) at 95°C for 4 minutes. Immediately the sample was cooled by placing the tube on ice for 2 minutes followed by a brief centrifugation to combine the contents. Library preparation step is as followes; to the tube, 2 µl of 1X library preparation buffer, 1 µl of library stabilization solution were added and thoroughly vortexed. The tube was consolidated by centrifugation and placing in a PTC-200 thermal cycler (MJ Research, Waltham, MA) at 95°C for 2 minutes. The sample was cooled by placing the tube on ice for 2 minutes, then combining by centrifugation and returning to ice. To the tube, 1 µl of library preparation enzyme was added, thoroughly vortexed and briefly centrifuged. The tube was placed in a PTC-200 thermal cycler and incubated with following conditions: 16°C for 20 minutes, 24°C for 20 minutes, 37°C for 20 minutes, and 75°C for 5 minutes. Tubes were removed from the thermal cycler and briefly centrifuged. For the amplification process: 15 µl of the library preparation was used for the subsequent amplification process by adding 7.5 µl of 10X Amplification Master Mix, 47.5 µl of water (molecular biology grade), and 5 µl of WGA DNA polymerase for a total volume of 75 µl. The tube was thoroughly vortexed, briefly centrifuged, and placed in a PTC-200 thermal cycler for amplification. The thermal cycler was setup as follows: an initial denaturation at 95°C for 3 minutes; followed by 28 cycles of denaturation at 94°C for 15 seconds, and annealing/extension at 65°C for 5 minutes. A 5 µl of

the final product, WGA amplified DNA, was resolved on a 1.5% Agarose gel to confirm the procedure was successful. The remaining volume of WGA DNA was purified using a GenElute™ PCR Clean-Up Kit from Sigma-Aldrich (Catalog Number NA1020). The WGA amplified DNA was quantified using Synergy H1 (Bio-Tek®, Winooski, VT, USA) microplate spectrophotometer and stored at -20°C. In order to obtain enough DNA for the subsequent SNP analyses, all WGA DNA samples were re-amplified following manufacturer's instructions using the GenomePlex WGA Re-amplification Kit (Sigma-Aldrich, Catalog Number WGA3). Re-amplified DNA samples were purified using the GenElute kits and concentrations were determined using the microplate spectrophotometer.

3.2.4 Identification of single nucleotide polymorphism (SNP) for *R. reniformis*

Putative SNPs were derived from reniform genomic DNA analysis in a previous study using nextRAD (Nextera-tagmented Reductivity-Amplifed DNA; SNPsaurus, Eugene, OR USA) technology (Dr. Jeffery D. Ray, USDA-ARS, unpublished data). Flanking sequences of 162 putative sequences are shown in Appendixes 1 and 2. From this list, 31 putative SNPs were selected for testing in the current study (Appendix 1). SNPs were selected specifically to be at different genomic locations (i.e. on different genomic contigs) as previously reported for the reniform nematode genome (RREN 1.0) at NCBI (https://www.ncbi.nlm.nih.gov/assembly/GCA_001026735.1/). The flanking sequences of the 31 selected SNPs (Table 3.2) were sent to LGC Genomics (Teddington, UK) where KASP (kompetitive allele-specific PCR) genotyping assays were designed for each SNP.

3.2.5 Kompetitive Allele Specific PCR (KASP) genotyping assay

Single base change in the genome or SNP can be detected by designing primers that amplify that particular base change. The letters [A/G] in parenthesis in the middle of the sequence as shown below gives an illustration of a SNP:

ACGCCCCGGGGGAAGGATAGAGGG**[A/G]**ATTCCCACTCTCCCCAGGGAAGC

The primers are designed in such a way as to specifically amplify one base or the other, in this case "A" or "G". KASP assays with dual emission fluorescent reactions are designed as where different wavelengths represent one or the other allele (i.e. "A" or "G" in the above case). Specific fluorescent emissions are read on a fluorimeter after a PCR amplification of the assay, and analyzed to determine which allele (or both alleles) are present in the sample. In this research, the LightCycler 480 real-time PCR equipment (Rouche Diagnostics Corporation, Indianapolis, IN, USA) was used to determine the fluorescent emissions and Rouche LightCycler® 480 software (ver. 1.5.1.62SP3) used to call alleles. Alleles were denoted as X or Y based on emission wavelengths. Mixtures of both alleles were denoted as "H or XY" for heterozygotes.

Of the 17 samples analyzed in this experiment, 4 were no-template controls and the remaining 13 were reniform nematode samples described earlier (Table 3.1). The Amplification Reaction Mix preparation provided each reaction with 10 µl of 1X KASP Master Mix, and 0.4 µl of 1X KASP Assay Mix (containing the allele specific primers unique to each SNP), and 9.6 µl of WGA DNA (at a concentration of 12.5 ng μl^{-1}). The PCR reactions were assembled in 96-well semi-skirted PCR plates with white wells and clear frames (4ti-0951, 4titude Ltd., Wotton, Surrey UK) using a Janus robot (Perkin Elmer, Shelton, CT). The plate was sealed with QPCR adhesive seals (9095-10055, KBio, Beverly, MA) and placed in a PTC-200 thermocycler (MJ Research, Waltham, MA). Thereafter the PCR reaction was conducted as follows: an initial denaturation at 94ºC for 15 minutes; followed by 10 cycles of denaturation at 94ºC for 20 seconds, and annealing/extension at 65ºC for 1 minute with a temperature reduction of -0.8 ºC

per cycle; and subsequent 26 cycles of denaturation at 94ºC for 20 seconds, and annealing/extension at 57ºC for 1 minute.

3.3 Results

Sufficient quantities and quality of DNA was obtained for SNP analysis using WGA and WGA re-amplification processes. In an ongoing project conduced at the USDA-ARS, Stoneville, MS, 162 putative reniform nematode specific SNPs (Appendix 1 and 2) that were not reported elsewhere were identified (Jeffery D. Ray, USDA-ARS, Stoneville, MS; personal communication). Nevertheless, none of these putative SNPs have been assessed to conclude their performance against reniform nematode populations. Of the 162 identified putative reniform nematode specific SNPs, 31 SNPs were designed and manufactured to function as dual emission fluorescent KASP (kompetitive allele-specific PCR) primers (Table 3.2). These KASP primers permitted the bi-allelic scoring of SNPs at specific loci including those in complex genomes. The 31 KASP SNP primer sets were tested on 13 reniform nematode isolates in this research. The 13 reniform nematode isolates tested in this research were collected from Louisiana, Mississippi, Arkansas, South Carolina, and Georgia (Table 3.1). Twenty six of the 31 SNPs tested, were able to amplify genomic DNA of reniform nematode isolates from different geographic locations with a success rate around 84%. Five SNPs failed to successfully amplify. Results from KASP genotyping assay are summarized in Table 3.3.

For the SNP analysis of the reniform nematode isolates from Louisiana, 25 SNPs were able to amplify, whereas 6 SNPs were unsuccessful in amplification. Of the 25 functioning SNPs, a total of 10 appeared to identify only one allele (four for allele X and six for allele Y), while seven identified both alleles only (heterozygous DNA). The remaining eight SNPs

identified allelic variants (i.e. genetic differences) among the Louisiana reniform nematode isolates.

SNP analysis of the samples from Mississippi revealed that seven SNPs were monomorphic while three identified only heterozygous loci out of the 26 amplified SNPs. Of the 26 SNPs, five failed to amplify and the results of one SNP assay could not be determined after amplification. The other remaining 15 SNPs identified genetic differences among the reniform nematode isolates from Mississippi.

Of the 31 SNPs tested on samples from Arkansas, only two did not amplify. Six SNPs out of the remaining 29 SNPs were monomorphic for the Y allele while the other four detected heterozygous loci in the tested samples. The remaining 19 SNPs were able to detect allelic variants in reniform nematode isolates from Arkansas.

Due to the limitations of samples, only a single isolate of reniform nematode were tested from South Carolina, and Georgia during this research. For the two samples each from South Carolina, and Georgia, four SNPs failed to successfully amplify genomic DNA. Of the remaining 27 SNPs that amplified, 9 SNPs identified allelic differences among samples from these isolates. Of the remaining assays, 12 were monomorphic and another five detected heterozygous loci in the tested samples.

The results from multiple isolates of reniform nematode from Louisiana, Mississippi, and Arkansas provided evidence for the existence of genetic differences between and among the geographic isolates. The 26 SNPs that, for the most part, amplified genomic DNA of reniform nematode isolates from different geographic locations, four (RREN_4410_3972, RREN_4834_4618, RREN_5033_5267, and RREN_269_9935) were able to distinguish genetic differences between and among isolates of reniform nematode from Louisiana, Mississippi, and

Arkansas. Even with limited numbers of samples, a genetic difference was observed with three SNPs between South Carolina, and Georgia isolates. Unfortunately, due to the limited number of isolates coupled with the limited number of SNPs tested, the degree of genetic differences among these isolates could not be properly elucidated.

3.4 Discussion

This research was conducted using the SNP analysis to evaluate the genetic diversity of *R. reniformis* from different geographical locations in Louisiana, Mississippi, Arkansas, South Carolina, and Georgia. There is evidence in the literature to provide information on the existence of genetic variability in variety of nematode species using first and second generation marker technology. Research using first and second generation marker technology to evaluate genetic variability was done on *Caenorhabditis elegans* and various plant parasitic nematodes including cyst nematodes (Caswell-Chen *et al.*, 1992; Folkertsma *et al.*, 1994; Kalinski and Huettel, 1988; Silva *et al.*, 2000), root-knot nematode (Guirao *et al.*, 1995; Semblat *et al.*, 1999; Tigano *et al.,* 2010; Khanal *et al.*, 2016), rice white tip nematode (Figueiredo *et al.*, 2013), and reniform nematode (Agudelo *et al.*, 2005; Tilahun *et al.*, 2008; Arias *et al.*, 2009). Even though several attempts were made to understand the genetic variability of the reniform nematode in the past, this research will be the first to report genetic variability in geographic isolates of reniform nematode with the use of SNP analysis.

Out of 162 putative SNPs identified, a total of 31 putative reniform nematode specific SNPs that were not reported elsewhere and selected from the reniform nematode genome, based on previous research (Dr. Jeffery D. Ray, personal communication) were used to confirm their functionality in this research (Appendix 1 and 2).

Table 3.2. Summary of SNP ID, contig, sequence position, fluorescence label for each SNP, GC content, and LGC Genomics reference number for the SNP-specific primers employed in this research.

SNP ID[w]	Contig	Sequence Position	Allele FAM[x]	Allele HEX[y]	GC% FAM	GC% HEX	GC% Common	LGC Genomics[z]
RREN_4410_3972	4,410	3,972	T	C	54.5	57.1	61.9	1140749440
RREN_1572_36933	1,572	36,933	T	C	45.8	52	59.1	1140749445
RREN_4410_3979	4,410	3,979	A	G	56	59.1	73.7	1140749464
RREN_43396_315	43,396	315	T	G	44	50	59.1	1140749374
RREN_523_19992	523	19,992	A	C	42.3	45.8	48	1140749391
RREN_367_3958	367	3,958	T	G	52.2	54.5	39.3	1140749398
RREN_4834_4618	4,834	4,618	A	G	42.3	45.8	48	1140749415
RREN_5033_5267	5,033	5,267	T	C	42.3	44	59.1	1140749422
RREN_845_36717	845	36,717	A	G	35.7	38.5	48	1140749439
RREN_3215_15723	3,215	15,723	T	C	42.3	44	37.9	1140749446
RREN_1660_513	1,660	513	T	C	30	35.7	33.3	216484048
RREN_4410_3946	4,410	3,946	A	G	65	68.4	59.1	216484047
RREN_7711_4758	7,711	4,758	T	C	42.3	45.8	48	216484024
RREN_514_63176	514	63,176	A	G	54.5	57.1	59.1	216484023
RREN_925_39379	925	39,379	C	G	33.3	31	48	216484000
RREN_514_63173	514	63,173	T	G	59.1	65	54.2	216483999
RREN_91287_201	91,287	201	A	C	44	45.8	33.3	216483976
RREN_91287_193	91,287	193	T	C	44	45.8	37.9	216484070
RREN_43396_339	43,396	339	A	C	29	30	59.1	216484049
RREN_1990_6847	1,990	6,847	A	G	37	44	46.2	216484046
RREN_20709_1089	20,709	1,089	A	C	33.3	37	37.9	216484025
RREN_258_12977	258	12,977	A	G	52	52.2	48	216484022
RREN_269_9935	269	9,935	A	G	24.2	30	46.2	216484001
RREN_456_104249	456	104,249	T	G	34.5	37	48	216483998
RREN_43396_325	43,396	325	T	G	37	40.7	59.1	216483977
RREN_901_49990	901	49,990	A	G	30	31	37.9	216484069
RREN_1886_12077	1,886	12,077	C	G	37	37	48	216484050
RREN_16875_158	16,875	158	T	C	24.2	30	73.7	216484045
RREN_251_23034	251	23,034	T	C	52.2	54.5	50	216484026
RREN_1895_31360	1,895	31,360	A	T	44	44	33.3	216484021
RREN_9137_320	9,137	320	T	C	35.7	42.3	37.9	216484002

[w]SNPs were assigned and chosen across the reniform nematode genome so that each SNP is not clustered together with the others

[x]End of primers were labelled with FAM and HEX fluorescence dyes which generate specific fluorescence signals which is detected and identified by LightCycler 480 software

[y]Percentage of Guanine and Cytosine in a SNP sequence

[z]LGC Genomics reference number for each SNP-specific primers used in this research

Table 3.3. Likely alleles as designated by LightCycler 480 software after the reaction of 31 single nucleotide polymorphism (SNP) analysis on isolates from Louisiana, Mississippi, Arkansas, South Carolina, and Georgia.

