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Virulence Phenotypes of Rotylenchulus reniformis: Evaluation of Host Status of Cotton and Utility of Single Nucleotide Polymorphisms (SNPs) for Identification

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VIRULENCE PHENOTYPES OF *ROTYLENCHULUS RENIFORMIS*: EVALUATION OF HOST STATUS OF COTTON AND UTILITY OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) FOR IDENTIFICATION

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by
Churamani Khanal
B.S., Tribhuvan University, Nepal, 2010
M.S., University of Arkansas, 2014
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I would like to dedicate this dissertation to my parents Pitambar Khanal and Jamuna Khanal and loving wife Durga Bhattarai

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ABSTRACT

Comparative reproduction and pathogenicity of reniform nematode (*Rotylenchulus reniformis*) populations derived from single-egg masses and collected form West Carroll (WC), Rapides (RAP), Morehouse (MOR), and Tensas (TEN) parishes in Louisiana were evaluated in microplot and greenhouse trials. Data from microplot trials showed significant differences among isolates of reniform nematode in both reproduction and pathogenicity on upland cotton (*Gossypium hirsutum*) cultivars Phytogen 499 WRF, Deltapine 1133 B2RF, and Phytogen 333 WRF. Across all cotton cultivars, MOR and RAP isolates had the greatest and the least reproduction values of 331.8 and 230.2, respectively. Reduction in plant dry weight, number of bolls, seed cotton weight, and lint weight was the greatest and the least for MOR and RAP isolate, respectively. The reproduction and pathogenicity of WC and TEN isolates were intermediate. In the greenhouse experiment, reproduction of MOR and RAP isolates across all cotton genotypes (three cultivars used in microplot experiment, cultivar Stoneville 4946 GLB2, and two resistant germplasm lines MT2468 Ren3 and M713 Ren5) was the greatest (reproduction value 10.7) and the least (reproduction value 7.9), respectively. Although reproduction values of reniform nematode were lower in the germplasm lines than the cultivars, the germplasm lines sustained greater plant weight loss. The variability in reproduction and pathogenicity among endemic populations of reniform nematode in both the microplot and greenhouse experiments adds further support to the hypothesis that virulence phenotypes of *R. reniformis* exist. In order to determine genetic variability in reniform nematode populations, 31 KASP SNP primers sets were evaluated against 13 reniform nematode isolates that include MOR and RAP isolates from Louisiana as well as other 11 isolates from Mississippi, Arkansas, Hawaii, and Alabama. Twenty-six SNP assays amplified genomic DNA of reniform nematode

isolates while five failed to successfully amplify. Five SNP assays identified genetic differences within and among populations of reniform nematode from Louisiana, Mississippi, and Arkansas. Similarly, eight SNP assays identified genetic differences among samples from Hawaii, and Alabama. The SNP markers developed in this study will be useful in resistance breeding programs as well as in the assessment of the genetic diversity, origin, and subsequent distribution of this nematode.

CHAPTER 1. INTRODUCTION

1.1 Cotton

Cotton (*Gossypium* spp.) is a very important textile fiber crop with an estimated worldwide production of 125 million bales (Dighe *et al*., 2009; Dabbert and Gore, 2014). The genus *Gossypium* comprises almost 50 species, four of which are commercial cotton crops (Wallace *et al*., 2009). Cotton is principally cultivated for the production of natural fiber for the textile industry. In addition to cotton fiber, cottonseed is also regarded as an economically important product of cotton. Approximately 6 million tons of cottonseed is produced annually in the United States (Koenning et al., 2004; Anonymous, 2017). Cottonseed is utilized in the animal feed industry as an excellent source of protein (Koenning *et al*., 2004). Similarly, a high quality vegetable oil, equivalent to or better than soybean oil suitable for human consumption, is produced from cotton (Mauney and Stewart, 1986; Thorp *et al*., 2014). Cottonseed oil is also a possible source of biofuel production (Thorp *et al.,* 2014).

Cotton production in the United States is intensive and highly mechanized (Koenning *et al*., 2004; Starr *et al.*, 2007). The United States is one of the leading cotton producers that accounts for almost a quarter of world lint supply (Koenning *et al*., 2004). In terms of cotton production, the United States ranks third after India and China (Meyer and MacDonald, 2016). Most of the United States cotton is upland cotton (*Gossypium hirsutum* L.) grown as an annual crop from seed planted each year (Anonymous, 2010). Commercial production of upland cotton is mainly carried out in the southern United States with concentrations in the Texas High Plains, the southeast, and the Mississippi Delta (Anonymous, 2010). According to United States Department of Agriculture, National Agricultural Statistics Service (USDA-NASS) report of 2017, Texas is the top cotton producer in the United States followed by Georgia, Mississippi,

Arkansas, Alabama, Oklahoma, Tennessee, Missouri, Arizona, North Carolina, and Louisiana (Anonymous, 2017). In 2016, upland cotton was planted in approximately 4 million hectares with production of approximately 16 million bales (Anonymous, 2017).

Cotton is one of the most important row crops in Louisiana (Lofton *et al.,* 2014). In 2016, upland cotton was planted on 57 thousand hectares in Louisiana with an estimated yield of 260 thousand bales (Anonymous, 2017). A bale of cotton contains 480 pounds of lint.

1.2 Cotton growth and development

Cotton is a woody perennial crop which passes through distinct developmental stages. An excellent overview of growth and development of cotton has been provided by Oosterhuis and Bourland (2001), and Mauney (2015). Briefly, under favorable conditions for germination, cotton seedlings emerge from soil in four to nine days after planting. Elongation of the apical meristem gives rise to a main stem which has monopodial and indeterminate growth habit meaning the terminal bud continues to grow as a central leader shoot. The main stem contains several nodes and internodes and branches develop from a bud located at a node in a leaf axil. Branches and leaves are spirally arranged on the stem in a three to eight phyllotaxy. Cotton produces vegetative and fruiting branches. Vegetative branches have monopodial growth while fruiting branches have sympodial growth meaning the fruiting branches terminate in a square. Fruiting branches are produced by both the main stem and vegetative branches. First square occurs 30-40 days after emergence. Fertilization followed by anthesis takes place near dawn in 20-25 days after the development of square. Flower consists of three bracts, calyx whorl (five sepals), corolla (five petals), and 4- or 5-locule ovary which contains six to nine ovules. Cotton is a self-pollinated crop, but cross pollination, predominantly by insects, can also occur. Boll maturity is reached in about 50 days after fertilization. Fibers are produced by extension of

epidermal cells of seed coat. Fibers are first elongated which is followed by secondary

thickening. Approximately 500 bolls are required to produce a kilogram of cotton fiber.

Depending on growing conditions, cotton is harvested in 130 to 160 days after planting.

1.3 Nematodes of cotton

Cotton is attacked by several plant parasitic nematodes. The predominant plant parasitic

nematodes of cotton are the southern root-knot nematode (*Meloidogyne incognita*), and the

reniform nematode (*Rotylenchulus reniformis*) (Koenning *et al.*, 2004; Starr *et al.*, 2011).

Amount of crop losses caused by southern root-knot and reniform nematodes is more or less

equally divided between the two (Weaver, 2015). Other economically important nematodes of

cotton are sting nematode (*Belonolaimus longicaudatus*), and Columbia lance nematode

(*Hoplolaimus columbus* Sher) (Weaver, 2015). Economic damage threshold of sting and lance

nematode on cotton is approximately one nematode per cubic centimeter of soil (Noe, 1993;

Crow *et al.*, 2000). Sting and lance nematode are distributed in very sandy soils (>80% sand) and

thus have little impact on cotton as very little cotton is grown in heavily sandy soil (Weaver,

2015).

1.4 The reniform nematode

Reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) was first observed

in Oahu island of Hawaii in 1940 attacking cowpea, pineapple, and several weeds (Linford and

Oliveira, 1940). The adult female of this nematode is kidney shaped and hence got the vernacular

name. The genus *Rotylenchulus* was proposed because of its morphological similarity to

Rotylenchus and life history similar to *Tylenchulus*. Reniform nematode belongs to the

superfamily Heteroderoidea and the family Nacobbidae. The genus *Rotylenchulus* has 10

previously described species (Robinson *et al.*, 1997) and a newly described species (Berg *et al.*,

2016). Of the 11 described species, only *R. reniformis* and *R. parvus* have been reported from the United States (Lehman and Inserra, 1990).

Reniform nematodes are categorized into five groups based on lip morphology and hyaline length of tail, with *R. reniformis* being the only species in group III (Robinson *et al*., 1997). Some diagnostic features of *R. reniformis* are the presence of males, adult female is 324-383µm long by 161-168 µm wide, 16-21µm long stylet, head sharply set off from the expanded neck region, posterior vulva (v>63%), later stage becomes immobile and develops feeding site, and posterior part of the body enlarges assuming reniform shape (Linford and Oliveira, 1940). Juveniles, young females, and males are vermiform in shape and dorsal gland opening is usually one stylet length behind stylet (Linford and Oliveira, 1940).

Following first report of occurrence from Hawaii, *Rotylenchulus reniformis* was reported from cotton and cowpea roots in Baton Rouge, Louisiana in 1941 (Smith and Taylor, 1941). Subsequently, scientists from other parts of the United States reported occurrence of this nematode. Currently, reniform nematode is the predominant parasitic nematode of upland cotton in the mid-south area of the United States (Stetina and Young, 2006; Robinson, 2007; Starr *et al*., 2011).

An excellent overview of life cycle of reniform nematode has been provided by Robinson *et al.* (1997). Briefly, a mature adult female lays eggs covered in a gelatinous mass. Upon embryogenesis, first stage juvenile (J1) is formed. J1 molts and becomes second stage juvenile (J2) which emerges from the egg by breaking the egg shell with the help of stylet. Subsequent molting gives rise to third and fourth stage juveniles (J3 and J4). The J4 becomes adult and differentiates into either male or female. Only the J4 female is infective while male remains non infective. The adult female infects the host root cortex, develops a feeding site, and becomes

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sedentary. Only the anterior part of the body is embedded in the root and posterior part swells as the nematode feeds and gains reproductive maturity. The mature adult female lays approximately 60 eggs covered in a gelatinous mass. This process completes the life cycle of reniform nematode. Multiple life cycles can occur in a single crop growing season.

Under optimum condition, life cycle of reniform nematode can be completed in as few as 25 days. In other words, the minimum life cycle can be roughly divided as 8 days for hatching, 8 days for completion of molting, and 9 days for laying eggs (Linford and Oliveira, 1940). The life cycle can be extended to several years if nematodes enter the state of anhydrobiosis (Birchfield and Martin, 1967; Gaur and Perry, 1991).

1.5 Crop losses by reniform nematode

Reniform nematodes are distributed in tropical and subtropical regions of the world attacking at least 314 plant species belonging to 77 families (Robinson *et al.,* 1997). The reason for its wide distribution may be attributed to the wide host range and the ability to survive in a state of anhydrobiosis for several years without host plants (Birchfield and Jones, 1961; Birchfield and Martin, 1967; Gaur and Perry, 1991). Using a stylet, the adult female nematode punctures host cells and delivers proteolytic and pectolytic enzymes to degrade cell wall (Dieterich and Sommer, 2009). Once inside host, nematode differentiates host cells to form multinucleated syncytial cells as a source of food. Upon infection, reniform nematode adversely affects plant growth, delays flowering and fruiting times, reduces number and size of the bolls, and decreases lint quality (Robinson, 2007). According to Dighe *et al*. (2009), cotton production in the United States has been greatly compromised by reniform nematodes. In upland cotton, loss to reniform nematode in 2016 was approximately 205 thousand bales including 8 thousand bales from Louisiana (Lawrence *et al*., 2017).

In Louisiana reniform nematode has become the major nematode pathogen of cotton and soybean over the last three decades (Overstreet and McGawley, 1994; Overstreet and McGawley, 1998; Overstreet, 2006, 2015). According to Robinson *et al*. (2007), Louisiana, Mississippi, and Alabama sustain almost three quarters of the United States cotton crop losses to the reniform nematode. Each year approximately 3 to 5% of the Louisiana cotton crop is lost as a result of reniform nematode pathology (Bell *et al.*, 2014; Lawrence *et al*., 2017).

1.6 Reniform nematode management

Some common management methods for reniform nematode include, but are not limited to, chemical, biological, crop rotation, tolerance, and resistance. Commonly used chemicals for reniform nematode management are fumigants (Vapam, Telone), non-fumigants (aldicarb, Vydate, Velum), and seed treatments (Avicta, Aeris). For economic and environmental reasons, the use of nematicides is not a preferred management option (Agudelo *et al*., 2005; Blessitt *et al*., 2012). Some biological control agents such as *Pasteuria penetrans*, *Bacillus firmus,* and *Paecilomyces lilacinus* have been effective to manage reniform nematode in lab conditions, however, their efficacy is greatly reduced under field conditions. Crop rotation with non-host crops such as corn and resistant soybean reduces the nematode populations greatly; however, the populations usually resurge quickly in a subsequent single year of cotton production (Robinson *et al*., 2007, Stetina *et al.*, 2007). Crop rotation with milo, peanuts, and sugarcane can also be used to reduce reniform nematodes (Overstreet *et al.*, 2014). Use of reniform nematode resistant cotton has been a most widely desired method of reniform nematode. More than three decades of research in developing reniform nematode resistant upland cotton cultivars has not been successful. Because no commercial cotton cultivars that are resistant to reniform nematode are

available, the best management options currently available are the use of tolerant cultivars and crop rotation.