SNP ID[x]	LA1[y]	LA2	MS1	MS2	MS3	MS4	MS5	MS6	AR1	AR2	AR3	SC1	GA1
RREN_4410_3972	H	H	H	X	X	UNK	Y	H	H	X	UNK	UNK	H
RREN_1572_36933	H	Y	H	H	H	H	H	Y	-	-	Y	UNK	H
RREN_4410_3979	X	X	X	X	H	X	X	X	H	H	X	X	X
RREN_43396_315	-	-	-	-	X	-	-	-	H	H	X	-	-
RREN_523_19992	H	H	H	H	H	H	H	H	H	H	H	H	H
RREN_367_3958	Y	UNK	Y	Y	Y	X	UNK	X	Y	UNK	Y	X	Y
RREN_4834_4618	X	H	H	H	-	X	Y	X	Y	Y	Y	X	X
RREN_5033_5267	X	Y	H	H	-	X	Y	H	UNK	UNK	H	UNK	X
RREN_845_36717	H	H	H	H	H	H	H	H	H	H	H	H	H
RREN_3215_15723	X	H	UNK	UNK	-	X	X	H	H	H	UNK	Y	UNK
RREN_1660_513	H	H	H	H	-	H	H	H	Y	X	Y	H	H
RREN_4410_3946	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_7711_4758	Y	Y	Y	Y	X	Y	Y	Y	H	H	Y	Y	Y
RREN_514_63176	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_925_39379	H	H	H	H	H	Y	Y	H	-	-	H	H	H
RREN_514_63173	Y	H	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_91287_201	-	-	-	-	Y	-	-	-	Y	Y	Y	Y	-
RREN_91287_193	-	-	-	-	H	-	-	-	H	H	H	-	-
RREN_43396_339	-	-	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_1990_6847	X	X	X	X	X	X	Y	X	H	UNK	X	UNK	X
RREN_20709_1089	H	X	UNK	UNK	UNK	UNK	UNK	UNK	Y	UNK	Y	UNK	X
RREN_258_12977	X	X	X	X	X	X	X	X	Y	X	X	X	X
RREN_269_9935	Y	H	H	H	H	Y	UNK	X	Y	Y	X	H	Y
RREN_456_104249	X	X	X	X	X	X	X	X	H	X	X	X	X
RREN_43396_325	-	-	-	-	Y	-	-	-	Y	Y	H	-	-
RREN_901_49990	Y	Y	X	X	X	Y	X	H	H	H	Y	X	X
RREN_1886_12077	X	X	X	X	X	X	X	X	Y	X	X	X	X
RREN_16875_158	-	-	-	-	Y	-	-	-	Y	Y	Y	-	-
RREN_251_23034	X	X	Y	Y	UNK	UNK	Y	H	Y	Y	X	X	H
RREN_1895_31360	H	H	H	H	H	H	Y	Y	H	H	H	H	H
RREN_9137_320	H	H	H	H	H	Y	UNK	Y	UNK	Y	-	Y	Y

[x]SNPs were assigned and chosen across the reniform nematode genome so that each SNP is not clustered together with the others

[y]Samples were collected from different locations in Louisiana (LA), Mississippi (MS), Arkansas (AR), South Carolina (SC), and Georgia (GA) and propagated/maintained in tomato

[z]X, Y, and H represent LightCycler 480 calls for FAM, HEX, and both fluorescent labels, respectively. "UNK" indicates LightCycler 480 could not distinguish the fluorescence while "-" indicates the failure of SNP on that particular isolate

These selected SNPs were used to design bi-allelic KASP genotyping assays and tested on genomic DNA of 13 reniform nematode isolates obtained from Louisiana, Mississippi, Arkansas, South Carolina, and Georgia to detect genetic differences among the isolates (Table 3.2).

In this research, 26 out of the 31 tested SNPs were able to amplify genomic DNA with a success rate of 84%. This high level of success rate in the SNPs tested in this research was comparable to similar studies conducted in plants with similar success rates of 78.5% to 88.4% (Cockram *et al.*, 2012; Saxena *et al.*, 2012; Semagn *et al.*, 2014; Graves *et al.*, 2016). With the success rate we obtained from the 31 SNPs used in this study, we could assume a similar success rate for the 131 putative SNPs that were not tested during this study (Appendix 2). After testing all 162 putative SNPs, information about the SNPs for identification of genetic variability of the reniform nematode will be available to the science community. The sequence information documented for the tested 31 SNPs in this study using KASP assays shown for each SNP can be utilized to prepare reniform nematode specific SNP assays together with the LGC Genomics Reference number shown in Table 3.2. As mentioned in the Results, five out of the 31 SNPs had very poor amplification and did not function properly. Optimizing PCR conditions might be useful to achieve better amplification of these failed SNPs. Results revealed that in many instances, the SNPs that were used in this research recognized the two SNP alleles as well as the heterozygous state (Table 3.3). Nevertheless, some SNPs only identified one allele or the other and most only identified heterozygous alleles (Table 3.3). Lack of genetic diversity at that genomic location probably indicates a monophorphic SNP whereas heterozygotes likely indicate the genetic variation within the samples. In future studies, increasing the number of different

isolates used is an approach to overcome this problem and thereby likely increase the detection frequency of all alleles.

Chapter 2 in this dissertation described difference in reproduction and pathogenicity among the reniform nematode isolates from 4 geographical locations in Louisiana with the use of greenhouse and microplot experiments using soybean genotypes. Those experiments conducted in both greenhouses and the microplots environments, revealed differences in reproduction and pathogenicity among the tested reniform isolates. According to results from those experiments the isolate from Morehouse parish designated as MOR had the least reproduction and the greatest pathogenicity compared to the isolate from West Carroll (WC) parish. The isolate from Tensas (TEN) and Rapides (RAP) parishes had moderate levels of reproduction and pathogenicity with slight dissimilarities among soybean genotypes. Therefore it was evident from those experiments that there are occurrences of variability among geographic isolates of reniform nematodes in Louisiana. Similarly to Chapter 2 in this dissertation a parallel research conducted by C. Khanal, have found significant differences in reproduction and pathogenicity with his greenhouse and microplot experiments using cotton as the host. Among the same isolates of reniform nematode from Louisiana, he has reported that Morehouse isolate (MOR) having the greatest reproduction and pathogenicity on cotton whereas Rapides isolate (RAP) having the least. He has stated that the isolate from Tensas (TEN) and West Carroll (WC) parishes had moderate levels of reproduction and pathogenicity (C. Khanal, personal communication). These findings from greenhouse and microplot research could be supported with similar research conducted by McGawley *et al.*, 2010:2011 using soybean and cotton. All of this research indicates the presence of variability in reproduction and pathogenicity of geographical isolates of the reniform nematode.

In the current research we found that eight SNPs of the 26 assays were able to identify non-heterozygous differences among the reniform nematode isolates from WC and TEN in Louisiana. Therefore this indicates the occurrence of molecular variability in isolates of reniform nematode from Louisiana. Due to the fact that common reniform nematode isolates used in both studies discussed above had different levels of reproduction and pathogenicity together with genetic differences in SNP analysis, we could make an assumption that these tested SNPs might have an association with biological functions in reniform nematode. This assumption could be further strengthen by the findings from Salem *et al.* (2012) stating that some SNPs are associated with biological functions in an organism. Therefore more emphasis should be given to explore the association of SNPs with biological functions in future experiments. When the available reference sequence for reniform nematode (RREN 1.0 assembly at NCBI) becomes more complete and fully annotated, genes in the areas around the SNPs that identified genetic differences in this study can be examined for potential biological functions. When considering the geographic origin of the reniform nematodes used in this study, most of the SNPs tested were polymorphic among and within the reniform nematodes from different locations. When further analyzing these polymorphisms, it was evident that SNPs polymorphic for reniform nematodes isolate in one geographic location would not be polymorphic in an isolates from another location (Table 3.3). Therefore this was enough to provide evidence for the presence of genetic variability within and among different locations. Therefore, the use of SNP assays could be a valid technique to identify genetic variability of reniform nematodes present in diverse geographical locations. Having a large number of SNPs spread throughout the reniform nematode genome would be beneficial to pin point the reniform nematode isolate having the greatest level of genetic diversity.

Studies conducted in the past to understand the amount of genetic variability in geographic isolates of reniform nematode either had contradicting results (Agudelo *et al.* 2005; Tilahun *et al.* 2008) or used markers that lack wide range distribution in the genome (Arias et al., 2009; Leach *et al.*, 2012). Therefore, SNP markers are much more efficient and powerful (Salem *et al*., 2012) for detecting genetic diversity of *R. reniformis* compared to those previously published techniques in the literature. To further understand and to confirm the existence of genetic variability of *R. reniformis* observed in this research, extensive research should be conducted. This could be achieved by using larger number of reniform nematode isolates collected from wider geographical locations in multiple states of the USA and samples around the world. When the future research enable to link SNPs association with reproduction and pathogenicity functions by understanding the specific location of SNP in a gene and subsequent gene function, these markers will be beneficial for the breeders to develop high yielding crops resistent to *R. reniformis*.

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CHAPTER 4. SUMMARY AND CONCLUSION

In many locations in the southern United States including Louisiana, resistance found in soybean and cotton cultivars/breeding lines are not consistent in their response to different geographical isolates of the reniform nematode *Rotylenchulus reniformis* (Xavier *et al*., 2014; Bhandari *et al*., 2015C. Overstreet, personal communications). Therefore the experiments described in this dissertation were conducted to address this phenomenon. To understand the pathogenicity and reproduction of the reniform nematode, responses of commercial cultivars and resistant germplasm lines of soybean were evaluated on indigenous isolates of the reniform nematode in Louisiana under microplot and greenhouse environments. These experiments were conducted during 2016 and 2017 with single egg-mass populations of *R. reniformis* isolated from West Carroll (WC), Rapides (RAP), Tensas (TEN) and Morehouse (MOR) parishes of Louisiana. Data from both full-season microplot trials and 60 day greenhouse trials, averaged over 2 trials, displayed significant differences in reproduction and pathogenicity of the nematode with the commercial cultivars of soybean, REV 56R63, Pioneer P54T94R, and Dyna-Gro 39RY57. In the microplot experiments, there was a significantly reduced population density (46.8%) in the isolate from the MOR parish compared to the isolate from the WC parish. The isolate from MOR was also the most pathogenic and resulted in significant reductions in soybean plant and pod weights compared to that of the control, 29.8% and 44.6%, respectively. Similar trend in reproduction and pathogenicity of *R. reniformis* had been documented by McGawley *et al*., 2011. In the greenhouse trials the susceptible cultivar Progeny P4930LL and the resistant germplasm lines PI 90763 and PI 548316 were tested together with the same cultivars used in the microplot trials. Similar to the microplot trials, the MOR isolate had the least level of reproduction with a 33% reduction compared to that of WC, the isolate with the greatest level of

reproduction. In both microplot and greenhouse trials, the soybean cultivar REV 56R63 had a significant reduction in reniform numbers compared to cultivars Pioneer P54T94R and Dyna-Gro 39RY57. The cultivar REV 56R63 demonstrated a resistant level similar to that of the moderately resistant germplasm line PI 548316 with all tested isolates. This resistance found in the cultivars REV 56R63 was previously reported by Robbins *et al*., 2015. In the greenhouse trials the resistant germplasm line PI 90763 was able to hold its resistance compared to tested cultivars and germplasm lines against all reniform nematode isolates.

In the past, literature have shown the occurrence of morphometric, physiological, and genetic variabilities within *R. reniformis* populations with the use of morphometric data, and first and second generation marker technologies (Nakasono, 2004; Dasgupta and Seshadri, 1971; Germani, 1978; Rosa *et al*., 2003; Soares *et al*., 2003, 2004; Agudelo *et al*., 2005; Tilahun *et al*. 2008; Arias *et al*., 2009, McGawley and Overstreet, 1995; McGawley *et al*., 2010; McGawley *et al*., 2011). The experiments discussed in chapter three with the utility of single nucleotide polymorphism (SNP) analysis, were conducted to understand the genetic variability among 13 geographic isolates of *R. reniformis* from Louisiana, Mississippi, Arkansas, South Carolina, and Georgia using third generation molecular maker technology (Gao *et al*., 2016). After going through the extraction of genomic DNA from gravid female nematodes for each reniform nematode population, DNA was increased quantitatively using the process of whole genome amplification to obtain a sufficient amount of genomic DNA. Thirty one putative SNPs were chosen from the previously assembled genomic DNA of the reniform nematode and were tested using kompetitive allele-specific PCR (KASP) genotyping assay. Out of the 31 tested SNPs, 26 SNPs, with a success rate of 83.9%, were able to amplify genomic DNA of reniform nematode isolates from all locations while the remaining SNPs failed to amplify for the most part.

Repeating the experiments multiple times while optimizing the PCR conditions would be useful to achieve a better amplification of these failed SNPs. Altogether from the SNPs that were able to amplify genomic DNA, four SNPs identified as SNP_515, SNP_521, SNP_522, and SNP_537 were able to detect genetic differences between and among isolates of reniform nematode from Louisiana, Mississippi, and Arkansas. Even though there are several reports indicating genetic variability in *R. reniformis* (Arias *et al*., 2009; Leach *et al*., 2012), this research will be the first to report genetic variability in genomic DNA among *R. reniformis* using SNPs in kompetitive allele-specific PCR genotyping assay. Further studies should be conducted together with more SNPs and more reniform nematode isolates across diverse geographical locations to fully understand SNP polymorphism and its association with biological function in this pathogen. Findings described in this dissertation would be beneficial in resistance breeding programs to develop high yielding crops resistant to reniform nematode as well as in the evaluation of the genetic diversity of the nematode *Rotylenchulus reniformis.* This findings might also be beneficial for providing soybean cultivar recommendations for growers in different geographical locations.