CHAPTER 2. REPRODUCTION AND PATHOGENICITY OF ENDEMIC POPULATIONS OF *ROTYLENCHULUS RENIFORMIS* ON COTTON

2.1 Introduction

The reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) was first

observed in Hawaii in 1940 attacking cowpea, pineapple and several weeds (Linford and

Oliveira, 1940). Subsequently, this nematode was reported from cotton and cowpea roots in

Baton Rouge, Louisiana in 1941 (Smith and Taylor, 1941). Currently, reniform nematode is the

predominant parasitic nematode of upland cotton in the mid-south area of the United States

(Stetina and Young, 2006; Robinson, 2007; Starr *et al.*, 2011). The female nematode infects

cotton roots producing approximately 60 eggs per egg mass in as less as 25 days (Linford and

Oliveira, 1940). Upon infection, reniform nematode adversely affects plant growth, delays

flowering and fruiting times, reduces number and size of the bolls, and decreases lint quality

(Robinson, 2007). Because of crop damage caused by reniform nematode, cotton production in

the United States has been greatly compromised (Dighe *et al.* 2009). Each year approximately

205 thousand bales of United States upland cotton is lost to reniform nematode (Lawrence *et al.*,

2017).

With an aim of durable management of reniform nematode in upland cotton production,

studies on identification of resistant cultivars and germplasm lines have been conducted since

early1960s. Birchfield and Brister (1963) evaluated 24 upland cotton cultivars in the greenhouse

and found that none were resistant to reniform nematode. Yik and Birchfield (1984) reported that

Gossypium longicalyx was a non-host while *G. stocksii, G. somalense*, and *G. barbadense* 'Texas

110' showed high levels of resistance. Robinson *et al.* (1999, 2007) emphasized the absence of a

source of resistance to the reniform nematode as a major constraint of upland cotton production

in the United States. In 2007, two cotton germplasm lines, LONREN-1 and LONREN-2, having

a resistance gene introgressed from *G. longicalyx* Hutch. & Lee were released by the USDA-

ARS, Texas Agricultural Experiment Station and Cotton Incorporated (Bell *et al.*, 2014a).

Reniform nematode resistance genes Ren_1 (previously Ren^{lon}), Ren_2 (previously Ren^{ari}), and

$Ren_2{}^{GB713}$ have been identified from *G. longicalyx*, *G. aridum* (Rose & Standl.) Skov, and *G.*

barbadense L. GB713 (PI 608139), respectively (Dighe *et al.*, 2009; Romano *et al.*, 2009; Fang

and Stetina, 2011; Bell *et al.*, 2014b). Subsequently, reniform nematode resistant germplasm

lines TAM RKRNR-9, TAM RKRNR-12, and BARBREN-713 were released (Starr *et al.*, 2011;

Bell *et al.*, 2014b). All of these germplasm lines suppressed reniform nematode reproduction by

40-90% (Starr *et al.*, 2011; Bell *et al.*, 2014a, 2014b). Similarly, a joint effort by USDA, ARS,

Mississippi State University, and College Station, Texas released several sources of reniform

nematode resistant germplasm lines that include TX 110, M713 Ren1, M713 Ren2, M713 Ren5,

MT2468 Ren1, MT2468 Ren2, and MT2468 Ren3 (Wallace *et al.*, 2009; McCarty *et al.*, 2012,

2013).

     Because no commercial cotton cultivars that are resistant to reniform nematode are

available, the best management options currently available are the use of tolerant cultivars and

crop rotation. A field study conducted by Blessitt *et al.* (2012) to evaluate cotton cultivars in

Mississippi identified six cultivars viz. Cropland Genetics 3520 B2RF, DynaGrow 2520 B2RF,

Stoneville 5242 BR, Stoneville 5599 BR, Deltapine 488 BG/RR, and Fibermax 960 B2R as

tolerant to reniform nematodes. Tolerant cultivars are those that have the capacity to support

reproduction while sustaining satisfactory yields (Schafer, 1971; Blessitt *et al.*, 2012). Crop

rotation with non-host crops such as corn and resistant soybean reduces the nematode

populations greatly; however, the populations usually resurge quickly in a subsequent single year

of cotton production (Robinson *et al.*, 2007). Use of chemicals can possibly lead to better management, however, the use of nematicides is no longer a viable management option because of economic and environmental reasons (Agudelo *et al.*, 2005; Blessitt *et al.*, 2012).

The success of management of a disease is based on understanding of variability in a pathogen (Werlemark *et al*., 2006; Silva *et al*., 2008). A few studies have reported the existence of variability in geographic populations of reniform nematode. A report from the 1960s indicated that Louisiana populations of *R. reniformis* were physiologically different from other reniform nematode populations suggesting the existence of races (Birchfield, 1962). Subsequent reports demonstrated physiological variation in reproduction and pathogenicity of geographic populations of reniform nematode (McGawley and Overstreet, 1995; McGawley *et al.*, 2010, 2011). Studies have also been conducted to determine the amount of genetic variability in reniform nematode populations. While Agudelo *et al.* (2005) did not find any obvious genetic variability in reniform nematode populations collected from ten states in the United States, other genetic studies reported variability in geographic populations of reniform nematode (Tilahun *et al.*, 2008, Arias *et al.*, 2009; Leach *et al.*, 2012). A better understanding of variability in populations of reniform nematode can help scientists develop a better management strategy. The main objective of this research was to determine whether or not there was reproductive and pathological variation in populations of *R. reniformis* endemic in Louisiana.

2.2 Materials and methods

2.2.1 General procedure

Isolates of reniform nematode were collected from West Carroll (WC), Rapides (RAP), Morehouse (MOR), and Tensas (TEN) parishes in Louisiana, confirmed morphometrically as *R. reniformis*, and used to establish single-egg mass cultures. Axenic cultures were maintained

under greenhouse conditions on tomato (*Solanum lycopersicum* L. cultivar Rutgers PS, Seedway; Hall, New York 14463). Reniform nematode isolates from four parishes were employed in greenhouse and microplot studies with the most widely planted upland cotton cultivars Phytogen 499 WRF, Deltapine 1133 B2RF, and Phytogen 333 WRF (Anonymous, 2015). The cultivars Phytogen 499 WRF, Deltapine 1133 B2RF, and Phytogen 333 WRF hereafter will be abbreviated as PHY499, DP1133, and PHY333, respectively. Exact details of greenhouse and microplot studies are presented below under the appropriate subheadings.

A soil mixture consisting of three parts Commerce silt loam soil (fine-silty, mixed, superactive, nonacid, thermic Fluvaquentic Endoaquepts) and one part sand, and pots, unless stated otherwise, used in all experiments was steam sterilized for 5 hours at 135°C prior to use. In each test, two cotton seeds were planted to a depth of 2.5 cm and thinned to one per pot after germination. Soils were 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 7-d-old seedling. The infestation level for the microplot experiments was 50,000 vermiform life stages per microplot. Similarly, the infestation level for the greenhouse experiments was 4,000 vermiform life stages per pot, which is equivalent to the number of vermiform life stages per gram of soil in microplot trials. Half of the inoculum was added to soil in microplots at 10 days after planting and the remainder at 21 days. Standard fertilization, weeding and insect management practices were employed in all trials.

In all cases, nematodes were extracted from a 250 cm^3 subsample of soil from each pot and processed 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 magnification. Eggs were extracted from whole root systems in greenhouse

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experiments by agitating root in 0.6% NaOCl for 10 min to dislodge eggs from egg masses (Hussey and Barker, 1973) and counting at 40x magnification. All plant materials were dried at 30-35 °C for two weeks and weighed.

2.2.2 Microplot studies

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 20 kg of soil mixture. The entire microplot area was bounded by aluminum Quonset hut skeletal frame which was open at both ends. The skeletal frame was covered with one layer of 6 mm polyethylene to protect plants in microplots from excessive summer rainfall and one layer of 20% reflective foilcloth for optimal sunlight. This cover was equipped with overhead fans and an irrigation system. The entire system allowed for maintenance of near field conditions (McGawley *et al.*, 2010). A total of 75 microplots were established to evaluate 3 widely planted upland cotton cultivars (PHY499, DP1133, and PHY333), 4 isolates of reniform nematode, a non-inoculated control for each cultivar and 5 replications. Establishment of plants, inoculation with nematodes, and processing of plant and nematode materials were the same as that described above. Additional plant data collected in microplot studies included number of harvestable bolls per plant, seed cotton weight, and lint weight.

2.2.3 Greenhouse studies

This study involved six genotypes of cotton: three cultivars (PHY499, DP1133, and PHY333), one susceptible cultivar (Stoneville 4946 GLB2), and two germplasm lines showing moderate to high levels of resistance (MT2468 Ren3 and M713 Ren5). The cotton genotypes Stoneville 4946 GLB2, MT2468 Ren3, and M713 Ren5 hereafter will be abbreviated as ST4946, MT2468, and M713, respectively. Terra cotta pots with a top diameter of 15 cm 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 the nematode, a non-inoculated control for each genotype and 5

replications. The experiment was terminated after 60 days and nematode life stages in soil and

roots were quantified as described above.

2.2.3 Data analysis

Each experiment employed a factorial treatment structure and was established as a

randomized block design with five replications. Each experiment was repeated once. Analysis of

variance was conducted using test as a fixed effect and there were no significant trial by

treatment interactions in any of the tests described herein. Therefore data from all like trials was

combined for analysis. Data were examined by analysis of variance (ANOVA) for a factorial

design using the "Fit Y by X" module of SAS JMP Pro, version 13.0 (SAS Institute; Cary, NC).

Means of data were separated by Fisher's LSD at $P \leq 0.05$.

2.3 Results

2.3.1 Microplot studies

Data from two microplot trials were combined for analysis because of the absence of year

by treatment interactions. Reniform nematode isolates, across all cotton cultivars, produced

significant differences in reproduction and effect on plant dry weight, seed cotton weight, and

lint weight (Table 1). Significant interactions were not observed between cotton cultivars and

reniform nematode isolates for any of the growth parameter measured. Statistical main effects of

the 4 isolates of the nematode across the 3 cultivars are presented in Table 2. Population density

at harvest ranged approximately from 288 to 415 thousand individuals per 500 cm^3 of soil,

representing reproductive values (number of vermiform stages per microplot divided by the

infestation level) of 230.2 to 331.8.

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Table 1. Main and interaction effects (*P* values) of four isolates of *Rotylenchulus reniformis* endemic in Louisiana on three cotton cultivars in a microplot environment[x].

Source	DF	Vermiform life stages	Plant weight	Number of bolls	Seed cotton weight	Lint weight
Cultivar (C)[y]	2	0.8956	0.0025*	0.0398*	0.0005**	0.0004**
Isolate (I)[z]	3	<0.0001**	<0.0001**	<0.0001**	<0.0001**	<0.0001*
C × I	6	0.7831	0.5983	0.2375	0.7521	0.7732

[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 2× 3 factorial with ANOVA ($P \leq 0.05$); * and ** indicate *P* values significant at the 0.05% and 0.01% levels, respectively.
[y] Cultivars were Phytogen 499 WRF, Deltapine 1133 B2RF, and Phytogen 333 WRF that were recommended for use in Louisiana in 2015.
[z] Isolates were each derived from a single-egg mass from roots of cotton from West Carroll, Rapides, Morehouse, and Tensas parishes in Louisiana.

Across all cotton cultivars, reproduction of the MOR isolate was significantly greater than that by the other 3 isolates. Reproduction by the RAP was least while TEN and WC had intermediate reproduction. The isolate from MOR parish significantly reduced plant dry weight, number of bolls, seed cotton weight, and lint weight compared to those of the non-inoculated control and other three nematode isolates. Cotton plant dry weight of inoculated plants ranged from 138g to 278g compared to 305.6g for the non-inoculated control. MOR, WC, TEN, and RAP isolates lowered plant dry weight of cotton by 55%, 25%, 21%, and 9%, respectively. The RAP isolate produced the least reduction in plant dry weight, number of bolls, seed cotton weight, and lint weight, although not always significantly different from the control.

Statistical main effects of the 3 varieties of cotton across the 4 isolates of the nematode are presented in Table 3. Across all 4 isolates of the nematode, dry weight for DP1133 was reduced significantly more than were those for the 2 Phytogen cultivars. Results for numbers of

Table 2. Main effect of isolate of *Rotylenchulus reniformis* on vermiform life stages, plant weight, number of bolls, seed cotton, and lint weights across three cultivars of cotton in a full season microplot environment[w].

Isolate[x]	Vermiform life stages (1000's) per 500 cm^3 of soil[y]	Reproduction value[z]	Plant weight (g)	Number of bolls	Seed cotton weight (g)	Lint weight (g)
WC	337.3 b	269.8	228.4 b	16.5 b	82.6 b	35.6 b
RAP	287.8 c	230.2	278 ab	23.9 a	99.7 b	45.2 ab
MOR	414.8 a	331.8	138 c	9.5 c	48.5 c	19.7 c
TEN	333.3 bc	266.6	241.9 b	17.3 b	89.5 b	39.6 ab
Control	0	0	305.6 a	27.7 a	117.4 a	55.5 a

[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 cotton were Phytogen 499 WRF, Deltapine 1133 B2RF, and Phytogen 333 WRF.

[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 in Louisiana.

[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]Reproduction values were calculated by dividing the estimated number of vermiform stages per microplot (20 kg of soil) by the infestation level of 50,000 vermiform life stages.

bolls, and seed and lint weights were similar, except for PHY499 where numbers of bolls were not significantly different, averaging 15.7.

2.3.2 Greenhouse studies

Because there was no significant trial by treatment interactions, data from the two greenhouse trials were also combined. For almost all parameters, there were highly significant genotype and isolate main effects as well as genotype by isolate interactions (Table 4). Genotype

Table 3. Main effect of three cultivars of cotton on plant weight, number of bolls, seed cotton, and lint weights across four isolates of *Rotylenchulus reniformis* in a full season microplot environment[x].

Cultivar[y]	Plant weight (g)[z]	Number of bolls	Seed cotton weight (g)	Lint Weight (g)
Phytogen 499 WRF	268.7 a	20.2 ab	100.9 a	45.3 a
Deltapine 1133 B2RF	196.7 b	15.7 b	64.4 b	28.2 b
Phytogen 333 WRF	251.4 a	21.1 a	98.2 a	44.3 a

[x]Data were combined over two full season trials and are means of ten replications. Plant material was dried at 30-35 °C.
[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.

Table 4. Main and interaction effects (*P* values) of four isolates of *Rotylenchulus reniformis* endemic in Louisiana on six genotypes of cotton in a greenhouse environment[x].

Source	DF	Vermiform life stages	Eggs/root system	Root weight	Shoot weight	Plant weight
Genotype (G)[y]	5	<0.0001**	<0.0001**	0.0027*	<0.0001**	<0.0001**
Isolate (I)[z]	4	<0.0001**	<0.0001**	<.0001**	<0.0001**	<0.0001**
G × I	20	<0.0001**	<0.0001**	0.5167	0.0001**	0.0002**

[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 5 × 4 factorial with ANOVA ($P \leq 0.05$); * and ** indicate *P* values significant at the 0.05% and 0.01% level, respectively.
[y] Genotypes were the cultivars Phytogen 499 WRF, Deltapine 1133 B2RF, Phytogen 333 WRF, and Stoneville 4946 GLB2 and the germplasm germplasm lines MT2468 Ren3, and M713 Ren5.
[z] Isolates were each derived from a single-egg mass from roots of cotton from West Carroll, Rapides, Morehouse, and Tensas parishes in Louisiana.

main effects on nematode vermiform stages and eggs as well as shoot and plant dry weights were

significant at the 1% level and root dry weight was significant at the 5% level. Isolate main

Table 5. Main effect of isolates of *Rotylenchulus reniformis* on vermiform life stages, eggs per root system, root, shoot, and plant weights across six genotypes of cotton in a greenhouse environment[w].

Isolate[x]	Vermiform life stages (1000's) per 500 cm^3 of soil[y]	Reproduction value[z]	Eggs/root system	Root weight (g)	Shoot weight (g)	Plant weight (g)
WC	11.4 b	9.1	4484 a	2.0 b	7.5 b	9.5 c
RAP	9.8 b	7.9	4093 a	2.2 b	8.2 b	10.3 b
MOR	14.4 a	10.7	4713 a	1.9 b	6.6 c	8.6 d
TEN	11.1 b	8.8	4228 a	2.1 b	7.8 b	9.9 bc
Control	0 c	0	0 b	2.7 a	9.1 a	11.7 a

[w]Data were combined over two 60-day trials and are means of ten replications. Plant material was dried at 30-35 °C. Genotypes were the cultivars Phytogen 499 WRF, Deltapine 1133 B2RF, Phytogen 333 WRF, and Stoneville 4946 GLB2 and the germplasm lines MT2468 Ren3, and M713 Ren5.
[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 in Louisiana.
[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]Reproduction values were calculated by dividing the estimated number of vermiform stages per pot (1.6 kg of soil) by the infestation level of 4,000 vermiform life stages.

effects were significant at the 1% across both nematode and plant parameters. Genotype by

isolate interactions were significant at the 1% levels for both nematode and plant values except

root dry weight where they were not significant.

Statistical main effects of the six genotypes of cotton across the 4 isolates of the

nematode in the greenhouse environment are presented in Table 5. Reniform nematode

population density in soil ranged from approximately 10 thousand to 14 thousand individuals per 500 cm^3 of soil with corresponding reproductive values of 7.9 to 10.7. The numbers of eggs per root system was similar among the isolates and averaged 4 to 5 thousand per root system. Across all cultivars, all isolates of the nematode caused significant reductions in root weight compared with controls, but there were no differences among the isolates. Results for weights of shoots was similar except that the MOR isolate caused greater reductions than the other 3 isolates. The isolate of *R. reniformis* from MOR parish also caused a reduction in final plant weight which was greater than that caused by the other 3 isolates, 8.6 g compared with 9.5 g for the WC isolate, 10.3 g for the RAP isolate, and 9.9 g for the TEN isolate.

The two Phytogen cultivars, along with DP1133 and ST4946 supported the highest, but not significantly different, numbers of nematodes which ranged from 9,688 to 13,022 individuals per 500 cm^3 of soil (Table 6). Significantly fewer numbers, 7,315, were recovered for MT2468 Ren3. Vermiform stages per 500 cm^3 of soil averaged 4,573 for M713 Ren5 and were significantly less than the averages across the 4 isolates for the other genotypes.

Egg production by the nematode, across isolates, was similar and not significantly different for PHY499, DP1133, PHY333, and ST4946 (Table 6). Respectively, eggs per root system averaged 4,667, 4,534, 4,637 and 5,181. Significantly fewer, 1,360 and 642, were collected from roots of MT2468 Ren3 and M713 Ren5.

There were highly significant genotype by isolate interactions which influenced both soil and root stages of the nematode (Table 4). Individual treatment means illustrating soil population levels of the nematode across the 6 genotype / 4 isolate combinations are presented in Figure 1. Nematode numbers associated with PHY499 ranged from 12,795 for the TEN isolate to 15,520 per 500 cm^3 of soil for the MOR isolate with intermediate values of 13,232 for WC and 14,454

Table 6. Main effect of genotypes of cotton on vermiform life stages, eggs per root system, root, shoot, and plant weights across four isolates of *Rotylenchulus reniformis* in a greenhouse environment[x].

Genotype[y]	Vermiform life stages[z]	Eggs/root system	Root weight (g)	Shoot weight (g)	Plant weight (g)
Phytogen 499 WRF	11200 a	4667 a	2.2 abc	8.0 b	10.3 b
Deltapine 1133 B2RF	9688 ab	4534 a	2.4 a	9.2 a	11.6 a
Phytogen 333 WRF	10174 a	4637 a	2.3 ab	8.2 b	10.4 b
Stoneville 4946 GLB2	13022 a	5181 a	2.1 bc	7.9 b	9.9 b
MT2468 Ren3	7315 b	1360 b	2.0 c	6.6 c	8.6 c
M713 Ren5	4573 c	642 b	2.1 bc	7.1 c	9.1 c

[x]Data were combined over two full season trials and are means of ten replications. Plant material was dried at 30-35 °C.
[y]Genotypes were the cultivars Phytogen 499 WRF, Deltapine 1133 B2RF, Phytogen 333 WRF, and Stoneville 4946 GLB2 and the germplasm lines MT2468 Ren3, and M713 Ren5.
[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.

for RAP and no significant differences in numbers among the 4 isolates. An average soil population level of 15,592 individuals per 500 cm^3 of soil was estimated for the WC isolate on DP1133 and this was significantly greater than the 8,907 and 11,829 for the RAP and MOR isolates but not the 12,112 per 500 cm^3 with the TEN isolate. With the genotype PHY333, nematode populations in soil averaged 13,728, 10,520, 14,768 and 11,856 nematodes per 500 cm^3's for the WC, RAP, MOR and TEN isolates, respectively. These population levels did not differ significantly from one another. For ST4946 there were significantly greater numbers of the MOR and TEN isolates (20,856 and 17,728, respectively) in soil than for the other 2 isolates with averages of 8,672 for WC and 12,056 for RAP. There were no significant differences in
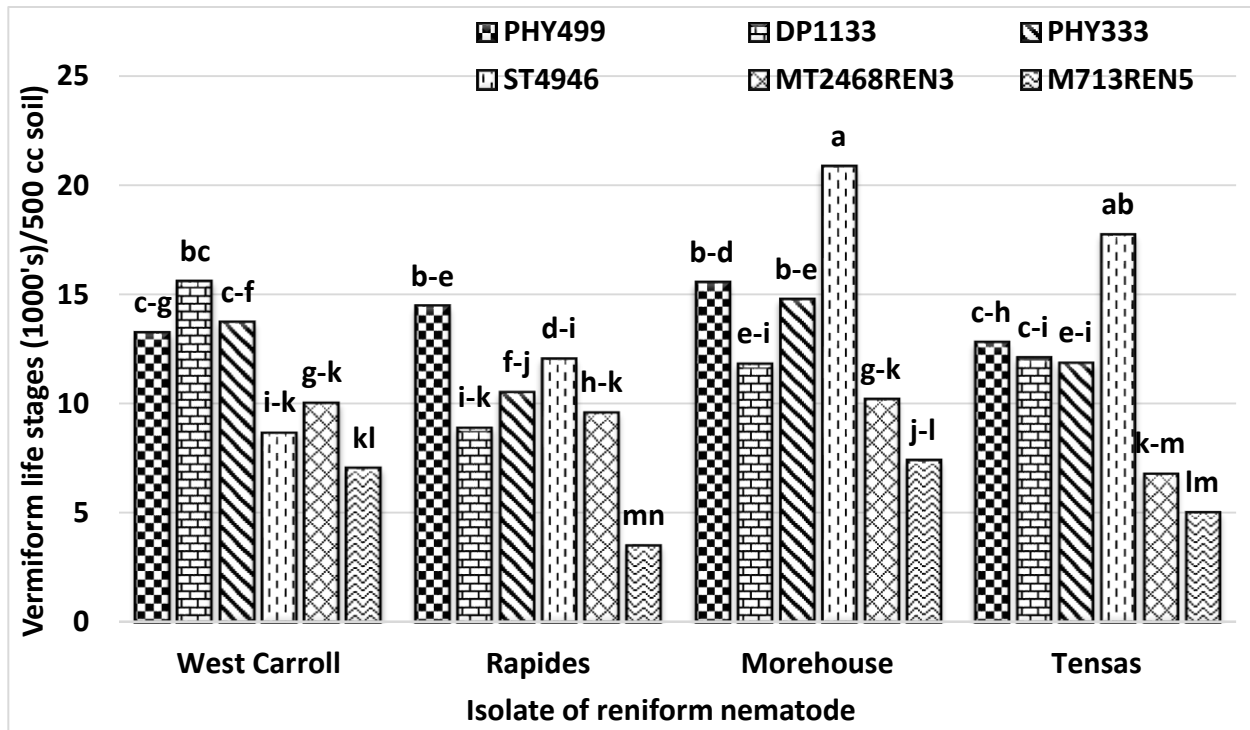
Figure 1. Vermiform life stages of *Rotylenchulus reniformis* per 500 cc of soil, after 60 days in a greenhouse environment from cotton genotypes Phytogen 499 WRF (PHY499), Deltapine 1133 B2RF (DP1133), Phytogen 333 WRF (PHY333), Stoneville GLB2 (ST4946), MT2468 Ren3, and M713 Ren5. Data are means of 10 replications averaged over two trials. Bars with common letters are not significantly different based on Fisher's LSD test (*P*<0.05)

population levels of the nematode in soil for any of the isolates with the genotype MT2468.

Numbers per 500 cm^3 of soil ranged from 6,776 for the TEN isolate to 10,192 for the MOR

isolate. Overall, the lowest populations of the nematode in soil were found with the genotype

M713. Nematode numbers for the RAP and TEN isolates were similar and not significantly

different averaging 3,488 and 4,992, respectively. Greater population levels, 7,016 for the WC

isolate and 7,369 for MOR were associated with M713.

Individual treatment means showing numbers of eggs of the nematode recovered per root

system across the 6 genotype / 4 isolate combinations are presented in Figure 2. The histograms

illustrate clearly that significantly greater numbers of eggs were produced by all isolates of the

nematode on the 4 named cultivars, PHY499, DP1133, PHY333 and ST4946, than on the
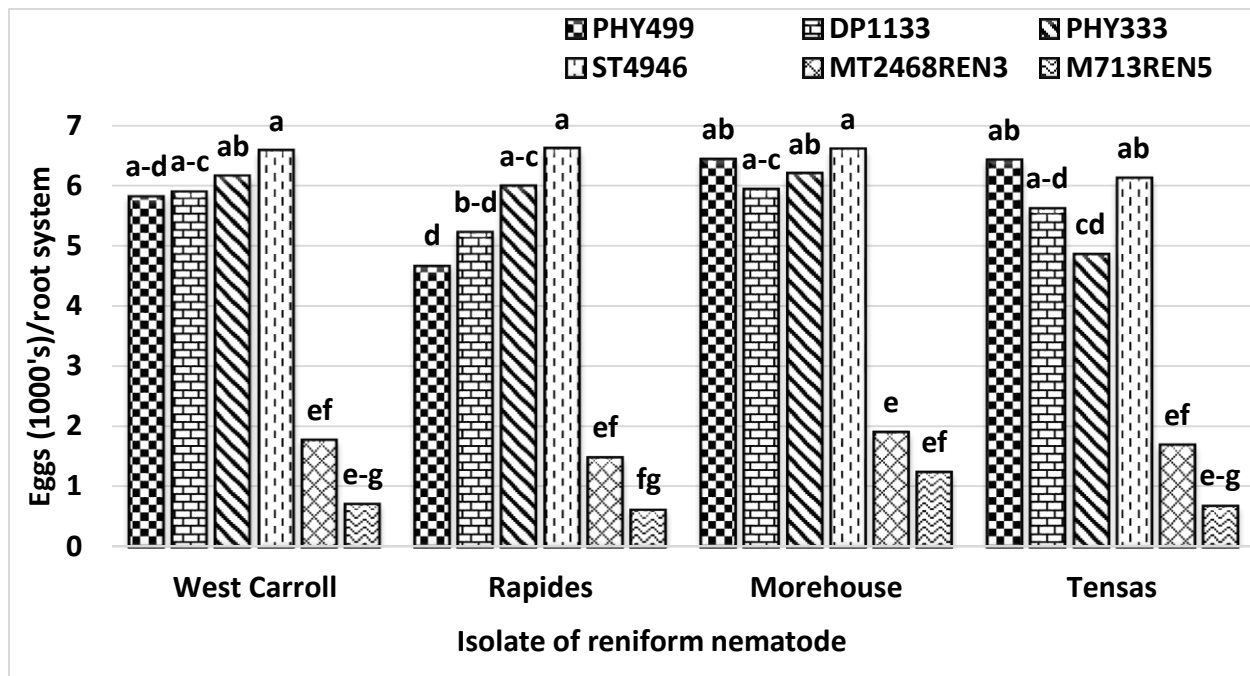
Figure 2. Egg stages of *Rotylenchulus reniformis* from whole root systems of cotton genotypes Phytogen 499 WRF (PHY499), Deltapine 1133 B2RF (DP1133), Phytogen 333 WRF (PHY333), Stoneville 4946 GLB2 (ST4946), MT2468 Ren3, and M713 Ren5 after 60 days in a greenhouse environment. Data are means of 10 replications averaged over two trials. Bars with common letters are not significantly different based on Fisher's LSD test ($P < 0.05$).

germplasm lines MT2468REN3 and M713REN5. For PHY499, there were significant

differences in egg numbers per root system across the 4 isolates of the nematode where RAP had

significantly higher reproduction than MOR and TEN, but not WC with means averaging 5,816,

4,664, 6,432 and 6,424 for WC, RAP, MOR and TEN, respectively. For DP1133, there were also

no significant differences in numbers of eggs with very similar numbers estimated for each

isolate; 5,896 for WC, 5,224 for RAP, 5,936 for MOR and 5,616 for TEN. Numbers of eggs per

root system of PHY333 were not significantly different for the WC, RAP and MOR isolates,

respectively averaging 6,152, 5,984 and 6,192. Numbers of eggs per root system from PHY333

averaged 4,856 for the TEN isolate of the nematode and this was significantly lower than that

numbers from the MOR and WC isolates but not the RAP isolate. Numbers of eggs for each of

the 4 isolates of the nematode were very similar for ST4946 and not significantly different with

means averaging 6,576 for WC, 6,608 for RAP, 6,600 for MOR and 6,120 for TEN. Mean

numbers of eggs from roots of MT2468 averaged 1,760 for the WC isolate of *R. reniformis*,

1,472 for RAP, 1,888 for MOR and 1,680 for TEN and there were no significant differences in

these averages. Similarly, there were no significant differences in numbers of eggs recovered

from M713 for each of the isolates of the nematode. Averages for eggs per root system were 704,

608, 1,228 and 672 for WC, RAP, MOR and TEN, respectively.