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APPENDIX 1: SEQUENCES OF SNPS EMPLOYED IN THIS RESEARCH

Flanking sequence information for SNP assays tested in this study. The "SNP ID" givers information on organism abbreviation "RREN" *Rotylenchulus reniformis* (nematodes), the reference assembly contig (first number) and the sequence location (second number) of the SNP position.

S.N.	SNP ID	SNP	Surrounding Sequence (approximately 100 bp each side)
1	RREN_4410_3972	[T/C]	AAGCAGACAGCGAAAAAGCCCCACTCGTGACCGCGTAGAGGGATAGCGAGGAA GGGATGGGGAGGCGACGAGCGAAAAAGACGCCCCGGGGGAAGGA[T/C]AGAGG GAATTCCCACTCTCCCCAGGGAAGCAAGTACGGGGAAACCACTCAGATGCGATG AGAACGAAGGGTTTTCGCTTAGGAAAAGGCAATGCGAGAGGAT
2	RREN_1572_36933	[T/C]	TTTTGGACATCTTTCGCTTCTCCTGGACAATTTTCTATCTTTCGAATATTTTGGAC TTTTTTGGACACCTTATGATTGACCATTTACAGCCCCATCC[T/C]GCTGGCCAAGC GGTCTCCTACTCGGCCCAGCAGAAGAACCTGTTGATGTGGGCGGTGGCCGTCGG CTCCATGCTCGGCACTTTCCCCTTCGCCTGGCTCTAC
3	RREN_4410_3979	[A/G]	CAGCGAAAAAGCCCCACTCGTGACCGCGTAGAGGGATAGCGAGGAAGGGATGG GGAGGCGACGAGCGAAAAAGACGCCCCGGGGGAAGGATAGAGGG[A/G]ATTCC CACTCTCCCCAGGGAAGCAAGTACGGGGAAACCACTCAGATGCGATGAGAACG AAGGGTTTTCGCTTAGGAAAAGGCAATGCGAGAGGATTCGCTGT
4	RREN_43396_315	[T/G]	ACTGTAAACAGGAATTCGCATATTCTGAGACCACCATCGTGTAGAGCATGGTCG ATAATAATAAGGAAGTGACATCCTTTTTTGGCACAAACCCCTG[T/G]TTAAATTT TGAGTGAATTTTTAAAATATTTTTTCCACGTGCTTCAAGCACGGGTCATCGGTGC TAAAAATGTCTTTTGGTCAACAAAGCTCAATAAGTTAAA
5	RREN_523_19992	[A/C]	CGGTAACCGAACGGCAGCGTTTCCATCCCCGGTCTTATACGACCCTTCTCGTAGT GAGGTCTATAATTTTTGTGTGCCTCATAGATGTAAAGATCGG[A/C]CAGCGGGGT GCGTCCAATGTGGGGAAAGGGCACATGAATGGTTGAGTCATTTCCCGGGAACAC GAACACGTCATCAGCTCCATCAGCACCGGCTCCATCATT

Appendix 1 Continued.

6	RREN_367_3958	[T/G]	AAAGATCCATTGAAGACTATGACAGCGACGATCTTGAAGGAGAGGAGGAGGAT GACAGTTTGCCAAGAGTTTGGACTGTTTTCCATCGCTATGAGGA[T/G]TTCTATGC GCTAGAGGACAGGCTCCGGGAGCAGTACGGGAACACGCTGAGGATGAGCACAC TGCCGGACCGGAGACCAACTCTACAACTGCTACAATTGGGC
7	RREN_4834_4618	[A/G]	CCATATTTTTGGGGTTGGTTGGTGGTCATGGATTATGTTTTGGGGTTGGTTGGTG GTCATGGATTATGTTTTTGGGGATGTTTTATGGTCATGGACA[A/G]TGTTTTTGGG GATAGTTGGTGGTGATGGACCAAGTTTTTGTGGATAGTTAGTGGTCATGGACAG TGTTTTGGGGATGGTTGGTGGTTGGTATTATTTCGTCT
8	RREN_5033_5267	[T/C]	ACTTCCATCTCCAAGTTGTTTATAGAGATGTTTGCCGAGTTCAGTGGGATTCGTG ATATTCGAAGGGGGCACTGTTACACGCACCTCCTGCCCTGTT[T/C]GCCATACAA CTCGCATATATTGCTGTTCCATAGTTCCCAAGGATGGCCATGAATGGGGATAAA CCATCACTGCAAGCCCCACATACCAATCTGAATTGAATT
9	RREN_845_36717	[A/G]	GACTTTCTGCATGGCTTTGAGGAGTAAAATTCTTGCCTAAAATTACAATCTTGTT TTATTAGTTTTTTATTCAAAAAAATAGCTTACAGCAGAGGTC[A/G]TGAACAATG AGATGATGATGTTGGAGCACGCGTGCTCCTCGACCTGAATTATGAAAAAGTTTA TTTTTCTCGAATAAAAATATCTAATTTATAAAAACATAC
10	RREN_3215_15723	[T/C]	TTTTTCATGAGCACTCTTTTCGTTTTCTTCAACACTTTTTCCTGAGCAATCTCTCG TTTCAACGAACACTTTTTTTATGGGGGTAAACCGTACAATA[T/C]AAGAGCCGAC TTATTTTGTACAGCGTACACTCTACACTGTATTGCAAAATAGAATTAAAAAATAA AAATAGTCTGGTACTTAGGTATATAGTTTACGTGACC
11	RREN_1660_513	[T/C]	GGGTGCGGTAGTCGGTTCGGCTTATGGCGTAGAGCTGATCAGTGAGAACCAGAT CCCGTCCGCACTCCAAGACATATTCCAAATTGGTGATGCTTAA[T/C]GAAAATGT TTGATCTTCTGACATGAACCAATCATCAAACATGGTTCCTTTCATTTCCTGCATC AACACGTACCGGCATTGTGTGTCCGCTTTGGGGCGGTTC
Appendix 1 Continued.			

12	RREN_4410_3946	[A/G]	GGAAAGTGTCGCTCTCGACGAAAAGGAAGCAGACAGCGAAAAAGCCCCACTCG TGACCGCGTAGAGGGATAGCGAGGAAGGGATGGGGAGGCGACGA[A/G]CGAAA AAGACGCCCCGGGGGAAGGATAGAGGGAATTCCCACTCTCCCCAGGGAAGCAA GTACGGGGAAACCACTCAGATGCGATGAGAACGAAGGGTTTTCG
13	RREN_7711_4758	[T/C]	GGAGGTGAGAGAGTGTAGAGTGGTGAAGTGGAGGTGAGAGAGTACGGTATGTG AGAGAGTACGGTATGTGACAGTACCGTGTTGTCCACGACCACGA[T/C]ACACTCT TTGTTGGTTGCCTTCACAACTCGGCAAACGGCCTCAATATCGATGACTTTCAGCA ATGGGTTGGACGGTGTTTCGAACCAGACCATCTATGGATG
14	RREN_514_63176	[A/G]	TGCCTCCAAATCCTCGGATTTTTCAGAAATTCGTCAAAATTTTATTGGCATTTTTT CTGTGTAGAGAGTTTATTGGAAGTCGGGAGGTGTGGCTGAA[A/G]AGCATCTCCA ACCTGTTGCCGCGGCACATCCTCAAGGCCTCATTGGCACTGCAGTCGGTGGTGC ACCAGTACGAGCCGGACGCCATGATGCCAATCCCGTCA
15	RREN_925_39379	[C/G]	GAGGCAAGAGGCATCGAACAAATGGATCAATCTGTCCCTACTTCCGGAAGCCAG CAAATGAAGCTGATCTGGTGCTAAATTTAACCTTATGTATTCA[C/G]TTGGAAAT AGCAAAAATTGATAAAATGAAAAAATGGACTAACCAGCCGAAGGATAGTTGTA TTCCAGACACAACACCTCGCTGTCATGTGCCTCTAATTCGA
16	RREN_514_63173	[T/G]	TTTTGCCTCCAAATCCTCGGATTTTTCAGAAATTCGTCAAAATTTTATTGGCATTT TTTCTGTGTAGAGAGTTTATTGGAAGTCGGGAGGTGTGGCT[T/G]AAGAGCATCT CCAACCTGTTGCCGCGGCACATCCTCAAGGCCTCATTGGCACTGCAGTCGGTGG TGCACCAGTACGAGCCGGACGCCATGATGCCAATCCCG
17	RREN_91287_201	[A/C]	CTCTGAATTCCTCGTATTATGAAAATGAGTACAGCTATTCGCAAGTCTTACCATA CATATATTCTAATTAATAGTTTTCCTTCTACCGATGTTCCTC[A/C]CTCTCTGAATT CCTCGTATTATGAAAATGAGTACAGCTATTCGCAAGTCTTACCATACATATATTC TAATTAATAGTTTTCCTTCTACCGATGTTCCTCCGC
Appendix 1 Continued.			

18	RREN_91287_193	[T/C]	CTCTGAATTCCTCGTATTATGAAAATGAGTACAGCTATTCGCAAGTCTTACCATA CATATATTCTAATTAATAGTTTTCCTTCTACCGATGTTCCTC[T/C]GTATTCACCTC TCTGAATTCCTCGTATTATGAAAATGAGTACAGCTATTCGCAAGTCTTACCATAC ATATATTCTAATTAATAGTTTTCCTTCTACCGATGT
19	RREN_43396_339	[A/C]	CTGAGACCACCATCGTGTAGAGCATGGTCGATAATAATAAGGAAGTGACATCCT TTTTTGGCACAAACCCCTGGTTAAATTTTGAGTGAATTTTTAA[A/C]ATATTTTTT CCACGTGCTTCAAGCACGGGTCATCGGTGCTAAAAATGTCTTTTGGTCAACAAA GCTCAATAAGTTAAAATTAAATAAAGAAAAAAAATGCAG
20	RREN_1990_6847	[A/G]	TAGGTGCTCGATTTCCCGACCATCCATTATGTCCGCCGTTCCTTTTCCGCTCGAGT GCTAGCCGGATGCTATATATTGTCCGGACTGTGTAGAGTAT[A/G]GCCAAGAAGA TTGTGAGCAGAATGGCCAGATAGCAGAAAAGATGAGTCCAGATGCTGTTCCCCA AGTTTTTGCAAAGATAGGCAAGCGGGTTGTGCGGCTCA
21	RREN_20709_1089	[A/C]	AATAGGCCAATGCCTTTTTTTCTGCTCATATGAAATTCGACATTTTTGCCTTTTTG GTGGAGTTGGGGTGTATTCAGAAGAGCTTGATTTTTGATCG[A/C]CTTAAATAAA GGATATTTACAAATTTAGAACATATTTTCTTACCATTTCCCTGTTCGGATTCATCG GAACTCTCGGATTCGCCTTCTCCATCTGACGACACT
22	RREN_258_12977	[A/G]	AGTGTTCTGTTAGACAGTATAGGCAATTAGTTAGTATTTTCACCATTTGCTCTGC ATCACCGTTCGGCTAATGGCTAGATGAAGGGATATGCTCCCC[A/G]CGGGCTTGA ATATATGTCTGCACGGCGGTGGGATTCGAACCCACGTCCCGGGATTTAGCGGTC CCGTGTGATAGACCACTACACCACGCCGCCGACTCTACA
23	RREN_269_9935	[A/G]	GAGCCTTGCAATAGTGAACTATGTATCAAGGGAATCAAAGAACTAAAAAATTGG TTGAAAAAATTTTAGCAATGGAAAAAAACTTGAATAAATTGCA[A/G]AGAGAAT CAGCTAAGATCTGGTCGGGATAAGAGTTGACAACATCTTAAATAGTAACGATTT TTTGTCATTAGAAAGAAAATAATCACTTGTTATAAAAGTAA
Appendix 1 Continued.			

24	RREN_456_104249	[T/G]	TTTAACCGTCCCATTAAATTTTTAGCCGTCCCATCACAGTTTAACCGTCCCATCA AATTTTTAACAGTCCAACCAGCTTCAATTTCCGACAAAATTA[T/G]TTTGTCAACA GAATAGAAATATATAATCGCGGAACATGTTGAACCGGGAAGTACGATTGTGTCG GATGGATGGCGCTCTTATGGCGGTATTAGAGCTCTACA
25	RREN_43396_325	[T/G]	GGAATTCGCATATTCTGAGACCACCATCGTGTAGAGCATGGTCGATAATAATAA GGAAGTGACATCCTTTTTTGGCACAAACCCCTGGTTAAATTTT[T/G]AGTGAATTT TTAAAATATTTTTTCCACGTGCTTCAAGCACGGGTCATCGGTGCTAAAAATGTCT TTTGGTCAACAAAGCTCAATAAGTTAAAATTAAATAAA
26	RREN_901_49990	[A/G]	AATTCGCATATTCTGACAACACCATCGTGTAGAGAGCAATAAGTAGTAAGGAAG TGATATCCTTTTTTGGCATAAACCCCTGCTGGTTAAAATATTA[A/G]TGAATTTTC AAACCAAATTTTCCACATGCCTTAAGCGCGGGTCATCGGTGGGTCACAGGCCAT TATGGTCAGCCAAATTTCAAAAAACAAACTAGAGTAAAC
27	RREN_1886_12077	[C/G]	GTCCAATGTGTGGAAAGGGTACATGGATGGTGGAGTCATTTCCCGGGAACACGA ACACGTCATCAGCTCCACCAGCATCACCACCATCATTGTACAG[C/G]ATGGGATC GCTAACAAAGAAAATTTCCTAGATTTAACTAAAGGTAAAAAGACTCACGCTTCT CCATCAATATCATAATGTTCCAAGACGAGGGCCTTCACCG
28	RREN_16875_158	[T/C]	CAGTAACATCAACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCGCCTTAACACCGC CGGGTCCAACAACTGCGCCAGCCACCGGGGACATTCGAGCCA[T/C]ATGTTCAAT TTGTTCATTCATACCATCTATTTCAACTGCTCAAAGCAGTAACATCAACTCTCTT CTCTCCGTTCGCCTCCTCCTGCCGCCTTAACACCGCCG
29	RREN_251_23034	[T/C]	CGGCGGTTCCGCCAGCTTTGCCTGCCAAAAAATCGGCAAAATGGTCGATGGACA CAGATGGATGCAGGCATTCGATGGGTTTGGTGTAGAGCGCCGG[T/C]CTAACACA TTTCAGGGCGCCAGGGCAAGAGCAGTTCACCTGCTCAATCCATTGCAGAAAGGT AGGGGAGGGGGCCATTTTTTCAGAATTGGAAGTGTAATGG
Appendix 1 Continued.			

30	RREN_1895_31360	[A/T]	GTAGAGAGCAAAAAGAGATTAATTAAAACCTAAATTTGTCCATGCCCGACTGAG TTGAAAAAGAAAATTTATAGACACGAATAGTTGTAGATGAGGG[A/T]TAGAAGA AATGGTGTAGTATTTTGAGGAAAAGATCGAAAGAAAACGTGAGACAAAGGGAA ATTTTAGTTTCGAATACTTTTCTAACATCAATCAAAGGCTCT
31	RREN_9137_320	[T/C]	ACGGATAGACCCATATCTATCCAAGGTCCATATTTGGATTTCAACAGACATTCCC ACCCATATACGGATAGACCCATATCTATCCAAGGTCCATATT[T/C]GGATTTCAA CAGGTATTCACATCCATATACGGATAGACCAATTTTCCCGCCTCTACCCCCATCC CAAGCCTCATGCACACCCATCAAGTTCGAGCAGTACAA

APPENDIX 2: 131 PUTATIVE SEQUENCES OF SNPS NOT USED IN THIS RESEARCH

Flanking sequence information for untested SNP. The "SNP ID" provides a common name including organism abbreviation "RREN" *Rotylenchulus reniformis*, the reference assembly contig (first number), and the sequence location (second number) of the SNP position.

S.N.	SNP ID	SNP	Surrounding Sequence (approximately 100 bp each side)
1	RREN_8_16622	[T/C]	GGCGCTCTACGACCTGTACACCCAGCCGGCCACCAAGTGCGGCCCCTTCCTCGT CGGCCTCCTGCTCGGCGTGTTCACCCTCCGTCCTCCTCCTTCCGCT[T/C]CCTCCTC CCCGTCTTCCGCTTCCTCCGCCTCCTCCCTACTCTTCTGGATCGGCTTCCTCCTTG CGCTGGGCACCATCTACGGCATTCTGCCGGAGTATTG
2	RREN_8_16659	[T/G]	TGCGGCCCCTTCCTCGTCGGCCTCCTGCTCGGCGTGTTCACCCTCCGTCCTCCTCC TTCCGCTCCCTCCTCCCCGTCTTCCGCTTCCTCCGCCTCCTCCC[T/G]ACTCTTCTG GATCGGCTTCCTCCTTGCGCTGGGCACCATCTACGGCATTCTGCCGGAGTATTGG CACCCGGACCAGGGGGTCACCCTCTACAACACCCTC
3	RREN_12_209115	[A/C]	TGCGCTCCATTGCACATTCTAAAATAGCGAAAATGGGATGTTGTTGATGCCCTAT AAGATGGAAATTGTGTTAAATTGACCCACAACCCATGCTTTTAAG[A/C]TCTCAG TTCTAGTTACCGGTTTTAAATGGAAAATATGTAAATTATTACATTACCATCGCTA TTTATGGCAACACAAGTGCCAATCTTATTGCGAAGATAC
4	RREN_24_69296	[A/G]	GGACGCGTAGAGGTCATCGACGCACGAGGGTGAGTCATTTGCATATTGTATGAC GGATTAAAATGAAAATTGGGCACTGATTAACCAGCTTGGAACCATC[A/G]TGAA TATGAAGCGAATTCCTTATTATAGAATGTTACGGGAAGAGTGAATGAACAGAGA AAAAGAAGTCAAACAACAATATATTTTTAACCCAGGGTTCTC
5	RREN_24_69332	[C/G]	ATTTGCATATTGTATGACGGATTAAAATGAAAATTGGGCACTGATTAACCAGCTT GGAACCATCGTGAATATGAAGCGAATTCCTTATTATAGAATGTTA[C/G]GGGAAG AGTGAATGAACAGAGAAAAAGAAGTCAAACAACAATATATTTTTAACCCAGGG TTCTCATCGAAAAATTTAAAAAATCAGGACATCATGACCAA
6	RREN_32_65082	[A/C]	AGCATTATTTTCTGTATATTTTTGCTTCTTACAGGTCTACCCTGACAAGGTTTCTA AATTTGGCTGATCAAAGTCGCGGTTGACCCACCGATGACACGTG[A/C]TTAAAGC ACGTGGAAAAAATATTTTAAAAATTCACTAATATTTTAGCAAGGGGTTTGTGCC AAAAACGGATGTCACTTCTTTATTATTATCGACCATGCT
7	RREN_61_242003	[A/T]	TGTAGAGCACCCTCACAAAGCATCGTATATTTTTGGCTTTTTTGGCCCGCTAACA TAGTGAGGGGTTGAAAAATCGGTTTCAATTTTAAATGAACGGTCG[A/T]TCATCC

Appendix 2 Continued.

			ACGCGAATGACCTGTCCATTGGATGACATCCACCTTTCAATGTAAAATAATATTT AAACATGATTATTTTTTGCTTTATATTCATCAAATTATC
8	RREN_61_242024	[T/C]	ATCGTATATTTTTGGCTTTTTTGGCCCGCTAACATAGTGAGGGGTTGAAAAATCG GTTTCAATTTTAAATGAACGGTCGTTCATCCACGCGAATGACCTG[T/C]CCATTG GATGACATCCACCTTTCAATGTAAAATAATATTTAAACATGATTATTTTTTGCTTT ATATTCATCAAATTATCATATAAAATAATTCCCGGACA
9	RREN_125_72000	[T/C]	GCCGATGCCGAGAGCACTGGGCGCAAAGCCAATCAACGAAGGGAACAACCAAC CAGGAGGCCAAATACAAAACGTACTACACCAGCACCCGCAACAACGA[T/C]CAC TACCACAGTGGCAACCCCCACAGCAACCACTACCAAAGCTCCGGAAACCCCGAG CACTGTCACAACTCGCCCTCAAACTCTCACCACAGTCACAACT
10	RREN_125_72023	[A/G]	CAAAGCCAATCAACGAAGGGAACAACCAACCAGGAGGCCAAATACAAAACGTA CTACACCAGCACCCGCAACAACGACCACTACCACAGTGGCAACCCCC[A/G]CAG CAACCACTACCAAAGCTCCGGAAACCCCGAGCACTGTCACAACTCGCCCTCAAA CTCTCACCACAGTCACAACTACAAAACCACCGATAACCCCAGG
11	RREN_125_72052	[A/C]	CCAGGAGGCCAAATACAAAACGTACTACACCAGCACCCGCAACAACGACCACT ACCACAGTGGCAACCCCCACAGCAACCACTACCAAAGCTCCGGAAAC[A/C]CCG AGCACTGTCACAACTCGCCCTCAAACTCTCACCACAGTCACAACTACAAAACCA CCGATAACCCCAGGCATCTCTACAGTCAGTCCACCAGTTGTGA
12	RREN_128_40796	[A/G]	TCAATTTTTCAAGCAACAATTTACGAAATTTATTTCTTATTTGGAATTTTTTGATT GATTTTCGCCATTTTCGTCACCTCTGCAGAATTCTTTGAGTTCA[A/G]CGCTAAAT TTCAGTTCCTCTACACGAGACAAAGGGTTGCGAAATGGTTCTTGGACCAGCCGG AACCAGCCGGAAACCGGAACCAGCCAGACACATTCCAT
13	RREN_159_85688	[A/T]	CCAATTCACTCCTCTCTCTCCCTATCCTCTCTGTTCTTTCTGGTCACTCTAAATTTC TCTTTCTCCCCCTCCGCTACATATCTATCCTTCTCTCCATCCT[A/T]TACCACACTC TCTCGTCCTCTTTCCGGTCATTCTCAATGTGTTTCTCCCCATTTTCCCCACTCTCTC AACCCTCCTCTGCATCTCTACCCGATCTCTCCA
14	RREN_190_41184	[T/C]	ACGGATGATCCGCGTGGGTGAACGACCGTTCGTTTGTTTTTGAAACCGATTTTTC TACCCTTCACTTCCTGTGCGGGCCAAAAAAGCCAAAAATATACGA[T/C]GCAATG TGAGGGTGCTCTACAAGATAGGATGGGTTAAATTATGATCTGAATACTCACACC TCGGTATGTTCCCTTGTTAGGTAATTTATAGAGGGTATAG
15	RREN_190_43474	[A/G]	GAAGAGCGACGACTATCCCCTTTGTAAAGAAGATTCGTTTCGAAATATTCTACT GACTAACTTGTAAGAAAGTGGCGAAAGCATAAATTATATTCCCAAG[A/G]CCGA

Appendix 2 Continued.

			GTAAGTTCACGTATGCTCATTTTACTTATTAGTACATATGATTCATGATTGGGAT TACAGTATGCCATTATCTATATCCTGGACAAGCTCTAAGGT
16	RREN_190_43475	[T/C]	AAGAGCGACGACTATCCCCTTTGTAAAGAAGATTCGTTTCGAAATATTCTACTG ACTAACTTGTAAGAAAGTGGCGAAAGCATAAATTATATTCCCAAGA[T/C]CGAGT AAGTTCACGTATGCTCATTTTACTTATTAGTACATATGATTCATGATTGGGATTA CAGTATGCCATTATCTATATCCTGGACAAGCTCTAAGGTG
17	RREN_202_14786	[A/G]	AACTTAATCAAGAGCATCAACGGTGCAGAGCCACCAAAGAACAACAGTCCGCT GATCTTCAATGGTGTACCGTGCCTAGACAACAGCAAGTGTGCCAACA[A/G]GCTG AATGCCTTGTTCCACCAACGACCAACCGGCAAACCTGTCAATACTGGCCGGGCC GCCAAACGACTAATCACCGCACAGGCCAAAGCCGCGGAGCAC
18	RREN_208_6302	[A/C]	GCACCCTCATATTCCATCGTATATTTTTGGCTTTTTCTCCCCGCTCAGGAAGTGA AGGGTAGAAAAATCGATTTTAAAAATGATCGGTCGTTCATCCACA[A/C]GGACC ATCCACCTATTGGATGAAATCCACCTTACTATGTATATAATAATGATTAATCATG ACTGTTTTCTGGCTTATATTCATCCTTTTAGCTTAGCAAT
19	RREN_228_25614	[A/G]	GCATGGTACGACCGACGCCATTCCAAAGGGTGTGTGGATTTGCGTGGATGAACG ACCGTTTATTTTTAAAATCAAAATTTCTACCCTTCACTATTTGAGC[A/G]GGCCAA AAAAGCCAAAAATATACGATGCAATGTGAGGGTGTTCTACAAAATAGGATGGG TTAAATTTCGATCTGAGTGGTGACAGCTCGATATGTTCCCT
20	RREN_228_25645	[T/C]	GTGTGGATTTGCGTGGATGAACGACCGTTTATTTTTAAAATCAAAATTTCTACCC TTCACTATTTGAGCAGGCCAAAAAAGCCAAAAATATACGATGCAA[T/C]GTGAG GGTGTTCTACAAAATAGGATGGGTTAAATTTCGATCTGAGTGGTGACAGCTCGA TATGTTCCCTTGTGAATTTTTTAAAGGAGGTGCTTAGTTGC
21	RREN_242_77265	[T/C]	CGGAACTCACCAGCGAAGAGTACGAAGAAAAGTCAATATATAAAGGACGAAGA AAGCCCCAAAAACCATCACGTGTAGAGCGGAGCAGACTCAGTTTTAT[T/C]ACAT TTGTGAACAACATAACTGCGCCAACAACCCGCAGTGAACAAGGGTATTCCTTGA CACAGTTTTTTTATGTTTTCATTTGATTTGTGGAAATTTGGA
Appendix 2 Continued.			