There were also highly significant genotype by isolate interactions indicated in Table 4

that influenced the cotton shoot and plant dry weights. Inspection of individual treatment means

for both plant parameters reveal a similar pattern and, therefore, only those for plant weight are

presented as Figure 3. Overall, the figure illustrates clearly that the isolate of the nematode from

MOR parish was the most pathogenic and it was significantly more so on the germplasm lines

MT2468 and M713. Relative to the non-inoculated control at 60 days after inoculation, weights

of PHY499 plants were reduced significantly by the reniform nematode isolates from WC, MOR

and TEN parishes but not by the one from RAP. Control plant weight averaged 11.9 g, those for

WC, MOR and TEN were 10.1, 8.8 and 9.6 g, respectively and that for RAP was 10.9 g. Three

of the 4 isolates, WC, RAP and TEN, did not cause significant reductions in plant weight for

DP1133 when compared with the average for the control. Respectively, these plant dry weights

averaged 11.2, 12.2, 11.6 and 12.6 g. The WC and TEN isolates caused significant damage to

PHY333 and the RAP and MOR isolates did not. The mean plant weight for non-inoculated

PHY333 was 11.7 g. Weights for PHY333 inoculated with isolates from WC and TEN were 9.2

and 9.9 g, respectively and those for RAP and MOR were 10.4 and 10.9. With ST4946 the

isolates from RAP and MOR reduced weights of plants significantly below the 11.6 g value of
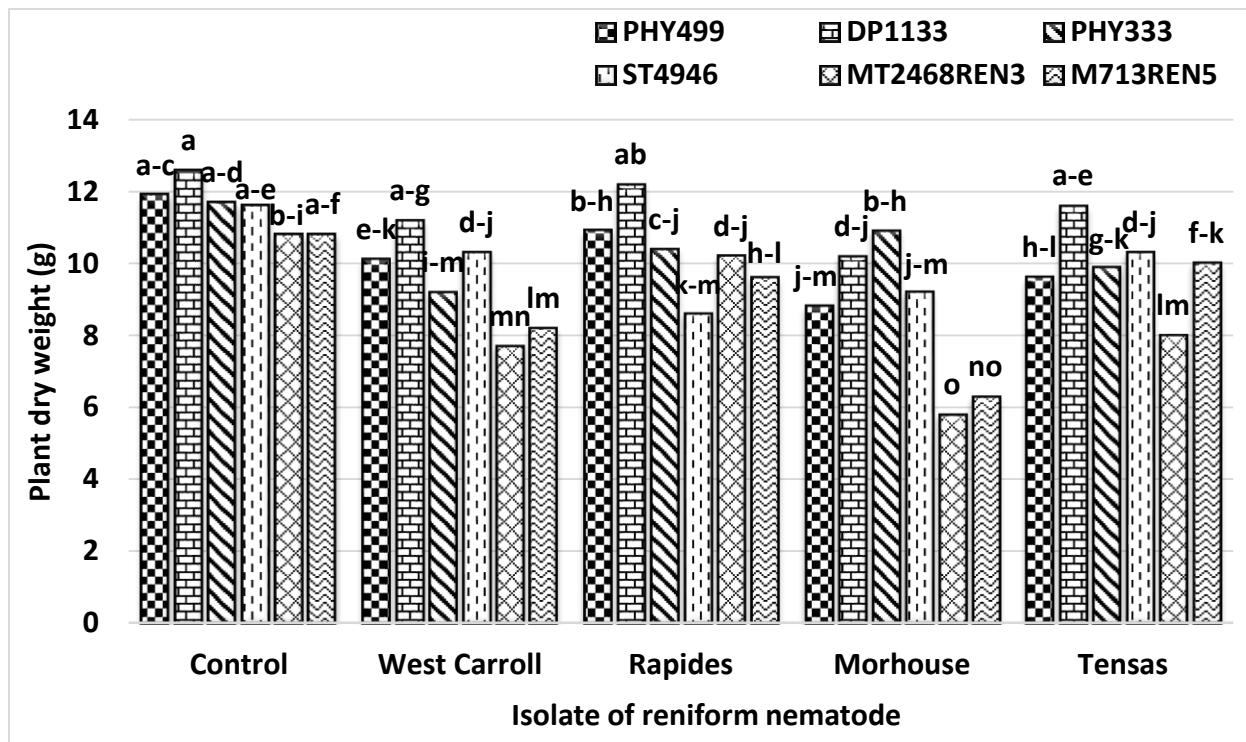
Figure 3. Effect of four isolates of *Rotylenchulus reniformis* on plant dry weight of cotton genotypes Phytogen 499 WRF (PHY499), Deltapine 1133 B2RF (DP1133), Phytogen 333 WRF (PHY333), Stoneville 4946 GLB2 (ST4946), MT2468 Ren3, and M713Ren5. Nematode isolates each were derived from a single-egg mass isolated from West Carroll, Rapides, Morehouse, andTensas Parishes of Louisiana. Plant material was dried at 30-35 °C. Data were combined over two 60-day trials and are means of ten replications. Data were analyzed with ANOVA and Fisher's LSD test (P≤0.05). Means followed by a common letter are not significantly different.

the non-inoculated control. Isolates from WC and TEN did not reduce weights of ST4946

significantly. Weights were 8.6 g for RAP, 9.2 g for MOR, and 10.3 g for WC and TEN. For

both MT2468 and M713, three of the four isolates of the nematode reduced weights of plants

significantly relative to the control. Plant weights for non-inoculated MT2468 and M713 each

averaged 10.8 g. For MT2468 significant reductions in plant weight were caused by WC, MOR,

and TEN isolates with mean plant weights of 7.7, 5.8, and 8.0 g, respectively. Plant weight for

the RAP isolate was 10.2 g and not significantly different from the control. For M713 the isolates

which caused significant plant damage were from WC, RAP, and MOR parishes. Respectively,

plant weights were 8.2, 9.6, and 6.3 g and the isolate which did not elicit significant plant

damage was from TEN parish and the final plant weight averaged 10.0 g.

2.4 Discussion

Nematologists have documented the existence of reproductive, pathogenic, and/or genetic

variability in a range of plant parasitic nematodes including burrowing nematode (Ducharme and

Birchfield, 1956; Huettel and Yaegashi, 1988), stem and bulb nematode (Seinhorst, 1957),

soybean cyst nematode (Riggs *et al.*, 1981; Niblack *et al.*, 2002), and root-knot nematode

(Barker *et al.*, 1985; Noe, 1992; Van der Beek *et al.*, 1999; Khanal *et al.*, 2016). The existence of

morphological, physiological, and/or genetic variability among geographic isolates of *R.*

reniformis have been proposed by some studies as well. Dasgupta and Seshadri (1971)

designated two races (Race A, Race B) of *R. reniformis* based on their differential reproduction

in castor, cotton, and cowpea. A study published in 1983 in Japanese and translated in English by

Nakasono in 2004 designated 3 distinct biological types (male-numerous, male-rare, and male-

absent) of *R. reniformis* that originated from Japan and the United States. McGawley and

Overstreet (1995) studied 17 populations of reniform nematode collected from Louisiana,

Arkansas, Hawaii, Mississippi, and Texas in greenhouse and laboratory tests and found variation

among populations with respect to reproduction on and/or damage to cotton and soybean. A

study conducted by Agudelo *et al.* (2005) on selected cotton and soybean cultivars involving 13

amphimictic populations of reniform nematode collected from major cotton growing area in the

United States showed that considerable variation in reproduction and morphology exists within

and among the geographic populations. Agudelo *et al.* (2005) further evaluated the ribosomal

internal transcribed spacer region-1 (ITS1) of populations of reniform nematodes from the

United States as well as from Brazil, Colombia, Honduras, and Japan. They found that all the

populations, except one from Japan, did not differ genetically for the studied nuclear region. The population from Japan, that was parthenogenetic, showed a considerable amount of nucleotide variation (41/348 bp) suggesting that a difference in genotypic make up can introduce considerable variation into a population. They suggested further that other molecular markers such as amplified fragment length polymorphism and microsatellites would be useful in assessing variation in nematode populations. In contrast to the results from Agudelo *et al.* (2005), Tilahun *et al.* (2008) found significant amount of variation in ITS1 and 18S ribosomal DNA of seven reniform nematode populations in Alabama. Such contrasting results have created confusion about the suitability of ribosomal DNA for assessment of genetic variability in this nematode.

As the genetic variability studies specifically focused on ITS and 18s rDNA has been elusive, Arias *et al.* (2009) readily distinguished reniform nematode populations from Texas, Louisiana, Mississippi, and Georgia using microsatellite markers. Furthermore, a greenhouse experiment by Arias *et al.* (2009) supported the notion of variability in geographic isolates. Studies of the variability of geographic isolates described heretofore were short duration greenhouse or lab studies that did not always employ populations derived from single-egg mass cultures, and the experiment may or may not have been repeated. In contrast to the methodology employed in previous research, McGawley *et al.* (2010, 2011) conducted microplot tests involving cotton and soybean to assess reproduction and pathogenicity of reniform nematode populations derived from single-egg mass cultures collected from Alabama, Arkansas, Hawaii, Louisiana, Mississippi, and Texas. They collected data from two full-season microplot trials and found significant differences among isolates of reniform nematode in both reproduction and pathogenicity. Microplot trials conducted by McGawley *et al.* (2010, 2011) reported that

reniform nematode inoculated cotton and soybean cultivars, respectively, sustained 38.6% and 27.9% plant dry weigh reduction compared to those of the non-inoculated controls. Further research including several isolates of reniform nematode endemic in Louisiana are necessary to support that reniform nematode is more damaging to cotton than soybean. Greenhouse study conducted by Bhandari *et al.* (2015) reported significant variability in reproduction and pathogenicity of Louisiana populations, although not derived from single egg mass cultures, of reniform nematode on susceptible cotton genotypes and resistant germplasm lines.

Research detailed in this report provides an indication of the amount of variation in endemic populations of reniform nematode from cotton growing regions in Louisiana. Over the course of this research, two microplot and two greenhouse experiments were conducted to determine the pathogenicity and reproduction of four endemic populations of reniform nematode on cotton genotypes. Data obtained from both greenhouse and microplot experiments demonstrated that the MOR and RAP isolates caused the greatest and the least damage, respectively. Furthermore, least reproduction and lower pathogenicity of reniform nematode isolates, especially MOR and RAP, was evident on the germplasm lines rather than on commercial cultivars (Figure 1-4).

This research is the first report that employs a series of microplot and greenhouse experiments to demonstrate reproductive and pathogenic variation among populations of *R. reniformis* endemic to Louisiana. Evidence strongly suggesting the existence of virulence phenotypes in endemic populations makes it essential for breeding programs aimed at developing reniform nematode resistant cultivars employ as many isolates as possible in order to produce durable sources of resistance.

Parallel research conducted by a fellow nematology student here at LSU, Mr. Manjula Kularathna, employs the same populations of reniform nematode, but uses soybean 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 soybean genotypes, the MOR isolate exhibited the lowest level of reproduction, but caused the greatest amount of damage. Conversely, with cotton, the MOR isolate exhibited the greatest level of reproduction and caused the greatest level of damage. Across all cotton and soybean genotypes, respectively, MOR isolate reduced plant dry weight by 54.8% and 29.8% relative to those of the non-inoculated controls. This difference in pathogenicity of MOR isolate on cotton and soybean is possibly a function of host.

CHAPTER 3. SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS PROVIDES AN INSIGHT ON GENETIC VARIABILITY IN *ROTYLENCHULUS RENIFORMIS*

3.1 Introduction

The reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) has established itself as the major plant parasitic nematode of cotton and soybean in the United States (Stetina and Young, 2006; Robinson, 2007; Arias *et al*., 2009; Leach *et al*., 2012; Allen *et al*., 2017). Approximately 12% of cotton and 5% of soybean yields are lost to reniform nematode in the United States cotton belt with Louisiana, Alabama, and Mississippi sustaining the greatest damage (Lawrence *et al*., 2017; Allen *et al*., 2017).

Current practice of reniform nematode management is based primarily on use of chemicals or crop rotation. Chemical control is not always the preferred method of reniform nematode management because of health and environmental issues (Agudelo *et al*, 2005). Selection of a suitable crop for rotation can help reduce losses caused by the nematode, however, reniform nematode populations resurge quickly to a damaging level in a single crop growing season when a susceptible crop is planted (Robinson *et al*., 2007). Use of host plant resistance has been the most desirable, but least available method of reniform nematode management in cotton. Unfortunately, reniform nematode resistant cotton cultivars are not available. Some cotton breeding lines and soybean cultivars showing moderate to high level of resistance to reniform nematode are available, however, their yield performance is not always similar when tested across different geographic locations (Yik and Birchfield, 1984; Robinson *et al*., 1997; Robinson *et al*., 2004; Weaver *et al*., 2007). It is well known that durable host plant resistance is mainly dependent on amount of variability present in a pathogen (Riggs *et al*., 1981; Noe, 1992; Van der Beek *et al*., 1999; Niblack *et al*., 2002). The inconsistency in suppression of

reproduction of reniform nematode in different geographic locations by some available resistant cultivars/breeding lines have been reported (Robinson *et al.*, 1997; Robinson *et al.*, 2004; Yik and Birchfield, 1984; Weaver *et al.*, 2007; Wallace *et al.*, 2013; Bell *et al*., 2015). This inconsistency could have been caused by the existence of physiological and genetic variability in geographic isolates of reniform nematode. Utilization of molecular techniques to better understand the genetic variability in reniform nematode populations would be helpful in developing durable reniform nematode resistant cultivars.

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 (SNP) have been used in the last three decades to study genetic variations and characterization of population of many organisms (Grover and Sharma, 2016). The first generation of molecular markers such as RFLPs are not currently much used for genetic variability assays because they are complex, costly, and identify lower rates of polymorphism (Gao *et al*., 2016; Yang *et al*., 2017). SSRs are often considered the second generation of genomic markers and are useful in determining a higher polymorphism rate (Gao *et al*., 2016). However, SSR markers are often considered cumbersome to use in high throughput genotyping protocols and they may not be widely and evenly distributed in the genome (Salem *et al*., 2012). The third generation of markers (Gao *et al*., 2016), SNPs, are superior in studying genetic variability as they are abundant and widely distributed in the genome (Salem *et al.*, 2012). Advent of next generation sequencing (NGS) technology has enabled more efficient study of genetic variation in an organism using SNP analysis (Gao *et al*., 2016). The utility of SNPs allows genomic sequences of an organism to be compared in a rapid, reliable, and highly

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efficient way (Graves *et al*., 2016; Yang *et al*., 2017). SNP analyses have been useful in

discriminating species of many organisms including fungi, bacteria, virus, nematode, plants, and

animals (Faga *et al*., 2001; Morais *et al*., 2006; Rattei, *et al*., 2007; Samson-Himmelstjerna *et al*.,

2007; Figueiredo *et al*., 2013; Lu *et al*., 2013; Ojeda *et al*., 2014; Gao *et al*., 2016; Linlokken *et

al*., 2017; Yang *et al*., 2017).

The existence of morphometric, physiological, and genetic variabilities within *R.

reniformis* populations have been documented from different 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 (McGawley and Overstreet, 1995;

Agudelo *et al*., 2005; Tilahun *et al*. 2008, Arias *et al*., 2009, McGawley *et al*., 2010; McGawley

et al., 2011). The variability among geographic populations of *R. reniformis*, particularly genetic

variability, has not always been obvious. A study conducted by Agudelo *et al*. (2005) showed no

variation in the first internal transcribed spacer (ITS1) region of reniform nematode collected

from ten states in the United States. In contrast to the report by Agudelo *et al*. (2005), Tilahun *et

al*. (2008) found fairly substantial variation in ITS1 as well as in the 18S region of reniform

nematode populations from Alabama. Regarding the lack of correlation between phenotypic and

genotypic variation, Agudelo *et al*. (2005) suggested that use of microsatellite markers could

provide a more reliable way to evaluate populations. Studies conducted by Arias *et al*. (2009),

and Leach *et al*. (2012) using microsatellite markers reported genetic variability in geographic

isolates of reniform nematode. With the advent of NGS technology, it is possible to analyze

whole genomic DNA of reniform nematode in much more detail using a robust molecular assay

such as SNPs. Analysis of SNP data would be useful in determining genetic variability within

isolates and among geographic populations of reniform nematode. To date, there have not been

any published reports of SNP molecular markers specific to reniform nematodes. The major objective of this research was to identify SNP molecular markers and evaluate their use in determining genetic variability among reniform nematode isolates.

3.2 Methodology

3.2.1 Isolation and extraction of nematode

A total of 13 geographic populations of reniform nematode, two from Louisiana, six from Mississippi, three from Arkansas, and one each from Hawaii, and Alabama, were used to evaluate putative SNPs and measure genetic variability (Table 7). Each population was derived from single egg mass and maintained on tomato (*Solanum lycopersicum* L. cultivar Rutgers PS, Seedway; Hall, New York 14463) in a greenhouse (Table 7). From each population, 300 to 400 gravid females were excised from tomato roots with the help of stereoscopic microscope, sterilized needles, and tweezers. The excised gravid females from each population were placed in distilled water in petri plates and subsequently transferred to labelled 2 ml Eppendorf tubes.

3.2.2 Genomic DNA extraction from nematode

From nematode samples DNA was extracted using a Maxwell 16 (Promega, Madison, WI, USA) automated DNA isolation machine. To Eppendorf tubes containing the nematodes in a water solution, 500 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) was added. The tubes were briefly vortexed vigorously. The tubes were then incubated for 2 hours at 60 °C with gentle shaking at 350 rpm. The tubes were then vortexed for 5 seconds to mix the solution. The solution obtained was processed using Maxwell 16 FFS Nucleic Acid Extraction System, Custom (Catalog No. X9431). The supernatant, which is essentially genomic DNA, obtained at the end of process was transferred to labelled 1.5 ml Eppendorf tubes. The genomic DNA was quantified on

Table 7. Sample ID, origin, population type, and source of reniform nematode populations used for SNP analysis.

Sample ID	Origin of sample[x]	Isolate[y]	Source[z]
LA1	Rapides, Louisiana	Single egg mass	Nematode advisory service, LSU
LA2	Morehouse, Louisiana	Single egg mass	Nematode advisory service, LSU
MS1	Humphreys, Mississippi	Single egg mass	Salliana Stetina
MS2	Holmes, Mississippi	Single egg mass	Salliana Stetina
MS3	Holmes, Mississippi	Single egg mass	Salliana Stetina
MS4	Stark, Mississippi	Single egg mass	Gary Lawrence
MS5	Elizabeth, Mississippi	Single egg mass	Salliana Stetina
MS6	Washington, Mississippi	Single egg mass	Salliana Stetina
AR1	Kibler, Arkansas	Single egg mass	Robert Robbins
AR2	Hawkins, Arkansas	Single egg mass	Robert Robbins
AR3	Fayetteville, Arkansas	Single egg mass	Robert Robbins
HI1	Oahu, Hawaii	Single egg mass	Brent Sipes
AL1	Auburn, Alabama	Single egg mass	Kathy Lawrence

[x]Location from where reniform nematode populations were originally collected.
[y]Reniform nematode populations were maintained on tomato in greenhouse either as single-egg mass or mixed populations.
[z]Persons or lab that originally collected and supplied the nematode samples.

Synergy H1 (BioTek®, Winooski, VT, USA) microplate spectrophotometer at 260 nm UV absorption. DNA from the single egg reniform nematode samples were isolated and whole genome amplified.

3.2.3 Whole genome amplification of genomic DNA

In order to conduct multiple SNP analyses, a larger amount of genomic DNA was required. Sufficient amounts of genomic DNA for each reniform nematode population was obtained by the process of whole genome amplification (WGA) using GenomePlex® Complete Whole Genome Amplification kits (Sigma-Aldrich, St. Louis, MO; Cat. No. WGA2) following manufacturer's instructions. The genomic DNA isolations served as the DNA template for whole genome amplification. The procedure consisted of three broad steps: 1) Fragmentation, 2) Library Preparation, and 3) Amplification. Briefly, for Step 1 (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 consolidate the contents. For Step 2 (Library Preparation): to the tube from Step 1, 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 placed in a PTC-200 thermal cycler (MJ Research, Waltham, MA) at 95 °C for 2 minutes. The sample was cooled again by placing the tube on ice for 2 minutes, then consolidated by centrifugation and returned to ice. A 1 µl of library preparation enzyme was added to the tube, thoroughly vortexed and briefly centrifuged. The tube was placed in a PTC-200 thermal cycler (MJ Research, Waltham, MA) 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 Step 3 (Amplification): the 15 µl library sample obtained in Step 2 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 set with following conditions: 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 aliquot of the final product, WGA amplified DNA, was run out 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 from each reniform nematode population was quantified on Synergy H1 microplate spectrophotometer and stored at -20°C. In order to obtain enough DNA for the subsequent SNP analyses, all WGA DNA samples were reamplified using the GenomePlex WGA Reamplification Kit (Sigma-Aldrich, Catalog Number WGA3) following manufacturer's instructions. Reamplified DNA samples were purified using the GenElute kits and concentrations determined on the microplate spectrophotometer.

3.2.4 Identification of SNPs

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 Appendix 1 and 2. The 31 putative SNPs shown in Appendix 1 were selected for testing in the current study. SNPs were specifically selected to be at different genomic locations (i.e. on different contigs) as reported for the reniform 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 8) were sent to LGC Genomics (Teddington, UK) where KASP (kompetitive allele-specific PCR) genotyping assays were designed for each SNP.

Table 8. Summary of SNP ID, contig, sequence position, fluorescence label for each SNP, GC content, and LGC Genomics reference number for the SNP-specific primers used in this research. The SNP and flanking sequences are shown in Appendix 1.

SNP ID	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	0216484048
RREN_4410_3946	4,410	3,946	A	G	65	68.4	59.1	0216484047
RREN_7711_4758	7,711	4,758	T	C	42.3	45.8	48	0216484024
RREN_514_63176	514	63,176	A	G	54.5	57.1	59.1	0216484023
RREN_925_39379	925	39,379	C	G	33.3	31	48	0216484000
RREN_514_63173	514	63,173	T	G	59.1	65	54.2	0216483999
RREN_91287_201	91,287	201	A	C	44	45.8	33.3	0216483976
RREN_91287_193	91,287	193	T	C	44	45.8	37.9	0216484070
RREN_43396_339	43,396	339	A	C	29	30	59.1	0216484049

(Table continued)

SNP ID	Contig	Sequence Position	Allele FAM[x]	Allele HEX[y]	GC% FAM	GC% HEX	GC% common	LGC Genomics[z]
RREN_1990_6847	1,990	6,847	A	G	37	44	46.2	0216484046
RREN_20709_1089	20,709	1,089	A	C	33.3	37	37.9	0216484025
RREN_258_12977	258	12,977	A	G	52	52.2	48	0216484022
RREN_269_9935	269	9,935	A	G	24.2	30	46.2	0216484001
RREN_456_104249	456	104,249	T	G	34.5	37	48	0216483998
RREN_43396_325	43,396	325	T	G	37	40.7	59.1	0216483977
RREN_901_49990	901	49,990	A	G	30	31	37.9	0216484069
RREN_1886_12077	1,886	12,077	C	G	37	37	48	0216484050
RREN_16875_158	16,875	158	T	C	24.2	30	73.7	0216484045
RREN_251_23034	251	23,034	T	C	52.2	54.5	50	0216484026
RREN_1895_31360	1,895	31,360	A	T	44	44	33.3	0216484021
RREN_9137_320	9,137	320	T	C	35.7	42.3	37.9	0216484002

[w]SNPs were assigned and chosen across the reniform nematode genome so that each SNP is not clustered together with the others.
[x]Tail of primers were labelled with FAM and HEX which generate specific fluorescence signals that is detected by LightCycler 480 software.
[y]Percentage of Guanine and Cytosine in a SNP sequence.
[z] LGC Genomics reference number for each SNP-specific assay tested in this research.

3.2.5 KASP genotyping assay

SNPs indicate a single base change in the genome which can be detected by designing primers that amplify the respective base change. For example in the middle of the sequence below the letters in brackets "[T/C]" represent a SNP, where one allele is a "T" and another allele is a "C" and heterozyogtes represent a mixture of both alleles:

AAGACGCCCCGGGGGAAGGA**[T/C]**AGAGGGAATTCCCACTCTCC.

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

A total of 17 samples, that included 4 no-template controls and the 13 reniform nematode samples described above, were tested. The Amplification Reaction Mix was prepared so that each reaction had 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. PCR was conducted as follows: an initial denaturation at 94°C for 15 minutes; followed by 10 cycles of denaturation at 94°C for 20 seconds, and annealing/extension at 65°C for 1 minute with a temperature reduction of -0.8 °C per cycle; and subsequent 26 cycles of denaturation at 94°C for 20 seconds, and annealing/extension at 57°C for 1 minute.

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3.3 Results

The procedures of WGA and WGA reamplification successfully produced sufficient quantities and quality of DNA for SNP analysis. This technology allows the molecular analysis of minute quantities of DNA derived from individual nematodes, thereby facilitating the analysis of genetic diversity among and within nematode populations. One approach is to examine genetic diversity among samples using molecular markers such as SNPs. However, to date, no SNPs have been reported specifically for reniform nematodes.

As part of an on-going project, 162 putative reniform nematode specific SNPs were identified (Jeffery D. Ray, USDA-ARS, Stoneville, MS; personal communication) but have not been reported elsewhere. However, none of these putative SNPs had been previously evaluated to determine if they actually perform as intended. Therefore a subset of 31 putative SNPs were designed and manufactured to function as dual emission fluorescent KASP[TM] (kompetitive allele-specific PCR) primers (Table 8, LGC Genomics reference number). These KASP primers enable the bi-allelic scoring of SNPs at specific loci including those in complex genomes. The 31 KASP SNP primers sets were evaluated against 13 reniform nematode isolates.