22	RREN_242_77303	[A/G]	TATAAAGGACGAAGAAAGCCCCAAAAACCATCACGTGTAGAGCGGAGCAGACT CAGTTTTATTACATTTGTGAACAACATAACTGCGCCAACAACCCGCA[A/G]TGAA CAAGGGTATTCCTTGACACAGTTTTTTTATGTTTTCATTTGATTTGTGGAAATTTG GATAATTATAATAAGAAAATATTCACCGATTATTTCTAAT
23	RREN_269_61835	[A/T]	TGTTCCTGCAGCCCATGGATTCCGATGAACAGTTTGTAGAGCCAGGCGCCGATC AGAGCCCCGAGGAAGGGAATAGCCATTGGGATCCAGAAGTAGAAGT[A/T]GTTG TTGCTGGGGAAGGATAAGTACAGTATAGCAAGAGAGTATGTACAGTATGCAAG GTCAATAGTCCCAATGGAACCTAAACACTTCCCAGCCAAGTCC
24	RREN_295_80337	[A/G]	CGTGGGTAAACGACCGTTCGTTTGTTTTTGAAACCAATTTTTCTACCCTTCACTAT CTGTGCGGGCCAAAAAAGCCAAAAATATACGATGAAATGTGAGG[A/G]TGCTCT ACAAGATAGGGTGCGTTAAATTTAGATCTGAGTGGTCACACCTCGGTATGTTCC CTTGTCAGTACTAAAAACACTGAAAAACTACTGTACTGAT
25	RREN_297_7718	[T/G]	CCCGGTCCCAGGAGCTTGCCTCGTTGGGCATCCCCGGACAAGACCCGCAGTCCA TGGTGGTCTCTGCCGAGCGGATCATGTACCAGCACGCGATTGATCT[T/G]TGCCA GTCGGCCGCTTTGGATGAGCTCTTTGGCAACCCGCAGTTGTGCCCCAAACGCTAC CAGACCGCACACATGATGCTGCACACGCTGCTCTACACGG
26	RREN_301_61722	[T/C]	GGTGATGTGGTCGTGGCATTGGCACCGTCAGTTCCAGATGGGGAAGGCGGCGTA GAGGTGGAAGTGGTGGCAGAGGTGGTAGGGGGCACCGAAGTGGTGG[T/C]ATTG AGGCATTCGCCCAACGGAATCCCTTCGTTGTCTGTCTGGTTGACCAAAACACGG ATCCCATTCTGAAAAAGCAAAAATCCATTGAGTTAGGGTCAC
27	RREN_308_72119	[A/T]	AATATTTCATCGATGATGTAGAGCATTGGATGTCCGATGGGGTATTGCGTTATAA AATTGATGAAAGGGTAGAGGCTGGTGACGTCGTAGTAGGAGATTT[A/T]CTCTCC TAACCGCAAAGCATGGAAAAGTTTAAGCGGTCCCGTTCGGCCTGAAAAATTCAA AAGGATTTTTTAAATATTACTTTAAAAATCTCACCTCCCA
28	RREN_336_82853	[T/C]	CCCCGGTAGAGAAATAGAGAAAGATTTGAGGGATTTATTTGTTCGTGTCGAAAA ACCGCCGATGAACGCGCGGACGACGAGTGCCACCGGCGTGGAAAAG[T/C]TGAA AATTTGCGTGTCATGGGTTTTGTTGAAAAAACAAATGTTTTGTATGGGAATTTGT GCTTTATTCATCTTATTATTAGTATGGATTATTTGTATTCT

Appendix 2 Continued.

29	RREN_349_16421	[T/C]	CACTCCGTAGAGCTGTGTCCAGTCAGCCTCCCATCAGCCAGCCAGCACATACCA ACGGGGAGTATTAGCCTCGTCAGTCCTTCTCCCCCAGCAGCCTGTC[T/C]TGCCTC TGCATCCGCCTTTTGTTGCGCCTCGGATCGACCGAAATGAATTGAACGCGTGTCC GTGTACTTTTCCAAAGCAGCATGAACAGAGAAAGAGAAA
30	RREN_371_58464	[T/C]	GACTCATAAATGGCTTGCGAATTTTGCGTTTGTACCCCTAGCTGTGGCTGCTGAA TGTATGTTTGGCTTCGGGGTTGCTGCTGAATAATTCGCTGCGCTC[T/C]CGCCTGC CTCACTACATGTTGCTGGGCTATTGGTTGCTGCACAACACCAGCCCTTGCTTGCC GGCTCCTACCCATGTTATGCCCCAACATATCATTCGCT
31	RREN_431_1809	[A/G]	AGAGTGCCAACCTTAGAGTCCCAAGTTGAAAACTCATGTTTTTAGTGATTTTTGA GCTTAGGTTTCTGTTACAAAAATGTAGAGCGTATGGAAAAACATG[A/G]TGTGAT AACAAAAAATTTTAGGCTTAGGTTAGGCCTAAGAAATTTTTTTGGGAAATTTCTA AAATTTCCGGGACACTAAGTGGGCCTAAGGCATTTGTGC
32	RREN_432_101500	[A/G]	TACATATTCATTGGCTAAAGCTGGTGCGTTTATTCAAGATTATTATTATTTTTCTG TTTTATAAGATAATTTGATAAAATACTCACTTTGGTGTAGAGCA[A/G]AGGGAAC ACAACACCAAAAAAGCGTTCGATGGTCAACGAAACAATGGCCATGCAACTGAT GTAGACGGGGGTGTTGAAGAGGTACTCGGTGAGGAGGCAG
33	RREN_514_63263	[A/G]	AGGTGTGGCTGAAGAGCATCTCCAACCTGTTGCCGCGGCACATCCTCAAGGCCT CATTGGCACTGCAGTCGGTGGTGCACCAGTACGAGCCGGACGCCAT[A/G]ATGC CAATCCCGTCATGGCAATGGGTGGACAGGTAGGGGGGCCAAGAAAATTCGCCA AATTCGGAAGAAAAATTAGACCAATTTTCCCATAAAATCGGGA
34	RREN_521_25222	[A/C]	GCTATTCTGTCCCTGCCAACTCTGGTTTGGCACCCATCGGCTCCCTTGGGGGCGC ATTCCTCCTTGCTCACCCTCTCTTTCTCTCTGTTCCCATTCTCTT[A/C]TCTTCTCTT CACCATCTTTCTTTTTCATTTCTCGCGAAATTGCGTTTATTTCTGCTCTCATTTCCT CCATCTCCACACGAGCGATCACCTCCGTCTCCAA
35	RREN_523_12130	[T/C]	TTCCATCTCCAAGTTGTTTATAGAGATGTTTGCCGAGTTCAGTGGGATTCGTGAT ATTCGAAGGGGGCACTGTTACACGCACCTCCTGCCCTGTTCGCCA[T/C]ACAACT CGCATATATTGCTGTTCCATTGTTCCCAGGGAAGGCCACGAATGGGGATAAACC ATCACTGCAAGCCCCACATACCAATCTGAATTGAATTGCA

Appendix 2 Continued.

36	RREN_523_13494	[T/C]	TGACTTCTTTCTGATGTTTTTTGCCTCCGTTAACAACGGGCTGACACTGGTAAAT GCTACTGGTGAATGTATATCCTTGTAGAGACGCTCCAATAGACGC[T/C]TTATGG ATGCCTTCATTGCACGTTACCGCCACAATGATGGTTTAAAAGAAAAACTGTTACT CACTACTCGACTTTTGGTTACGTTTTTGTTCACCTTTTT
37	RREN_526_81331	[A/C]	TCTGCTGTAAGCTATATTATCATTTTAACTCATAAAATTAATTTTCAACCGTTTTT ATGGACCATTAGATTTTAGTGTTTTTGCATGGTACGACCGACGC[A/C]ATTCCAA AGGGTGTGTGGATTTGCGTGGATGAACGACCGTTTATTTTTAAAATCAAATTTTC TACCCTTCATTATTTGAGCGGGCCAAAAAGGCCAAAAA
38	RREN_526_81371	[T/C]	ATTTTCAACCGTTTTTATGGACCATTAGATTTTAGTGTTTTTGCATGGTACGACCG ACGCAATTCCAAAGGGTGTGTGGATTTGCGTGGATGAACGACCG[T/C]TTATTTT TAAAATCAAATTTTCTACCCTTCATTATTTGAGCGGGCCAAAAAGGCCAAAAAC ATACGATGCAATGTGGGGGTGCTCTACAAGATAGGATGG
39	RREN_526_81423	[A/G]	ACCGACGCAATTCCAAAGGGTGTGTGGATTTGCGTGGATGAACGACCGTTTATT TTTAAAATCAAATTTTCTACCCTTCATTATTTGAGCGGGCCAAAAA[A/G]GCCAA AAACATACGATGCAATGTGGGGGTGCTCTACAAGATAGGATGGGTTTAATTTCG ATCTGAGTGGTGACAGCTCGATATGTTCCCTTGTAAGAACT
40	RREN_526_81432	[T/C]	ATTCCAAAGGGTGTGTGGATTTGCGTGGATGAACGACCGTTTATTTTTAAAATCA AATTTTCTACCCTTCATTATTTGAGCGGGCCAAAAAGGCCAAAAA[T/C]ATACGA TGCAATGTGGGGGTGCTCTACAAGATAGGATGGGTTTAATTTCGATCTGAGTGG TGACAGCTCGATATGTTCCCTTGTAAGAACTTTATTTTCT
41	RREN_526_81448	[A/G]	GGATTTGCGTGGATGAACGACCGTTTATTTTTAAAATCAAATTTTCTACCCTTCA TTATTTGAGCGGGCCAAAAAGGCCAAAAACATACGATGCAATGTG[A/G]GGGTG CTCTACAAGATAGGATGGGTTTAATTTCGATCTGAGTGGTGACAGCTCGATATGT TCCCTTGTAAGAACTTTATTTTCTCTAATACTCAAAAATC
42	RREN_558_68223	[T/C]	ATTCTGTCATTTCTTATGCAATTCCCTCCTCATTTGTCAACTATATAAGCAATGCA TTTTCAATCATTTGTCACTTCCATTCCCATTCCAGCTCCCATTT[T/C]CCTTATTCC AATTCATCTATTGTCCTATATTGTCTTCAATAAATTCTTCACGAGGACACAACAA TTTGGCGCAGTCACGAAAACGACTCTACGCAATGCC
Appendix 2 Continued.			

43	RREN_590_31647	[T/C]	TCTTACAATCAAAATCTGCCCTTGGGAGCTTTTTATGGAAATTTTTTCAAGGAAA ATCATGAAACTGGACAATAAGATTAAAACTCTGCCAGAACCACCA[T/C]CTGGCC AAGTTTGCGCTGCGGCGTCGTGACGGCGACTTTTTGTCCATCGAAGCAGCATTGT TCTTGGCCGAACAGGGCGCCGCTGTGGTGGTGGAACATC
44	RREN_625_19284	[T/C]	ACATCTGCATTTGGGGAAGGGGGCGAATTTTGTAATAATAGGCAAAAATCGAAG GTGAACAAGGGGGGTAGGAGATGGATGTTTTTCATCATCCATTACA[T/C]CCAAC ACATCCAATTCTGAAAAATGGCCCCTTCCCCAACTTTTCTGCAGTGGATCGAACA GCTGAATTGCTCATGCCCTGATGGTCTGAAGTGTGTTCGA
45	RREN_718_5249	[A/G]	ACAAGGCATGTAGAGGACGACGCAGAAGGGACCAATTGTACAGAATATTGATG ATCAAAGCCCCTAGCACGCCACCCTCCGAACCAGCAGGACCCACCTC[A/G]GGC TGAACAAAAGGATTAGAGTAAATAAAGAACTGGAATGAGTGTAATAATACCAT GTAGGGAACAAAAACGGCACTGGCCAAATATCCGCCAATTCCGG
46	RREN_721_22152	[A/G]	AAACTTTCGTATCGGATTCTCCTGCTGCTGTCCGTCAATTCCTGAACGACCCAAA AATTGAAGTGGACTTTATAGAACAACTAAACGAGGAATGCGTCCT[A/G]ATCCG ATACACACCACTAAAAGAATGGATCGAGGAGCACAACTGTTCAAACATTGTGCT CTCTCTATGGACAACGGCGGCGGCACGACTACATCTCCTCA
47	RREN_721_22224	[A/G]	TAGAACAACTAAACGAGGAATGCGTCCTAATCCGATACACACCACTAAAAGAAT GGATCGAGGAGCACAACTGTTCAAACATTGTGCTCTCTCTATGGAC[A/G]ACGGC GGCGGCACGACTACATCTCCTCAAGCTAATGCAAAAAGTGGCGTCCACTCCCAA CTGTGTACTCCTCTACACGGACACGGACAGTTTGATTTTCG
48	RREN_780_29960	[A/G]	TCCACACAATATAAGCACTTGGCCAAGGTCAAAATTTTCATTTTTACTCTAAATT TTTTTCTGACAATTTTTAACCCTTCAGCTTCTCGCCCCTCCTTCC[A/G]CCCACAG CGAACCATTGATGTGCCACGTCCTTTCCATGTCCATTTGTGCGATTCATGCCGCG CTTTGTGCTCTTTTGGAGTTGTCCATGTCCGAGTCCGC
49	RREN_800_50912	[T/C]	ACAAGGGAAGGACAAAACATGTTAATCGTGGATCAATTCGAGACCCGTATAATA TTAGAGCATCCGCAACTTCCGTCTTAAATTTTAATATTAGCTGCCC[T/C]CGTCCA CAAGAAAAAAAAGTTATGAATATTTATTTCATAATCCGCGATCCGCGCGGGTCA AGGGAGTGACATACCTATTCAGATTTCTAAAAATTACGTC
Appendix 2 Continued.			

50	RREN_929_9795	[A/C]	CGGAGGCGCGAGTTTGGGCTAGTACCGTAAAAATGGGAAAAAAATAGAGTTTA CGTTCCGAAAATAAATTGGGCCAAATTATATACCATAGTGTAGAGCT[A/C]GACG AGCTGAGTACGAATATGTAATTATTTTTTGGCGCAAACCACTTTAAACCGGTTTT TGAACCCTTTCAAGTTCTTATCCAAAAATGCAAAACACTTG
51	RREN_981_25656	[T/C]	CATCCAGCGGTTTGTTCGGAGCGTAAATCCGTATTCCTTCGCGACTTGTGCACCT GGACACGGATGTGTAGAGTTGTCCATGAGCGAAAGGTTCTTCGCT[T/C]AGGTCG ATCCCCAGCCTTTCGATGGTCTGACCTTGGGCCTTCGCGAATGTCATCGCGAAGG CTACCCGTACCGGGAACTGGAATCGTTCAAAGGGCACAT
52	RREN_1012_21981	[T/C]	AACTTTTCTCTCAAATCAGCAAAAATCAAAAAACATGGCAAAAAGCATGGCAAA TTTTAAAAAACATGGCATTTGCCATGTATACATGGTAATTTGGCCT[T/C]CCTGTT CAAAAATCCGATATAAAAGTGACCCCTTCTTCCGAAAATAATTCATTTCTTCGCG GGCATCTTCGTTGTTTCTACTTCTACATCTCTACACAAC
53	RREN_1120_48434	[C/G]	TTTTACTACTTTTTCTTTTGATTTTATGCATTTTTGCAAAAGTGTGCAAGTGAAAT TGCACAGAATTTAAAAGGGTTCAAAAACCGGTTTAAAGTGGTTG[C/G]CGCCAA AAAATATTTATATATTCGTGCTCAGCTCGACGAGCTCTACACGATGGTATATAAT TTTGGTCAGTTTGACTTCCGGAACATAAACTGCATTTTT
54	RREN_1123_57666	[A/C]	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTGGG AAGGTAAGCGGAAGGGTTTGGGAAGGTAAGCGGAAGGGTCTGGGAAGGA[A/C] GCGGAAGGGTTTGGGAAGGTAAGCGGAAGGGTCTGGGAAGGCTGTGTGGACGA ATTTTGGTAGCGCCGAAAGTCTGGATCTCCCTAAAAAAGAACGGACC
55	RREN_1175_25864	[T/G]	TACCAATTTTTCGATAATTTAACAAAAATGCTGTCATTTTTGATATGCATGCATG TGTATGCGCGTAGAGTGCGCGCGTGTGTGACAAGGGTATGCGCGT[T/G]ACAAA GAAGTACAAATTCACCATGTTAGTTGGTCAGTATCTTGGCTAAGTTGGTCTCGTC CTTGGTTAAATGCATCATCGTCGTCGTCTGAATTTTCCCT
56	RREN_1187_12082	[A/C]	TATTATCTCTTATAAATTAATTGCCAAACATTTTTATGACATTTCACATTAAAGTG TTTATGCATGGTACAATCGACGTCATCCCAAAGGGCGAATGATC[A/C]GCGTGGA TGAACGACCGTTCATTTTTAAAACCGATTTTTCGACCCCTCACTATCTGAGCGGG CAGAAAAAGCCAAAAATATACGATGTAATGTGAGGATG

Appendix 2 Continued.

57	RREN_1187_25663	[A/G]	GTGCCAAAGACGGCCGTACCAGCAAACGAGACCATTGTGCCTCCGCCCGCATCC GATCCCTCTTCTTCTTCCATTGCGACAGTGCAATCCGCTGGAACAC[A/G]CTGGC ACCCATCACGACCTTCACCAACCTCGGCCCTTTCCGCTCTGCACTCGGCCTTCCC ATCCGATACACAGTGACTGGCATCGCTTCCACGCCCAATT
58	RREN_1215_1686	[A/C]	TCGAATTCAATTTAAGATTCAATAAATTAGAATACCAAAAAACCTCTAGAAACT TTCATAAAGCTAATTATAAGCAGATAAACAATATATTCAAAAATAC[A/C]GACTG GAATACTCTCTTTTCAAATAGTATAGAAATTGATCATCTATATCAAACATTTAGC CACAATATCCATAAAACCATCGAAGATCATATTCCTATCA
59	RREN_1572_36973	[A/C]	TCTTTCGAATATTTTGGACTTTTTTGGACACCTTATGATTGACCATTTACAGCCCC ATCCCGCTGGCCAAGCGGTCTCCTACTCGGCCCAGCAGAAGAAC[A/C]TGTTGAT GTGGGCGGTGGCCGTCGGCTCCATGCTCGGCACTTTCCCCTTCGCCTGGCTCTAC ACCCGGCACGGTGCCCGCTGGGTTCTGTTCGGTGCCGG
60	RREN_1660_519	[A/T]	TGCGGTAGTCGGTTCGGCTTATGGCGTAGAGCTGATCAGTGAGAACCAGATCCC GTCCGCACTCCAAGACATATTCCAAATTGGTGATGCTTAATGAAAA[A/T]GTTTG ATCTTCTGACATGAACCAATCATCAAACATGGTTCCTTTCATTTCCTGCATCAAC ACGTACCGGCATTGTGTGTCCGCTTTGGGGCGGTTCCATA
61	RREN_1695_18038	[A/C]	AAAAAGTAATATTGTGCTGAATTTTATGCTCTATCTTCTGGGATTTATAATTCGG CCAAAAAATTGGAAATATCCCCTAAAACCTTATTTTTCAGAGTAA[A/C]TTTTTG GTAGAATTTAAATAAATAATAGAAGATTCTGCACAATGGCTTTTTATAGTTTTTG GCCCTAGCACCGATCAACCCTCTACACGGTATACCATTT
62	RREN_1695_18063	[A/C]	ATGCTCTATCTTCTGGGATTTATAATTCGGCCAAAAAATTGGAAATATCCCCTAA AACCTTATTTTTCAGAGTAACTTTTTGGTAGAATTTAAATAAATA[A/C]TAGAAG ATTCTGCACAATGGCTTTTTATAGTTTTTGGCCCTAGCACCGATCAACCCTCTAC ACGGTATACCATTTTAGTGGCGGACCAATTAATTGTGGT
63	RREN_1721_18343	[T/C]	GGCCCCCAAATGTTCCTGAATATGTACACGCCTCTCTCAATTTATAGTTTAGGTG TACCCTCGCGATTAAATTGTAAATACGCCCATGGCCACCGTCCTC[T/C]CTTTTCC TACACACATCTTGATAGAATATTTTCAATAGCTCTATTTTCAAAGTTGTGATAAA TAATCAACATTATTTCCCTATGATCAATATTCTCACTC

Appendix 2 Continued.

64	RREN_1741_30669	[T/C]	TCCCATTCCTGCACTTTTTTTTATATTTTCTGTTCCTCAATTACTTTTCTTCGCAAT CCAAATCCGTATTTAAGGGTGTTTCAATTTCTCTTTTTGCTCA[T/C]TTAAACTTG GACTTATCTTCACTGTCCCTCACCCAATTTACTAATTCGCCGTCCCAGCTCTACA CTATTCACTTTTGGTGGGTACACATCCCATTTCGAT
65	RREN_1874_61680	[A/G]	CTATTGATTTTAAGCATTTTTGGTTGTGTGCCAGTGAAATTTGCGTGGAACTAAA GGGGTTCAAAAACCGGTTTAAAGTGGTTTGCGCCAAAATATAATT[A/G]CATATT CGTACTCAGCTCGACGAGCTCTACACGATGGTATATAATTTGGGCCCGATTTATT TTCGGAACGTAAACTGCATTTTTACCCAGAATTTTATGC
66	RREN_1886_8608	[T/C]	TAATTTGTAAGGCTCCCGCAGGATTTCGCATCAAAACAAGATAATGCGAATTAT TTCGTGCGACCTTCAATTCTTTCGTGAACAGATGCTGAGTGACTAA[T/C]ACCAC ACTCATACCCCAATTGTGGGAACCCCGAGTGAAAACTGTGTCTAAAAATTGCTG ACGCATCCCGACCATCAAATCATCCAATACAACCAGCAGAT
67	RREN_1886_12029	[T/C]	GTCTATAATTTTTATGGGCCTCATAGGTGTAGAGATCGGCCAGCGGATTGCGTCC AATGTGTGGAAAGGGTACATGGATGGTGGAGTCATTTCCCGGGAA[T/C]ACGAA CACGTCATCAGCTCCACCAGCATCACCACCATCATTGTACAGCATGGGATCGCT AACAAAGAAAATTTCCTAGATTTAACTAAAGGTAAAAAGAC
68	RREN_1915_307	[T/C]	CGTCTAGAGACTCAATCGTGGAGACTCAATCGTGGAGACTCAATCGTAGAGACT CAATCGACCCCCACCGGATAAACGACGCTCTTCATCTTTTGCCAAC[T/C]CAAAT GTGAAAGATAAGCAGAAACTGAGAGAAACAAAACGGTAGAGAAAAGAGTATG AGAGAGAACATAAACGATAAAGAAAGAGTTCGTTTCGGAGGCT
69	RREN_1928_25815	[T/G]	CCATAGGTTATTTTTCGTTCTCTATTTTAAATTTTATTAATCACCCATTGCCTTATT ATTCAAATTTTACATTTTCTAAATACAAAAAAATTAAAGATGG[T/G]TTTCCGCTC TAAATTGTGCAATTTTTTATTATGTGAAATTTATCCGCGCTTCAATATTCATCATC TAAGGCATTTCCTCTACACATCCTCTCCAACCACA
70	RREN_1930_18838	[A/T]	ATTTTGTGCATTTTTGGAAAAGTGTACCAGTGAAATTGTACAGAACTTAAAAGG GTTCAAACCCAGGTTTAAAGTGGTTGCCGCCAAAAAATATTTACAT[A/T]TTCGT GCTCAGCTCGACGATCTCTACACGTTGGCAGATAATTTTGCCCAGTTTGGCTTCC GGAACATAAACTGCATTTTTTCCAAAAACAAAACATCCTT

Appendix 2 Continued.

71	RREN_2082_17935	[A/C]	CATTCTTTACAACTATCACAAAAATGGTGTCTATTGAACATTATTAAGAATTCGA CTAAGGGTATCGACACAGGATAAGCACCGCTTAAACCGGTGAATG[A/C]CCATTT TTTCGTTATACGGTATCGATTTAATGCATGGTACAGTAACCAATTCAATACCACA ATTTTTGTAACGTAACTTCTCTACACTATCCCCAATGAC
72	RREN_2082_17995	[T/C]	GGTATCGACACAGGATAAGCACCGCTTAAACCGGTGAATGCCCATTTTTTCGTT ATACGGTATCGATTTAATGCATGGTACAGTAACCAATTCAATACCA[T/C]AATTT TTGTAACGTAACTTCTCTACACTATCCCCAATGACCTTCTACCGTATGCTCCTTTT ATCGTGTCGTTTAGGATGACTGCATACCATATTCCAGCT
73	RREN_2193_23293	[C/G]	ATTGGCGACAGAGAAGAAGGCAGCGACTGTGGGGAGTTCGGCGACAGAGAAAA AACCAGCGACAATGACAGAGAAGCTTCTGGAGAAGAAGAATGGATTC[C/G]AGA CACAGCTTTAAGTAGTAAGAATGGTGCCACATAAATCATGCATAAAAAACCGGT ATCGGGGCAACCGAATAGTGGTCATGGACACCAAGAAATATGA
74	RREN_2229_14581	[A/G]	ATGAGGTGAGTCATAAAAGTTTTAACGATGGGTTGATTGGGTGTTATTGGACAC CATCAGAGGTAAAGGAGGACACGTGTACAGCCAACGCTATGTCGCA[A/G]AGAA ACCGGATGTCCCACCCAAGGATGTGTATCAGCTATCGGTATGGAATAAGAGGTG GAGATGAATGGATCAAAGATCTATTTGGACCGGAAGAACAGC
75	RREN_2245_19256	[A/G]	ATTCCGGTGCCCCCACGGAAGAAGTTATTCAAAGACAGCCCAAACCCGCACTGG TCATCCTTGATGATCTCCTTTATTCCATCGATCTCAAGTTTCTGGC[A/G]GATCTC TACACCAAGAAGAGTCACCATGGTAATTTTGGAATCGTTATGCTCACTCAGGAT TTGTTTGATCGTAAGATGAAGGTGGTTAGGCAAAATTCAA
76	RREN_2295_24784	[A/G]	TGCCTATCCACGTGTCCAAGCAGCTACTGCGGCTTTGGAAGCAATCCTTGAATG GCTGACCAACAACCCACAGTCTTCTGCGGTTGAAAAGATCACACTT[A/G]TGGTC TCTAATCCAAATGACCAAGGCCTCTACAAAGATCTACTTCAACGGGCTAAGCGT CAAATTGTAGGCTCCAGAACAGCAAGTCGCGCATCTTCCAG
77	RREN_2301_5490	[A/G]	TTGAATGCAAGAAACAATAGACAATGGCGAGCTGGGATGATAGCCAACTGCCA AGAATGAGGGAAAATCACTAGCTTATATACATGCAGGCGTGAAGGAA[A/G]GGG AAGGAAGAAAGAGAGAAAACAATACAATAGCGACAGACAAATGTGAAAGGGA ACATAATGCCAAACAAATACGAATGAACAATACATTTGAATCACA
Appendix 2 Continued.			