The 13 isolates of reniform nematode were collected from Louisiana, Mississippi, Arkansas, Hawaii, and Alabama (Table 7). Of the 31 SNPs tested, 13 amplified genomic DNA of reniform nematode isolates from different geographic locations while five SNPs, for the most part, failed to successfully amplify. A summary of results from KASP genotyping assays is presented in Table 9.

In the case of reniform nematode samples from Louisiana, 25 SNPs amplified while six SNPs failed to amplify. Of the 25 positive SNPs, twelve appeared to identify only one allele

while six identified both alleles (i.e. heterozygous DNA). The remaining seven SNPs identified

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

For nematode samples from Mississippi, two of the SNPs failed to amplify half of the

samples. Of the remaining 29 SNPs, eight were monomorphic (except for MS11 which the

LightCycler could not differentiate) while two detected heterozygous loci (except for MS10

which was not amplified). The other 15 SNPs identified genetic differences among the reniform

nematode isolates from Mississippi. The remaining three SNPs were not very good because of

inability to detect amplified samples by the LightCycler.

Table 9. Likely alleles as called by LightCycler 480 software after running 31 single nucleotide polymorphism (SNP) analysis on isolates from, Mississippi (MS), Arkansas (AR), Hawaii (HI), Alabama (AL), and Louisiana (LA)[xyz].

SNP ID	MS1	MS2	MS3	MS4	MS5	MS6	AR1	AR2	AR3	HI1	AL1	LA1	LA2
RREN_4410_3972	X	X	X	X	UNK	X	H	X	H	Y	H	X	X
RREN_1572_36933	X	X	UNK	UNK	X	X	Y	X	Y	H	H	Y	Y
RREN_4410_3979	Y	H	X	Y	X	X	X	X	X	X	X	X	X
RREN_43396_315	X	X	UNK	X	-	-	X	X	-	X	-	-	-
RREN_523_19992	H	H	H	H	H	H	H	H	H	H	H	H	H
RREN_367_3958	Y	Y	X	Y	X	X	X	UNK	X	Y	Y	Y	Y
RREN_4834_4618	Y	Y	X	X	X	H	H	Y	X	Y	X	H	H
RREN_5033_5267	X	X	H	UNK	UNK	X	Y	H	UNK	Y	UNK	X	H
RREN_845_36717	H	H	H	-	H	H	H	H	H	H	H	H	H
RREN_3215_15723	X	Y	UNK	H	H	Y	H	UNK	H	Y	UNK	Y	H
RREN_1660_513	X	X	H	UNK	H	UNK	H	UNK	H	H	H	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	Y	Y	Y	X	Y	Y	Y	Y	Y

(Table continued)

SNP ID	MS1	MS2	MS3	MS4	MS5	MS6	AR1	AR2	AR3	HI1	AL1	LA1	LA2
RREN_514_63176	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	X	Y
RREN_925_39379	H	Y	H	H	H	H	H	Y	H	H	H	H	H
RREN_514_63173	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	X	Y
RREN_91287_201	Y	Y	-	Y	-	Y	-	-	-	-	-	-	-
RREN_91287_193	H	H	-	H	-	Y	H	-	-	-	-	-	-
RREN_43396_339	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	-	-
RREN_1990_6847	X	X	Y	X	X	X	X	X	X	X	X	X	X
RREN_20709_1089	X	UNK	X	X	UNK	UNK	Y	X	Y	Y	UNK	X	X
RREN_258_12977	X	X	X	X	X	X	X	X	X	X	X	X	X
RREN_269_9935	Y	H	H	UNK	H	UNK	Y	UNK	Y	H	H	H	Y
RREN_456_104249	X	X	X	X	X	X	X	X	X	X	X	X	X
RREN_43396_325	Y	H	-	Y	-	-	-	H	-	-	-	-	-
RREN_901_49990	UNK	X	H	Y	H	UNK	H	X	X	X	UNK	Y	X
RREN_1886_12077	X	X	X	X	X	X	X	X	H	X	X	X	X

(Table continued)

SNP ID	MS1	MS2	MS3	MS4	MS5	MS6	AR1	AR2	AR3	HI1	AL1	LA1	LA2
RREN_16875_158	Y	Y	-	Y	-	-	-	Y	-	-	-	-	-
RREN_251_23034	UNK	UNK	H	UNK	Y	UNK	X	UNK	UNK	UNK	H	X	H
RREN_1895_31360	H	Y	H	Y	H	H	H	H	H	H	H	Y	Y
RREN_9137_320	H	UNK	H	Y	UNK	Y	UNK	UNK	Y	UNK	H	H	H

[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 Mississippi (MS), Arkansas (AR), Hawaii (HI), Alabama (AL), and Louisiana and 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.

Only 28 SNPs amplified nematode samples from Arkansas while three mostly failed to amplify. Eight SNPs were monomorphic while other three detected heterozygous loci in samples from Arkansas. Of the remaining 17 SNPs, nine identified allelic differences in reniform nematode isolates from Arkansas.

Five SNPs failed to successfully amplify genomic DNA on samples from Hawaii, and Alabama. Of the remaining 26 SNPs, two identified allelic differences among samples from Hawaii and Alabama.

Evaluation of multiple isolates of reniform nematode from Louisiana, Mississippi, and Arkansas resulted in detection of genetic differences between and among the geographic isolates. Of the 26 SNPs that, for the most part, amplified genomic DNA of reniform nematode isolates from different geographic locations, five (RREN_5033_5267, RREN_3215_15723, RREN_269_9935, RREN_901_49990, and RREN_251_23034) were able to identify genetic differences between and among isolates of reniform nematode from Louisiana, Mississippi, and Arkansas. Because only a single isolate of reniform nematode from Hawaii and Alabama were available, genetic difference among the isolates from these locations could not be elucidated. However, among the isolates from Hawaii, and Alabama, eight SNPs were able to detect genetic variability (Table 9).

3.4 Discussion

To date no SNPs have been reported in reniform nematodes. Herein 162 putative SNPs (Appendices 1 and 2) are reported of which a select group were tested to confirm their functionality. These SNPs were tested on a group of 13 geographic isolates of reniform nematode from Louisiana, Mississippi, Arkansas, Hawaii, and Alabama. This study employed SNPs, a third generation marker (Gao *et al*., 2016), to elucidate the genetic differences in

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geographic isolates of reniform nematode from Louisiana, Mississippi, Arkansas, Hawaii, and Alabama. Existence of genetic variability in *Caenorhabditis elegans* and various plant parasitic nematodes, including but not limited to 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) have been documented. Previous identifications, however, were based on use of first and second generation markers. This study is the first to report genetic variability among geographic isolates of reniform nematode using SNP analysis.

A subset of 31 SNPs (Table 8) were used to design bi-allelic KASP genotyping assays and tested on genomic DNA of 13 isolates of reniform nematode collected from various geographic locations in the United States in order to detect genetic differences among the isolates (Table 7). Most SNPs identified the two SNP alleles as well as heterozygotes across all samples (Table 9). However, some SNPs only identified one allele or the other and many only identified heterozygous alleles (Table 9). Monomorphic SNPs likely indicate a lack of genetic diversity at that genomic location where as heterozygotes likely indicate the samples were not necessarily pure. Increasing the number of different genotypes (i.e. isolates) tested would most likely increase the frequency of all alleles detected.

Five of the 31 SNPs evaluated did not work because of poor amplification. Better amplification could possibly be achieved by optimizing PCR conditions specifically for each SNP. Although some SNPs failed to amplify, 26 of 31 SNPs amplified reniform nematode DNA giving a success rate of 83.9%. This rate of success is comparable to similar studies conducted in plants with success rates of 78.5% to 88.4% (Cockram *et al.*, 2012; Saxena *et al.*, 2012; Semagn

et al., 2014; Graves *et al.*, 2016). Assuming a similar success rate for the putative SNPs not tested, then most of those SNPs shown in Appendix 2 should function.

Prior to this research no reniform nematode specific SNPs have been available. The sequence information shown for each SNP in Appendices 1 and 2 can be used to prepare reniform nematiode specific SNP assays. For the 31 SNPs tested in this study using KASP assays (Appendix 1), the sequence information can be used to design other types of SNP assays. Alternatively the LGC Genomics Reference number shown in Table 8 can be used to order the KASP assays used in this study directly from LGC Genomics.

Seven SNPs did appear to identify non-heterozygous differences among the reniform nematode isolates from Louisiana indicating there are identifiable molecular differences between the isolates. As described in Chapter 2 of this dissertation, significant differences in reproduction and pathogenicity among the reniform nematode isolates was evident from greenhouse and microplot experiments that employed cotton as host. Morehouse isolate (MOR) had the greatest reproduction and pathogenicity on cotton whereas Rapides isolate (RAP) had the least. A parallel research conducted by Manjula and colleagues (personal communication) involving the same isolates of reniform nematode and soybean as host found that MOR isolate had the least reproduction and the greatest pathogenicity. This study involving SNP analysis also indicated that significant variability does occur in isolates of reniform nematode from Louisiana. Six SNPs were found to be effective in distinguishing between the MOR and RAP isolates. Salem *et al.* (2012) reported that some SNPs are associated with biological functions in an organism. Because the isolates having different levels of reproduction and pathogenicity were also found to be genetically different in SNP analysis, some of the SNPs are possibly associated with reproduction and pathogenicity functions. The association of SNPs with biological functions in

reform nematode should be explored in future experiments. Although a reference sequence for reniform nematode is available (RREN 1.0 assembly at NCBI) the sequence has not been fully annotated. As the sequence annotation becomes more complete, genes in the areas around the SNPs that were found to identify genetic differences in this study (as well as future studies) can be investigated for potential relationships to reproduction and pathogenicity functions.

Several SNPs were polymorphic not only for reniform nematode isolates within a single geographic location but also across several locations. This suggests that a significant amount of genetic variability exists in reniform nematode isolates from within and among geographic locations. Variability in reproduction and pathogenicity in geographic isolate of reniform nematode have previously been reported in a microplot study involving cotton and soybean as hosts (McGawley *et al.*, 2010; 2011). Results from this study further supported the existence of variability in geographic isolates of reniform nematode.

Mostly the SNPs functioned and identified molecular differences among a broad range of nematode samples. Moreover, five SNPs detected genetic differences between and among the geographic isolates of reniform nematode (Table 9) indicating these SNPs could be valuable markers to distinguish reniform nematode isolates in larger geographic areas. A greater number of SNPs and utilization of a greater number of reniform nematode isolates would provide more robust data that can distinguish the pathogen from larger geographic areas. It was also observed that some SNPs polymorphic in one geographic isolate of *R. reniformis* were not always polymorphic in other geographic isolates. For example, RREN_514_63173 was polymorphic for reniform nematode isolates from Louisiana while it was monomorphic on reniform nematode from other geographic locations (Table 9). Finding of additional such SNPs would be useful in determining which geographic isolates of reniform nematode is more genetically diverse.

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Evaluation of a greater number of SNPs and isolates could lead to the development of a SNP panel that could be used to categorize reniform isolates from any location.

Several studies were conducted in the past to understand the amount of genetic variability in geographic isolates of reniform nematode, although with contrasting results. Agudelo *et al.* (2005) did not find any differences in ITS1 marker of reniform nematode from the United States, Brazil, Colombia, and Honduras except for a population from Japan. In contrast to the findings of Agudelo *et al.* (2005), Tilahun *et al.* (2008) found fairly substantial nucleotide difference in ITS1 marker of the reniform nematode collected from Alabama. Microsatellite analyses have also been employed to determine genetic variability in populations of reniform nematode (Arias et al., 2009; Leach *et al.*, 2012). Although microsatellite analysis is useful in determining polymorphism in a pathogen, SNPs are much more efficient and powerful markers (Salem *et al.*, 2012). Results from this research, by utilizing SNP analysis, provided a strong evidence for existence of genetic variability within and among geographic isolates of *R. reniformis*. An extensive characterization of genetic variability comprising larger number of reniform nematode isolates representing greater geography are necessary to further describe the genetic variability in this pathogen. The SNP markers developed in this study will be extremely useful in the assessment of the genetic diversity, origin, and subsequent distribution of this nematode. Additionally, once SNPs are found to be associated with genes for pathogenicity in reniform nematode, these markers will be of importance for the breeders involved in development of reniform nematode resistant crops.

CHAPTER 4. SUMMARY AND CONCLUSIONS

The reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) has become one of the major constraints of cotton production in the United States cotton belt. Approximately 205 thousand bales of the United States cotton was lost to this nematode in 2016 (Lawrence *et al.* 2017). Among the 16 states in cotton belt, cotton yield in Mississippi, Alabama, and Louisiana sustain greatest damage to this pathogen (Lawrence *et al.,* 2017).

Some common management options for reniform nematode in cotton include chemical, crop rotation, and biological. Chemical control is the most widely used method of reniform nematode management in cotton, however, it is least desirable because of health and environmental issues. Crop rotation with a non-host crop can help reduce the nematode population, however population usually resurges to a damaging level in a single season of susceptible crop plantation (Robinson *et al*., 2007). Use of host plant resistance is the most widely desired method of reniform nematode management. Unfortunately, reniform nematode resistant upland cotton cultivars are not available to date. Some upland cotton breeding lines showing moderate to high levels of reniform nematode resistance are available, but their performance is not consistent across wide geographic area (Yik and Birchfield, 1984; Robinson *et al*., 1997; Robinson *et al*., 2004; Weaver *et al*., 2007). This inconsistency, to the most part, is caused by existence of physiological and genetic differences in geographic populations of reniform nematode. A few studies conducted in the past using geographic isolates of reniform nematode have suggested the existence of virulence of phenotypes (McGawley *et al.*, 2010, 2011). Additionally, several studies involving internal transcribed spacer-1 (ITS1), 18S, and microsatellite marker analysis reported a significant amount of genetic variation in geographic isolates of reniform nematode while one study was unable to detect any differences making

genetic variability more elusive (Agudelo *et al*., 2005; Tilahun *et al*., 2008; Arias *et al*., 2009, Leach and Agudelo, 2012). Reniform nematode populations derived from single-egg mass and collected form West Carroll, Rapides, Morehouse, and Tensas parishes in Louisiana were used in this study to determine variability in this pathogen. Variability in reniform nematode was studied from two perspectives: (1) physiological variability as determined from reproduction and pathogenicity data obtained from microplot and greenhouse experiments, and (2) genetic variability determined from single nucleotide polymorphism (SNP) analysis. Two full season (150 days) microplot experiments were conducted using three most widely planted upland cotton cultivars (Phytogen 499 WRF, Deltapine 1133 B2RF, and Phytogen 333 WRF) that were recommended for use in Louisiana in 2015. Similarly, two 60-day greenhouse experiments were conducted to determine reproduction of four reniform nematode isolates endemic in Louisiana using three upland cotton cultivars that were used in microplot experiment, two cotton germplasm lines showing moderate to high level of resistance (MT2468 Ren3, and M713 Ren5), and one susceptible control. The reasons behind employing SNP analysis to determine genetic variability in geographic populations of reniform nematode are that SNPs are robust, more efficient, and most suitable to analyze genomic data obtained from next generation sequencing.

Results from microplot experiments suggest a significant difference in reproduction and pathogenicity of reniform nematode populations. In the microplot experiment, Morehouse and Rapides isolates, respectively, had the greatest and the least reproduction while West Carroll and Tensas isolates had intermediate levels of reproduction. Effect of reproduction was reflected in plant yield. The Morehouse isolate caused the greatest reduction in plant dry weight, number of bolls, seed cotton weight, and lint weight while the Rapides isolate caused the least.

Data from two greenhouse experiments showed similar reproduction results to that of microplot experiment. In other words, Morehouse isolates had significantly higher reproduction while Rapides isolate had significantly lower reproduction. Reproduction of West Carroll and Tensas isolates on cotton was intermediate. Differences in reproduction and pathogenicity of reniform nematode isolates was more pronounced on the germplasm lines in greenhouse experiments. Greenhouse and microplot studies conducted in the past have reported significant differences in reproduction and pathogenicity of geographic populations of reniform nematode suggesting existence of virulence phenotypes (McGawley *et al.*, 2010, 2011). Results from this experiment support the existence of virulence phenotypes in reniform nematode.

Kompetitive allele-specific PCR (KASP) genotyping assay was conducted to test 31 SNPs on 13 reniform nematode isolates collected from Louisiana, Mississippi, Arkansas, Hawaii, and Alabama. Of 25 SNPs that amplified reniform nematode isolates, five SNPs were of the most interest as they identified genetic differences between and among geographic isolates of reniform nematode from Louisiana, Mississippi, and Arkansas. This study is the first to report genetic variability in geographic isolates of reniform nematode employing SNP assay. Further studies comprising larger number of SNPs and greater number of reniform nematode isolates are necessary to understand SNP polymorphism and its association with biological function in this pathogen. The SNP markers developed in this study will be extremely useful in resistance breeding programs as well as in the assessment of the genetic diversity, origin, and subsequent distribution of this nematode.

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APPENDIX 1. FLANKING SEQUENCE INFORMATION FOR SNP ASSAYS TESTED IN THIS STUDY. THE "SNP ID" PROVIDES A COMMON NAME INCLUDING 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 5'-3' (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]TTAAATTTT

GAGTGAATTTTTAAAATATTTTTTCCACGTGCTTCAAGCACGGGTCATCGGTGCT

AAAAATGTCTTTTGGTCAACAAAGCTCAATAAGTTAAA

5   RREN_523_19992   [A/C]   CGGTAACCGAACGGCAGCGTTTCCATCCCCGGTCTTATACGACCCTTCTCGTAGT

GAGGTCTATAATTTTTGTGTGCCTCATAGATGTAAAGATCGG[A/C]CAGCGGGGT

GCGTCCAATGTGGGGAAAGGGCACATGAATGGTTGAGTCATTTCCCGGGAACAC

GAACACGTCATCAGCTCCATCAGCACCGGCTCCATCATT

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] ACTTCCATCTCCAAGTTGTTTATAGAGATGTTTGCCGAGTTCAGTGGGATTCGTGATATTCGAAGGGGGCACTGTTACACGCACCTCCTGCCCTGTT[T/C]GCCATACAACTCGCATATATTGCTGTTCCATAGTTCCCAAGGATGGCCATGAATGGGGATAAACCATCACTGCAAGCCCCACATACCAATCTGAATTGAATT

9 RREN_845_36717 [A/G] GACTTCTGCATGGCTTTGAGGAGTAAAATTCTTGCCTAAAATTACAATCTTGTTTTATTAGTTTTTTATTCAAAAAAATAGCTTACAGCAGAGGTC[A/G]TGAACAATGAGATGATGATGTTGGAGCACGCGTGCTCCTCGACCTGAATTATGAAAAGTTTATTTTTCTCGAATAAAAATATCTAATTTATAAAAACATAC

10 RREN_3215_15723 [T/C] TTTTTCATGAGCACTCTTTTCGTTTTCTTCAACACTTTTTCCTGAGCAATCTCTCGTTTCAACGAACACTTTTTTTATGGGGGTAAACCGTACAATA[T/C]AAGAGCCGACTTATTTTGTACAGCGTACACTCTACACTGTATTGCAAAATAGAATTAAAAAATAAAAATAGTCTGGTACTTAGGTATATAGTTTACGTGACC

11 RREN_1660_513 [T/C] GGGTGCGGTAGTCGGTTCGGCTTATGGCGTAGAGCTGATCAGTGAGAACCAGATCCCGTCCGCACTCCAAGACATATTCCAAATTGGTGATGCTTAA[T/C]GAAAATGTTTGATCTTCTGACATGAACCAATCATCAAACATGGTTCCTTTCATTTCCTGCATCAACACGTACCGGCATTGTGTGTCCGCTTTGGGGCGGTTC

12   RREN_4410_3946   [A/G]   GGAAAGTGTCGCTCTCGACGAAAAGGAAGCAGACAGCGAAAAAGCCCCACTCG
TGACCGCGTAGAGGGATAGCGAGGAAGGGATGGGGAGGCGACGA[A/G]CGAAA
AAGACGCCCCGGGGGAAGGATAGAGGGAATTCCCACTCTCCCCAGGGAAGCAA
GTACGGGGAAACCACTCAGATGCGATGAGAACGAAGGGTTTTCG

13   RREN_7711_4758   [T/C]   GGAGGTGAGAGAGTGTAGAGTGGTGAAGTGGAGGTGAGAGAGTACGGTATGTG
AGAGAGTACGGTATGTGACAGTACCGTGTTGTCCACGACCACGA[T/C]ACACTCT
TGTTGGTTGCCTTCACAACTCGGCAAACGGCCTCAATATCGATGACTTTCAGCA
ATGGGTTGGACGGTGTTTCGAACCAGACCATCTATGGATG

14   RREN_514_63176   [A/G]   TGCCTCCAAATCCTCGGATTTTTCAGAAATTCGTCAAAATTTTATTGGCATTTTT
CTGTGTAGAGAGTTTATTGGAAGTCGGGAGGTGTGGCTGAA[A/G]AGCATCTCCA
ACCTGTTGCCGCGGCACATCCTCAAGGCCTCATTGGCACTGCAGTCGGTGGTGC
ACCAGTACGAGCCGGACGCCATGATGCCAATCCCGTCA

15   RREN_925_39379   [C/G]   GAGGCAAGAGGCATCGAACAAATGGATCAATCTGTCCCTACTTCCGGAAGCCAG
CAAATGAAGCTGATCTGGTGCTAAATTTAACCTTATGTATTCA[C/G]TTGGAAAT
AGCAAAAATTGATAAAATGAAAAAATGGACTAACCAGCCGAAGGATAGTTGTA
TTCCAGACACAACACCTCGCTGTCATGTGCCTCTAATTCGA

71

16   RREN_514_63173   [T/G]   TTTTGCCTCCAAATCCTCGGATTTTTCAGAAATTCGTCAAAATTTTATTGGCATTT
TTTCTGTGTAGAGAGTTTATTGGAAGTCGGGAGGTGTGGCT[T/G]AAGAGCATCT
CCAACCTGTTGCCGCGGCACATCCTCAAGGCCTCATTGGCACTGCAGTCGGTGGT
GCACCAGTACGAGCCGGACGCCATGATGCCAATCCCG

17   RREN_91287_201   [A/C]   CTCTGAATTCCTCGTATTATGAAAATGAGTACAGCTATTCGCAAGTCTTACCATA
CATATATTCTAATTAATAGTTTTCCTTCTACCGATGTTCCTC[A/C]CTCTCTGAATT
CCTCGTATTATGAAAATGAGTACAGCTATTCGCAAGTCTTACCATACATATATTC
TAATTAATAGTTTTCCTTCTACCGATGTTCCTCCGC

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
TTGTGAGCAGAATGGCCAGATAGCAGAAAGATGAGTCCAGATGCTGTTCCCCA
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
TTTGTCATTAGAAAGAAAATAATCACTTGTTATAAAGTAA

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

74

28   RREN_16875_158   [T/C]   CAGTAACATCAACTCTCTTCTCTCCGTTCGCCTCCTCCTGCCGCCTTAACACCGCC

GGGTCCAACAACTGCGCCAGCCACCGGGGACATTCGAGCCA[T/C]ATGTTCAATT

TGTTCATTCATACCATCTATTTCAACTGCTCAAAGCAGTAACATCAACTCTCTTCT

CTCCGTTCGCCTCCTCCTGCCGCCTTAACACCGCCG

29   RREN_251_23034   [T/C]   CGGCGGTTCCGCCAGCTTTGCCTGCCAAAAAATCGGCAAAATGGTCGATGGACA

CAGATGGATGCAGGCATTCGATGGGTTTGGTGTAGAGCGCCGG[T/C]CTAACACA

TTTCAGGGCGCCAGGGCAAGAGCAGTTCACCTGCTCAATCCATTGCAGAAAGGT

AGGGGAGGGGGCCATTTTTTCAGAATTGGAAGTGTAATGG

30   RREN_1895_31360   [A/T]   GTAGAGAGCAAAAGAGATTAATTAAAACCTAAATTTGTCCATGCCCGACTGAG

TTGAAAAGAAAATTTATAGACACGAATAGTTGTAGATGAGGG[A/T]TAGAAGA

AATGGTGTAGTATTTTGAGGAAAAGATCGAAAGAAAACGTGAGACAAAGGGAA

ATTTTAGTTTCGAATACTTTTCTAACATCAATCAAAGGCTCT

31   RREN_9137_320   [T/C]   ACGGATAGACCCATATCTATCCAAGGTCCATATTTGGATTTCAACAGACATTCCC

ACCCATATACGGATAGACCCATATCTATCCAAGGTCCATATT[T/C]GGATTTCAA

CAGGTATTCACATCCATATACGGATAGACCAATTTTCCCGCCTCTACCCCCATCC

CAAGCCTCATGCACACCCATCAAGTTCGAGCAGTACAA

APPENDIX 2. 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 5'-3' (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

76

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]   AGCATTATTTCTGTATATTTTTGCTTCTTACAGGTCTACCCTGACAAGGTTTCTA

AATTTGGCTGATCAAAGTCGCGGTTGACCCACCGATGACACGTG[A/C]TTAAAGC

ACGTGGAAAAAATATTTTAAAAATTCACTAATATTTTAGCAAGGGGTTTGTGCC

AAAAACGGATGTCACTTCTTTATTATTATCGACCATGCT

7   RREN_61_242003 [A/T]   TGTAGAGCACCCTCACAAAGCATCGTATATTTTTGGCTTTTTTGGCCCGCTAACA

TAGTGAGGGGTTGAAAAATCGGTTTCAATTTTAAATGAACGGTCG[A/T]TCATCC

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

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

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

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]   TACATATTCATTGGCTAAAGCTGGTGCGTTTATTCAAGATTATTATTATTTTCTG
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

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]   ATTCTGTCATTTCTTATGCAATTCCCTCCTCATTGTCAACTATATAAGCAATGCA

TTTTCAATCATTTGTCACTTCCATTCCCATTCCAGCTCCCATTT[T/C]CCTTATTCC

AATTCATCTATTGTCCTATATTGTCTTCAATAAATTCTTCACGAGGACACAACAA

TTTGGCGCAGTCACGAAAACGACTCTACGCAATGCC

43   RREN_590_31647   [T/C]   TCTTACAATCAAAATCTGCCCTTGGGAGCTTTTTATGGAAATTTTTTCAAGGAAA

ATCATGAAACTGGACAATAAGATTAAAACTCTGCCAGAACCACCA[T/C]CTGGCC

AAGTTTGCGCTGCGGCGTCGTGACGGCGACTTTTTGTCCATCGAAGCAGCATTGT

TCTTGGCCGAACAGGGCGCCGCTGTGGTGGTGGAACATC

44   RREN_625_19284   [T/C]   ACATCTGCATTTGGGGAAGGGGGCGAATTTTGTAATAATAGGCAAAAATCGAAG

GTGAACAAGGGGGGGTAGGAGATGGATGTTTTTCATCATCCATTACA[T/C]CCAAC

ACATCCAATTCTGAAAAATGGCCCCTTCCCCAACTTTTCTGCAGTGGATCGAACA

GCTGAATTGCTCATGCCCTGATGGTCTGAAGTGTGTTCGA

45   RREN_718_5249   [A/G]   ACAAGGCATGTAGAGGACGACGCAGAAGGGACCAATTGTACAGAATATTGATG

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TGAACAAAAGGATTAGAGTAAATAAAGAACTGGAATGAGTGTAATAATACCAT

GTAGGGAACAAAAACGGCACTGGCCAAATATCCGCCAATTCCGG

46   RREN_721_22152   [A/G]   AAACTTTCGTATCGGATTCTCCTGCTGCTGTCCGTCAATTCCTGAACGACCCAAA

AATTGAAGTGGACTTTATAGAACAACTAAACGAGGAATGCGTCCT[A/G]ATCCG

ATACACACCACTAAAAGAATGGATCGAGGAGCACAACTGTTCAAACATTGTGCT

CTCTCTATGGACAACGGCGGCGGCACGACTACATCTCCTCA

47   RREN_721_22224   [A/G]   TAGAACAACTAAACGAGGAATGCGTCCTAATCCGATACACACCACTAAAAGAAT

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GGCGGCACGACTACATCTCCTCAAGCTAATGCAAAAAGTGGCGTCCACTCCCAA

CTGTGTACTCCTCTACACGGACACGGACAGTTTGATTTTCG

48   RREN_780_29960   [A/G]   TCCACACAATATAAGCACTTGGCCAAGGTCAAAATTTTCATTTTTACTCTAAATT

TTTTTCTGACAATTTTTAACCCTTCAGCTTCTCGCCCCTCCTTCC[A/G]CCCACAG

CGAACCATTGATGTGCCACGTCCTTTCCATGTCCATTTGTGCGATTCATGCCGCG

CTTTGTGCTCTTTTGGAGTTGTCCATGTCCGAGTCCGC

49   RREN_800_50912   [T/C]   ACAAGGGAAGGACAAAACATGTTAATCGTGGATCAATTCGAGACCCGTATAATA

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50   RREN_929_9795   [A/C]   CGGAGGCGCGAGTTTGGGCTAGTACCGTAAAAATGGGAAAAAAATAGAGTTTA