78	RREN_2380_20166	[T/C]	ACGGCAAGGTCTCCATTCCCGGTCTTATCCGCCCTTTCTCATAGTGTGGTCTATA ATTTTTGTGGGCCTGATAGGTGTAGAGATCAGACACTGGAGTTCG[T/C]CCGATC TGTGGAAACGGAACATTGATGGAAGAGTCGTTGCCCGCAAATACAAACACATCA TCATCCGCGTTAAAATTGTCCAGAGCAGGATCGCTAAATT
79	RREN_2380_20294	[A/C]	GAAGAGTCGTTGCCCGCAAATACAAACACATCATCATCCGCGTTAAAATTGTCC AGAGCAGGATCGCTAAATTGGTCACGAAATTTTATACCCAGAATAT[A/C]TACAC CCACATTTGACCATCGATGTCATAATGTTCCAAGACAATCGCTTTCACTGTTTCG AAATTGAGCAATTGATGGTTGGCATAGGAGAGGCGAAAGC
80	RREN_2496_36896	[T/G]	ATCACATTGCATCGTATATTTTTGGCTTTTTTGTCTCGCACAGATAGTGAAGAGT AGAAAAATCGGTTTTAAAAATAAACGAATGGTCGTTTACCCACGC[T/G]GATCAT CCGTCCGTTGAATGATAGCTATCTTACAATGAATAAAATTTTTTAACAATGACTA TTTTCTGGTTTTATATTCATCATTATATCATATAGAATA
81	RREN_2611_11791	[A/T]	AAAATTGTAGAGCGTGTGTCAAAACATAAAGTATGATCAAAAAATTTTGAGGTT AGGTTAGGCCTAAGAAAAATTTTCGGGAAATTTTCGAAAAATTTTC[A/T]GGTTT ATTGGAGGCCTAATAGAAATGTGGTTTGTAATTGATATTTTGAGCTGATTTTTTG TACTCAGGGGTTTTCGAGGGTGCTGAATCCGAATATGACA
82	RREN_2644_249	[T/C]	CATTCTCCGACTTGAAGGGAACGGGCGCGTTTGTCGGTATGTGGCCATCGAACC TAAAAATAATTTATACTAAATAAGTATAGTATTTTAATCCAAAAGA[T/C]GAACC TAAAAATAATTTATACTAAATAAGTATAGTATTTTAATCCAAAAGAAAGCTAAC CGAATGTTGACATACCTATCCTGAAGCCGCCAAAAACCCTG
83	RREN_3007_13959	[A/G]	TTTCGGTTTTAGGGCCAAAAACACTGATTTTCGGTTTTTAAAATTTGACGGCAGC GTGGTGTAGTGGTCTATCACACAGGACACAAATTCTGGGACGTGG[A/G]TTCGAA TCCCACCGCTGTGTTGGTCATATACTCAAGCCCGTGGGGAGCATATCCCTTCACG TAGCCATTAGCCGAGCGGTGATGCAAAGCAAATGGTGAA
84	RREN_3315_26470	[A/C]	GAGACTGCGGATGAGGAAGAGGGTGTGGAGGATGGAGCTGGGGGTGATGTACT GCTTGGAGATGATGGATTTGAAGAATTTAATGGTGCAGAGGAAGATG[A/C]GGA GGTGGAATTGGAAGGGATGGAAGATGAGGGGGAGGAAGATGAAGAGGAGGAT GAAGACACGTTTGACGCTGGCAGCGAGCTGGAGTCCAGGGCCCAG
Appendix 2 Continued.			

85	RREN_3415_20865	[T/C]	GCATGGGTCTCTGGAGGTGTAGAGCGTCAAAGCGGCCGCGTTTTTTATGGAAAT TGTTGAAAAAAGGTTTTTTCCGCGCGAATTTAAATGTGAATCTTTT[T/C]TGATTA GGGACAATGCGACACGTATGGCATTGATTAGGAAGTATATCAATCCCGGTACCA CTATTCATTCTGATTGTTAGAAAGCGTATGCAAACATGGA
86	RREN_3722_17382	[A/C]	CTCGCGTAACTCGTTCCGGCATCCGAGCATTATAAATAGTGAAAGGTGGCATAT CCAACGGACGACATGATGAAGCATGGGTTTGCCTTACCCATCTGAT[A/C]ACCTA GAGAAGGTTAAAAGGGAAAGGATATATTAGAGGAAAAGTTGAAAACTTACATT TTCTAAACCCTCTTCCGAGTCGGGAATTGTCATTTCGAAAGC
87	RREN_3853_3705	[A/G]	AACACTTTGCGAGCCTCTATCCACAAACGCTGTACTCCAGCAAAAGCTGATGGG GATTTTGGGTCATTGTAGAGTCGATCCAAGGTAGCCTTCACTGTGG[A/G]CCTAG AATTATAAAGTAATTCAAGGTTATTTTTCTCGAATGAAAAAATACCTAAATTTAT TTCAGTAAATTAAGGTTAGCTTTTTCTAAAGAAAAATCTT
88	RREN_4099_6882	[A/T]	TTGGGTGTACGGGGGGATGTAGAGGTGCAGGCTGACGGCCGGATCGGCGTGGG AAGGGTTTTCCATCCGGTGCAGGCCGATCTTGTCTGTGAGATCAAAG[A/T]TCAG TGATCAATATCAGCCGGCCTTTTTTCTCATTACCTATTTATATTCTGTGGGGATAT GACTTTTATTTAAATATTATTTAAAGCGGATACCAAAGTA
89	RREN_4280_20028	[T/C]	CACGTAGAGCTGTGTGTGAATTCGGGATTGAGCTAGTCGGGATTTCGGGATTTC GAGATTTCGGAATTTTGCCATTGTTGATCTCTAACCTCGACTATTT[T/C]GATTTG GAAACTGCGGAATTTCCCCAATTAAAAATATGACAATTTTTGAGGCATATAGGT AGTAGTGTGGAGTGAGTGAAGTAGGCTTCCTCACGATGCT
90	RREN_4396_2332	[T/C]	AACTAGATTAAGAAAATAGTGGGGTAGAGAAAAAAGAGAGACGAAATAATAGG TAGAGAAACTAGAGTAAGAAAATAGTGGGGTAGAGAAAACAGAGGGA[T/C]GA AATAATAGGTAGAGAATAGAGAAAACAGAAGACAGCGAAAGAAACAAATATA GATTAACGAGAGAAAGCAGAGAAAAAATATAGCCTTAGGCATGTAC
91	RREN_4396_2346	[A/G]	AATAGTGGGGTAGAGAAAAAAGAGAGACGAAATAATAGGTAGAGAAACTAGA GTAAGAAAATAGTGGGGTAGAGAAAACAGAGGGACGAAATAATAGGTA[A/G]A GAATAGAGAAAACAGAAGACAGCGAAAGAAACAAATATAGATTAACGAGAGA AAGCAGAGAAAAAATATAGCCTTAGGCATGTACAATGACCACCAACC

Appendix 2 Continued.

92	RREN_4491_8860	[A/T]	GACGAAACACCTAATTTGAAATGTTAAACCTTTTATTGTATTAAAGTTGCTGGGC AACCTTGCATAACTTGATGCAATTTTATTAATTACGTGTTTTTTT[A/T]ACTTCATT TTACTGCGTTTTCTATTGATTTTATGCATTTTTGGTTATTTTTTCAGTGAAATTTGC GTAAAGTAAATGGGTTCAAAAACCGGTTTAAAGTG
93	RREN_4741_9023	[C/G]	AGCTCTTCGGGAATAGACAAAACCCGAAAACGTAAATATGACACCGAAGAAGT CAATGGGATGCCAAATGACGAGGGTCCATGGCTCAGCAAAGCGGGTG[C/G]TGG AGTGAACAAAAAGCTGCTTTGGATGCTTTGCAAAAACAAGTATCAACTCGGATT CGATTTGTGCACCGACTGCAATCAGGCCCTCTACAAGAAGAAG
94	RREN_4834_4812	[A/G]	ATTTCGTCTCTCTTTTTTCTCTACCCCACTTATTTCTTACTCTAGTTTCTCTACCTC TTATTTCGTCTCTTTTTTCGCTAACCCAGTATATTCTTACTCT[A/G]GTTCTCTACC TATTATTTCATCTCTCCGTTTTCTCTGTCCCACTATTTTCTTACTCTAGCTTCTCTA CCCCACTATTTTCTTTCTCTAGTTTCTCTACCT
95	RREN_4834_7584	[T/C]	GTGCACTTTGGCACCTTAACTCTGCGTTTGGTTCATCCCACATCGCCAGTTCTGC TTACCAAAAATGGCCCACTTGGAGCTTCAGCATTCAATGCCTGGG[T/C]TCACAG AGAGTCAAGCAACCCTGGCTTCATACCCATTTAGAGTTTGAGAATAGGTTAAGG ACATTTCGTCCCCAAGTCCTCTAATCATTCGCTTTACCGA
96	RREN_4985_4041	[A/T]	TCCAAAAAACATGAAAATAATAAATGTAATAATTAAAAGAAAAAAAATATGTA CACATAGGCTGACGGGGACAATTACACAATCGGATTTTTGTACAAGA[A/T]AATA CCCAAGCATTTGGACAAGTATAATTTGAATATCGGTTTGGACAATAACCAAGAA TTTGGCCTGAAGTTGCAAGGCGATGAGTGGTGGAATAAAACT
97	RREN_5033_11813	[A/G]	GTGGAAAGGGCACATGGATGCTCGAGTCATTTCCCGGAAACACGAATACATCAT CAGCTCCACCAGCATCACCACCATCATTGTACAGCATGGGATCGCT[A/G]ACAAA GAAAATTTCCTAGATTTAACTAAAGGTAACAAACTCACGCTTCTCCATCAATATC ATAATGTGCCAAGACGAGGGCCTTCACCGTTTCAAAATTC
98	RREN_5033_11816	[A/G]	GAAAGGGCACATGGATGCTCGAGTCATTTCCCGGAAACACGAATACATCATCAG CTCCACCAGCATCACCACCATCATTGTACAGCATGGGATCGCTGAC[A/G]AAGAA AATTTCCTAGATTTAACTAAAGGTAACAAACTCACGCTTCTCCATCAATATCATA ATGTGCCAAGACGAGGGCCTTCACCGTTTCAAAATTCAAC

Appendix 2 Continued.

99	RREN_5385_4749	[A/G]	CTGTAACATGTTACCTTGCGTGCTGACAAGGGAACATACCGAGGTGTGACCACT CAGATCTTAATTTAACCCATTCTATCTTGTAGAGCACCCTCACATT[A/G]CATCGT ATATTTTTGGCTTTTTTGGCCCGCACAGATAGTGAAGGGTAGAAAAATCGGTTTC AAAAATAAACGGTCGTTCATCCACGCGGATCATTCGCCC
100	RREN_5385_4848	[A/C]	TGCATCGTATATTTTTGGCTTTTTTGGCCCGCACAGATAGTGAAGGGTAGAAAAA TCGGTTTCAAAAATAAACGGTCGTTCATCCACGCGGATCATTCGC[A/C]CTTTGG AATGGCGTCGATCGTACCATGCATAAACACAAAAATGTAATTCTCCATAATTTG GGTTGGAAATTATTCTATATGATATAACGATGAATATAAA
101	RREN_5497_2602	[A/G]	TGTCGGCTTGTCCCCTGCTCAATCGCTGCTACCGGCGAATAATTCCGTGTAGAGG GGCGGAAAGGCACTGAGCACATTTTGCGGATGAAAATTCTGTAAG[A/G]AAGTG TAAAAACAAATTGAATTTCGAAGCTTTGGATATATTCAAAAAAATTTAAACTAA TAACTTATCAGAACAAAGACGAGGAAAATGAAGAATGAAAT
102	RREN_5940_3890	[T/G]	AGACTGTAGAGACCGAAGAGGAGGCAGAGGAGAGAGTGGCAGAGCAGGTAGG CGAACTTGTGGCTGATCATTATCTTTTTGTGTCTGAAGGGGACAATCA[T/G]CGA AGATATTCTGGACAAGGACAAGACAGAACAGACTGGAAGGGCAATGCAACTGC AAGAAGCCGATCAGCGGCGGCACTGAACAACGCCATCATGTTGG
103	RREN_5940_3923	[A/T]	GAGTGGCAGAGCAGGTAGGCGAACTTGTGGCTGATCATTATCTTTTTGTGTCTGA AGGGGACAATCAGCGAAGATATTCTGGACAAGGACAAGACAGAAC[A/T]GACTG GAAGGGCAATGCAACTGCAAGAAGCCGATCAGCGGCGGCACTGAACAACGCCA TCATGTTGGCATTGGCACTGAACAGCATAGGCTGAAGGAGGG
104	RREN_6983_4756	[A/G]	GACAAGGGAACATACCGAGGTGTGACTATTCAGATCATAATTTAACCCATCCTA TCTTGTAGAGCACCCTCACATTGCATCGTATATTTTTGGCTTTTTT[A/G]GCCCGC ACAAATAGTGAAGGGTAGAAAATTCGTTTTTAAAAATAAACGGTCGTTCATCCA CGCGGATCATTCGCCCTTTGGAATGGCGTCGATCGTACCA
105	RREN_7324_5103	[T/C]	GCAGTGTGGCAACCAGGTGTTCTCTCCAAAGAGACAGAGAAGCAGATAATTATC GATTTTTTCCGAGGGTCAGAGACGCAGAAAGGACGCCAACTTTTGG[T/C]GGGA AAAGCGGCGTCACCTTTCCTGCCAGCCACCTCTCTACACTCTCTGTAAGACAGAG AGCGAAATTTATCGATTGATTTTGGGTTAGAGACGCAGAGG

Appendix 2 Continued.

106	RREN_7711_4765	[T/C]	GTGAGAGAGTGTAGAGTGGTGAAGTGGAGGTGAGAGAGTACGGTATGTGAGAG AGTACGGTATGTGACAGTACCGTGTTGTCCACGACCACGACACACTC[T/C]TTGT TGGTTGCCTTCACAACTCGGCAAACGGCCTCAATATCGATGACTTTCAGCAATG GGTTGGACGGTGTTTCGAACCAGACCATCTATGGATGATTAT
107	RREN_7711_4768	[A/G]	AGAGAGTGTAGAGTGGTGAAGTGGAGGTGAGAGAGTACGGTATGTGAGAGAGT ACGGTATGTGACAGTACCGTGTTGTCCACGACCACGACACACTCTTT[A/G]TTGG TTGCCTTCACAACTCGGCAAACGGCCTCAATATCGATGACTTTCAGCAATGGGTT GGACGGTGTTTCGAACCAGACCATCTATGGATGATTATGTA
108	RREN_8907_4239	[A/G]	GTGAACAGAACTTTAAATATGGCGGAGATCGAGTTGACAAAGAAACGCTTAAA AAACTTGACAAAATGCTCAGGAAACACCATCCTTTGGCAAAAGAATT[A/G]ATG AATTTCCACACACAATACCAGCGGGAATTAGCTCTAAACGGACCTGATGCCGTT GCAAACTACCGTTTCACGATTCTCGAGGCACGTGATGCACCGA
109	RREN_9458_2923	[T/C]	GGAATGGACAGCAGAAAGTTGGATGGCGGAAATGGACGGGAGAGAAAGTGAA GAGCAGAGGTGGACGGCGGACATGAGCGGCGGATTTAAACGGCGGAAG[T/C]G AACGGCAGGTGTCGGTGGCTGACGGCGGATAAGTGAACAGCGGAAATAGACGG TGGAGGGTTGACGGTGGAGCTTGACGGCGGACGTGGACAGGACGGA
110	RREN_10201_2603	[A/T]	AATGTTCGAAACAATAGGGAATGGATCTCACGATCCCTCGACTTTGAAGGGTAC TTTGGTCAGGGGACCAGGGATTAGCCCCTATTTAAAGCCGCTCCAA[A/T]AATGA GAGAGGCATGTTCTGTTCAATTCATTCATTCCAACTGCTCAAAGCAGCAACATCA ACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCGCCTTAACA
111	RREN_10854_3216	[T/C]	ACCACCTACACAACGAGAGCCTTATGCTTCCAGTCAAGGAGCACAACTACATGC TCAGCAAGCAGTTCCTGGCCAAATGCCGCCATCCACTTCATCCAAA[T/C]TTCCT CGCCACAAACAACGTCCCAGCCAGGCTCATGAAGCAGACCCTTCCGTCCAAGTT TTGGAAGGAAGTCGACCAAGCTCTACAGGCCGCGGACAACG
112	RREN_10854_3286	[A/G]	GGCCAAATGCCGCCATCCACTTCATCCAAACTTCCTCGCCACAAACAACGTCCC AGCCAGGCTCATGAAGCAGACCCTTCCGTCCAAGTTTTGGAAGGAA[A/G]TCGA CCAAGCTCTACAGGCCGCGGACAACGACCACAACAAATGCAGCGCCAACATCC ACACAACCACAGTCCAAACGGCCAAGCAGCGACAAAGGAGCAC

Appendix 2 Continued.

113	RREN_16875_122	[T/C]	AAGCAGTAACATCAACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCGCCTTAACAC CGCCGGGTCCAACAACTGCGCCAGCCACCGGGGACATTCGAGCCA[T/C]AGCCC CTATTTAAGCCGCTCCAACAATGAGAGAGGCATGTTCAATTTGTTCATTCATACC ATCTATTTCAACTGCTCAAAGCAGTAACATCAACTCTCTT
114	RREN_16875_146	[T/C]	AAGCAGTAACATCAACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCGCCTTAACAC CGCCGGGTCCAACAACTGCGCCAGCCACCGGGGACATTCGAGCCA[T/C]AATGA GAGAGGCATGTTCAATTTGTTCATTCATACCATCTATTTCAACTGCTCAAAGCAG TAACATCAACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCG
115	RREN_16875_190	[T/C]	AAGCAGTAACATCAACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCGCCTTAACAC CGCCGGGTCCAACAACTGCGCCAGCCACCGGGGACATTCGAGCCA[T/C]CAACT GCTCAAAGCAGTAACATCAACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCGCCTT AACACCGCCGGGTCCAACAACTGCGCCAGCCACCGGGGAC
116	RREN_23053_791	[T/C]	GCAAAGGTCGTTTTGGCATTCTTCACAATAAAATTTACCGTTAAATGCGTCTAAG CAATAATTGCAAATGGTTAAATGATGACGAAAATCATAGTTCCAT[T/C]TTAAAT TATTGCAGAAGCCGCGCTCTCTACAATCCCACTGAATATCAATAAACCATGATC CCTTTGTCACTGAAAATAAAGACTGTAGGAAATTTATGAT
117	RREN_23053_809	[T/C]	TTCTTCACAATAAAATTTACCGTTAAATGCGTCTAAGCAATAATTGCAAATGGTT AAATGATGACGAAAATCATAGTTCCATTTTAAATTATTGCAGAAG[T/C]CGCGCT CTCTACAATCCCACTGAATATCAATAAACCATGATCCCTTTGTCACTGAAAATAA AGACTGTAGGAAATTTATGATGCTGAGATTGTGGCTCCC
118	RREN_28983_551	[T/C]	ACATGTTTTTTCTTCGTTTTACTGCGTTTTCTATTGATTTTATGCATTTTTGTTAAG TGTACCATCGAAATTTGCGTGATAACTAAAGGGGTTCAAAAAC[T/C]GGTTTAAA GTGGTTTCCCCCAAAAAATAATTACATATTCGTACTCAGCTCGACGAGCTCTACA CGATGGTATAAATTCTACTCGATTTGTCTTCCGGTAC
119	RREN_36168_664	[A/C]	GAATTTAATTTGTTCAGCGCAACTCTCAAGCAAAATGCGGTGAATGTTCTGCGC AGGCGGAACTATAAAAGGGTGTTGATTTCGCACCGTAACCACCACC[A/C]GCCAT CACTCGCTCTACACGCAACAAACACGCACTCAACTCTCCGCGTTCCCAGCAGCC GCTTCAACAACACAACACTGTTCGTCGACCCTCTGGACAAG

Appendix 2 Continued.

120	RREN_37161_217	[A/G]	CCGAAATACTAAAAACACGTAAAAACTTCGAAGGATCATAACTCTGCTACAGCA TATCCATGCAAGACGAGCAATATACCAATCAATAGAGTAACATGTC[A/G]CGTA AAAACTTCGAAGGATCATAACTCTGCTACAGCATATCCATGCAAGACGAGCAAT ATACCAATCAATAGAGTAACATGTCCTCCACTAATCCCAGAA
121	RREN_37161_226	[C/G]	CCGAAATACTAAAAACACGTAAAAACTTCGAAGGATCATAACTCTGCTACAGCA TATCCATGCAAGACGAGCAATATACCAATCAATAGAGTAACATGTC[C/G]TTCGA AGGATCATAACTCTGCTACAGCATATCCATGCAAGACGAGCAATATACCAATCA ATAGAGTAACATGTCCTCCACTAATCCCAGAAAAGAACATG
122	RREN_43396_312	[T/C]	TTTTCTACTGTAAACAGGAATTCGCATATTCTGAGACCACCATCGTGTAGAGCAT GGTCGATAATAATAAGGAAGTGACATCCTTTTTTGGCACAAACCC[T/C]TGGTTA AATTTTGAGTGAATTTTTAAAATATTTTTTCCACGTGCTTCAAGCACGGGTCATC GGTGCTAAAAATGTCTTTTGGTCAACAAAGCTCAATAAG
123	RREN_47097_473	[C/G]	CATTGTTTTGTATGCTTGTATTTGTATTGTTTTCCCTCTTTCTTCCTTCCCTTTCCTT CACGCCTGCATGTATATAAGCTAGTGATTTTCCCTCATTCTT[C/G]GCAGTTGGTT GTCACCCCAGCTCGCCATTGTTTATTGTCTATTGTATCTTCAATAAACACTTCTTC TCGGGTTCGGACTTCACTTTGGGGTTGTCCTTAC
124	RREN_47097_533	[T/C]	CGCCTGCATGTATATAAGCTAGTGATTTTCCCTCATTCTTCGCAGTTGGTTGTCA CCCCAGCTCGCCATTGTTTATTGTCTATTGTATCTTCAATAAACA[T/C]TTCTTCTC GGGTTCGGACTTCACTTTGGGGTTGTCCTTACTCCTAAACTATCAATCTTCATTG GGCAGTGGCTGTCCTACATTTTTCTCCGATTATTACT
125	RREN_47097_539	[A/C]	CATGTATATAAGCTAGTGATTTTCCCTCATTCTTCGCAGTTGGTTGTCACCCCAG CTCGCCATTGTTTATTGTCTATTGTATCTTCAATAAACACTTCTT[A/C]TCGGGTTC GGACTTCACTTTGGGGTTGTCCTTACTCCTAAACTATCAATCTTCATTGGGCAGT GGCTGTCCTACATTTTTCTCCGATTATTACTTGGTAG
126	RREN_53141_284	[A/T]	GACACCACCATCGGAATCAGCATCTACGAAAACCCTCGAGTACTAAAAATTGGC TCAAAATATCAATAATAAACAACATTTCCATTAGGCCCCCAAAAAA[A/T]TTAGG CCCCCAAAAAACCTGAAATTTTTTCGAAAATTTCCCCAAAAATTTTCTTAGGCCT AACCTAAGGTCAAAATTTTTTGACTAATTTTATGTTTTCG

Appendix 2 Continued.

127	RREN_53141_365	[T/G]	AATAATAAACAACATTTCCATTAGGCCCCCAAAAAACCTGAAATTTTTTCGAAA ATTTCCCCAAAAATTTTCTTAGGCCTAACCTAAGGTCAAAATTTTT[T/G]GACTAA TTTTATGTTTTCGCACATGCTCTACAACTTTAAAAAATAAACCATGCCTCTAAAC CCCTTATAAACACTTCAAATAGTCCTGTCACGTATGATT
128	RREN_53845_394	[T/G]	ACGCTTATGGTAGAGGGGATACGATAACGCACTTACTGTAGAGGGAATGCCGCA ACACATTTAGGGTAGAAGGGATACGGTAACACACTTAAGGGAGGGG[T/G]AGGA TACGGTACCACAATACGTTAACACAATTGATGTGATACGGTACCACACCTTAAG TAGAGGGATACGGTAACACACTTATGGTAGAGGAAATACGAG
129	RREN_57146_374	[T/C]	TGCTGATTTGGGGATCCTTTGTCCGTCGGCCTTTTGTCCACAATCCGATGTCATA TTCGGATTCAGCAGCCTCGATAACCCCCGAGTACCAAAAATCAGC[T/C]CAAAAT ATCAATTACAAATAGCATTTCCATTAGGCCTCCAAAAAACCTGAAATTTTTTTGA AAATTTCCCGAAAATTTTTCTTAGGCCCAACCTAAGGTC
130	RREN_86325_225	[A/G]	CTCTCTAACTCTCCCTCGTCGTCTATACTCTCTCCCCAAACCACACTATCTTATAT TTTTTATGCAATATTCCATCCCCTCTTCACGCTTTTCCAATCAC[A/G]TACTCTCTC CCCAAACCACACTATCTTATATTTTTTATGCAATATTCCATCCCCTCTTCACGCTT TTCCAATCACTCCCCCATCGCTTACGACCATACCG
131	RREN_90419_227	[A/G]	TGAATAAGTGTGCCAGTGAAATTTGCAGTAACTAAAAGGGTTCAAAAACCGGTT TAAAGTGGTTTCCCCCAAAAAATAATTACATATTCGTACTCAGCTC[A/G]GTAAC TAAAAGGGTTCAAAAACCGGTTTAAAGTGGTTTCCCCCAAAAAATAATTACATA TTCGTACTCAGCTCGACGAGCTCTACACGATGGTATATAAT

APPENDIX 3: LETTER REQUESTING PERMISSION TO USE COMMON METHODOLOGY IN CHAPTER 3

Herath Kularathna
Department of Plant Pathology and Crop Physiology
302 Life Sciences Building
Louisiana State University
Baton Rouge, La 70808

Churamani Khanal
Department of Plant Pathology and Crop Physiology
302 Life Sciences Building
Louisiana State University
Baton Rouge, La 70808

Dear Mr. Khanal,

Would you please grant me permission to use common methodology from our collaborative research on chapter three of my dissertation? I look forward to hearing from you about your decision.

Thank you.

Sincerely,

.....

Herath Kularathna

APPENDIX 4: LETTER OF PERMISSION TO USE COMMON METHODOLOGY IN CHAPTER 3

Churamani Khanal
Department of Plant Pathology and Crop Physiology
302 Life Sciences Building
Louisiana State University
Baton Rouge, La 70808

Herath Kularathna
Department of Plant Pathology and Crop Physiology
302 Life Sciences Building
Louisiana State University
Baton Rouge, La 70808

Dear Mr. Kularathna,

I hereby grant you permission to use common methodology from our collaborative research on chapter three of your dissertation.

Thank you.

Sincerely,

.....
Churamani Khanal

VITA

Herath Mudiyanselage Manjula Thaminda Kularathna, son of H. M. Kularathna and I. S. Rathnayake, was born in 1981 in Bandarawela National Hospital, Sri Lanka. He obtained his Bachelor of Science degree in 2006 from University of Colombo, Sri Lanka. He majored in parasitology and was interested in studying the effects of plant parasitic nematodes on plants. He joined the Department of Plant Pathology and Crop Physiology in 2010 to work on his Master's degree on plant pathology under the supervision of Dr. Charles Overstreet. After completing his Master's degree in December 2013, he continued his research work in Dr. Overstreet's lab towards a Doctor of Philosophy degree in plant pathology with the emphasis on phytonematology. During his time at Louisiana State University (LSU) he was involved in different student organizations on and off campus. He was the President of the Plant Pathology and Crop Physiology Graduate Student Association during 2015-2016 and was an active committee member in the Sri Lankan Students Association at LSU. In 2011, he was invited to the honor society of Phi Kappa Phi for his academic achievements. He was a member in the Society of Nematologists and the Honor Society of Gamma Sigma Delta. In his graduate career, he was able to attend different national and international meetings to present the findings of his research. In 2013, he won second place in the student competition at the Beltwide Cotton Conference held in San Antonio, TX. In 2017, he was fortunate enough to attain the prestigious Nathan A. Cobb Nematology Foundation travel award to deliver his findings at the annual Society of Nematologists Meetings in Williamsburg, VA. He anticipates graduating with his Doctor of Philosophy degree in Plant Pathology and Crop Physiology in December, 2017.