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51   RREN_981_25656   [T/C]   CATCCAGCGGTTTGTTCGGAGCGTAAATCCGTATTCCTTCGCGACTTGTGCACCT

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52 RREN_1012_21981 [T/C] AACTTTTCTCTCAAATCAGCAAAAATCAAAAAACATGGCAAAAAGCATGGCAAA
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CAAAAATCCGATATAAAAGTGACCCCTTCTTCCGAAAATAATTCATTTCTTCGCG
GGCATCTTCGTTGTTTCTACTTCTACATCTCTACACAAC

53 RREN_1120_48434 [C/G] TTTTACTACTTTTTCTTTTGATTTTATGCATTTTTGCAAAAGTGTGCAAGTGAAAT
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AAAATATTTATATATTCGTGCTCAGCTCGACGAGCTCTACACGATGGTATATAAT
TTTGGTCAGTTTGACTTCCGGAACATAAACTGCATTTTT

54 RREN_1123_57666 [A/C] NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTGGG
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

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TGAACGACCGTTCATTTTTAAAACCGATTTTTCGACCCCTCACTATCTGAGCGGG

CAGAAAAAGCCAAAAATATACGATGTAATGTGAGGATG

57   RREN_1187_25663   [A/G]   GTGCCAAAGACGGCCGTACCAGCAAACGAGACCATTGTGCCTCCGCCCGCATCC

GATCCCTCTTCTTCTTCCATTGCGACAGTGCAATCCGCTGGAACAC[A/G]CTGGC

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ATCCGATACACAGTGACTGGCATCGCTTCCACGCCCAATT

58   RREN_1215_1686   [A/C]   TCGAATTCAATTTAAGATTCAATAAATTAGAATACCAAAAAACCTCTAGAAACT

TTCATAAAGCTAATTATAAGCAGATAAACAATATATTCAAAAATAC[A/C]GACTG

GAATACTCTCTTTTCAAATAGTATAGAAATTGATCATCTATATCAAACATTTAGC

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59   RREN_1572_36973   [A/C]   TCTTTCGAATATTTTGGACTTTTTTGGACACCTTATGATTGACCATTTACAGCCCC

ATCCCGCTGGCCAAGCGGTCTCCTACTCGGCCCAGCAGAAGAAC[A/C]TGTTGAT

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60 RREN_1660_519 [A/T] TGCGGTAGTCGGTTCGGCTTATGGCGTAGAGCTGATCAGTGAGAACCAGATCCC

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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

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63 RREN_1721_18343 [T/C] GGCCCCCAAATGTTCCTGAATATGTACACGCCTCTCTCAATTTATAGTTTAGGTG

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TAATCAACATTATTTCCCTATGATCAATATTCTCACTC

64   RREN_1741_30669   [T/C]   TCCCATTCCTGCACTTTTTTTATATTTTCTGTTCCTCAATTACTTTTCTTCGCAAT

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65   RREN_1874_61680   [A/G]   CTATTGATTTTAAGCATTTTTGGTTGTGTGCCAGTGAAATTTGCGTGGAACTAAA

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66   RREN_1886_8608   [T/C]   TAATTTGTAAGGCTCCCGCAGGATTTCGCATCAAAACAAGATAATGCGAATTAT

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67   RREN_1886_12029   [T/C]   GTCTATAATTTTTATGGGCCTCATAGGTGTAGAGATCGGCCAGCGGATTGCGTCC

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68   RREN_1915_307   [T/C]   CGTCTAGAGACTCAATCGTGGAGACTCAATCGTGGAGACTCAATCGTAGAGACT

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69   RREN_1928_25815   [T/G]   CCATAGGTTATTTTTCGTTCTCTATTTTAAATTTTATTAATCACCCATTGCCTTATT

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TAAGGCATTTCCTCTACACATCCTCTCCAACCACA

70   RREN_1930_18838   [A/T]   ATTTTGTGCATTTTTGGAAAAGTGTACCAGTGAAATTGTACAGAACTTAAAAGG

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GCTCAGCTCGACGATCTCTACACGTTGGCAGATAATTTTGCCCAGTTTGGCTTCC

GGAACATAAACTGCATTTTTTCCAAAAACAAAACATCCTT

71   RREN_2082_17935   [A/C]   CATTCTTTACAACTATCACAAAATGGTGTCTATTGAACATTATTAAGAATTCGA

CTAAGGGTATCGACACAGGATAAGCACCGCTTAAACCGGTGAATG[A/C]CCATTT

TTTCGTTATACGGTATCGATTTAATGCATGGTACAGTAACCAATTCAATACCACA

ATTTTTGTAACGTAACTTCTCTACACTATCCCCAATGAC

72   RREN_2082_17995   [T/C]   GGTATCGACACAGGATAAGCACCGCTTAAACCGGTGAATGCCCATTTTTTCGTT

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ATCGTGTCGTTAGGATGACTGCATACCATATTCCAGCT

73   RREN_2193_23293   [C/G]   ATTGGCGACAGAGAAGAAGGCAGCGACTGTGGGGAGTTCGGCGACAGAGAAAA

AACCAGCGACAATGACAGAGAAGCTTCTGGAGAAGAAGAATGGATTC[C/G]AGA

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74   RREN_2229_14581   [A/G]   ATGAGGTGAGTCATAAAAGTTTTAACGATGGGTTGATTGGGTGTTATTGGACAC

CATCAGAGGTAAAGGAGGACACGTGTACAGCCAACGCTATGTCGCA[A/G]AGAA

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75   RREN_2245_19256   [A/G]   ATTCCGGTGCCCCCACGGAAGAAGTTATTCAAAGACAGCCCAAACCCGCACTGG

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TACACCAAGAAGAGTCACCATGGTAATTTTGGAATCGTTATGCTCACTCAGGAT

TTGTTTGATCGTAAGATGAAGGTGGTTAGGCAAAATTCAA

76   RREN_2295_24784   [A/G]   TGCCTATCCACGTGTCCAAGCAGCTACTGCGGCTTTGGAAGCAATCCTTGAATG

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77   RREN_2301_5490   [A/G]   TTGAATGCAAGAAACAATAGACAATGGCGAGCTGGGATGATAGCCAACTGCCA

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78   RREN_2380_20166   [T/C]   ACGGCAAGGTCTCCATTCCCGGTCTTATCCGCCCTTTCTCATAGTGTGGTCTATA

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TGTGGAAACGGAACATTGATGGAAGAGTCGTTGCCCGCAAATACAAACACATCA

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79   RREN_2380_20294   [A/C]   GAAGAGTCGTTGCCCGCAAATACAAACACATCATCATCCGCGTTAAAATTGTCC

AGAGCAGGATCGCTAAATTGGTCACGAAATTTTATACCCAGAATAT[A/C]TACAC

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80   RREN_2496_36896   [T/G]   ATCACATTGCATCGTATATTTTTGGCTTTTTTGTCTCGCACAGATAGTGAAGAGT

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81   RREN_2611_11791   [A/T]   AAAATTGTAGAGCGTGTGTCAAAACATAAAGTATGATCAAAAAATTTTGAGGTT

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82   RREN_2644_249   [T/C]   CATTCTCCGACTTGAAGGGAACGGGCGCGTTTGTCGGTATGTGGCCATCGAACC

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83   RREN_3007_13959   [A/G]   TTTCGGTTTTAGGGCCAAAAACACTGATTTTCGGTTTTTAAAATTTGACGGCAGC

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84	RREN_3315_26470	[A/C]	GAGACTGCGGATGAGGAAGAGGGTGTGGAGGATGGAGCTGGGGGTGATGTACT

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85	RREN_3415_20865	[T/C]	GCATGGGTCTCTGGAGGTGTAGAGCGTCAAAGCGGCCGCGTTTTTTATGGAAAT

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86	RREN_3722_17382	[A/C]	CTCGCGTAACTCGTTCCGGCATCCGAGCATTATAAATAGTGAAAGGTGGCATAT

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87	RREN_3853_3705	[A/G]	AACACTTTGCGAGCCTCTATCCACAAACGCTGTACTCCAGCAAAAGCTGATGGG

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88   RREN_4099_6882   [A/T]   TTGGGTGTACGGGGGGATGTAGAGGTGCAGGCTGACGGCCGGATCGGCGTGGG

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TGATCAATATCAGCCGGCCTTTTTTCTCATTACCTATTTATATTCTGTGGGGATAT

GACTTTTATTTAAATATTATTTAAAGCGGATACCAAAGTA

89   RREN_4280_20028   [T/C]   CACGTAGAGCTGTGTGTGAATTCGGGATTGAGCTAGTCGGGATTTCGGGATTTC

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90   RREN_4396_2332   [T/C]   AACTAGATTAAGAAAATAGTGGGGTAGAGAAAAAGAGAGACGAAATAATAGG

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91   RREN_4396_2346   [A/G]   AATAGTGGGGTAGAGAAAAAGAGAGACGAAATAATAGGTAGAGAAACTAGA

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92   RREN_4491_8860   [A/T]   GACGAAACACCTAATTTGAAATGTTAAACCTTTTATTGTATTAAAGTTGCTGGGC

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93   RREN_4741_9023   [C/G]   AGCTCTTCGGGAATAGACAAAACCCGAAAACGTAAATATGACACCGAAGAAGT

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94   RREN_4834_4812   [A/G]   ATTTCGTCTCTCTTTTTTCTCTACCCCACTTATTTCTTACTCTAGTTTCTCTACCTC

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96   RREN_4985_4041   [A/T]   TCCAAAAAACATGAAAATAATAAATGTAATAATTAAAAGAAAAAAAATATGTA

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97   RREN_5033_11813   [A/G]   GTGGAAAGGGCACATGGATGCTCGAGTCATTTCCCGGAAACACGAATACATCAT

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98   RREN_5033_11816   [A/G]   GAAAGGGCACATGGATGCTCGAGTCATTTCCCGGAAACACGAATACATCATCAG

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99   RREN_5385_4749   [A/G]   CTGTAACATGTTACCTTGCGTGCTGACAAGGGAACATACCGAGGTGTGACCACT

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100

100   RREN_5385_4848   [A/C]   TGCATCGTATATTTTTGGCTTTTTTGGCCCGCACAGATAGTGAAGGGTAGAAAAA

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101   RREN_5497_2602   [A/G]   TGTCGGCTTGTCCCTGCTCAATCGCTGCTACCGGCGAATAATTCCGTGTAGAGG

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TAACTTATCAGAACAAAGACGAGGAAAATGAAGAATGAAAT

102   RREN_5940_3890   [T/G]   AGACTGTAGAGACCGAAGAGGAGGCAGAGGAGAGAGTGGCAGAGCAGGTAGG

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103   RREN_5940_3923   [A/T]   GAGTGGCAGAGCAGGTAGGCGAACTTGTGGCTGATCATTATCTTTTTGTGTCTGA

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104   RREN_6983_4756   [A/G]   GACAAGGGAACATACCGAGGTGTGACTATTCAGATCATAATTTAACCCATCCTA

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CGCGGATCATTCGCCCTTTGGAATGGCGTCGATCGTACCA

105   RREN_7324_5103   [T/C]   GCAGTGTGGCAACCAGGTGTTCTCTCCAAAGAGACAGAGAAGCAGATAATTATC

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106   RREN_7711_4765   [T/C]   GTGAGAGAGTGTAGAGTGGTGAAGTGGAGGTGAGAGAGTACGGTATGTGAGAG

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107   RREN_7711_4768   [A/G]   AGAGAGTGTAGAGTGGTGAAGTGGAGGTGAGAGAGTACGGTATGTGAGAGAGT

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GGACGGTGTTTCGAACCAGACCATCTATGGATGATTATGTA

108   RREN_8907_4239   [A/G]   GTGAACAGAACTTTAAATATGGCGGAGATCGAGTTGACAAAGAAACGCTTAAA

AAACTTGACAAAATGCTCAGGAAACACCATCCTTTGGCAAAGAATT[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

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

104

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

105

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
AACCTAAGGTCAAAATTTTTTGACTAATTTATGTTTTCG

127 RREN_53141_365 [T/G] AATAATAAACAACATTTCCATTAGGCCCCCAAAAAACCTGAAATTTTTTCGAAA
ATTTCCCCAAAAATTTTCTTAGGCCTAACCTAAGGTCAAAATTTTT[T/G]GACTAA
TTTTATGTTTTCGCACATGCTCTACAACTTTAAAAAATAAACCATGCCTCTAAAC
CCCTTATAAACACTTCAAATAGTCCTGTCACGTATGATT

107

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

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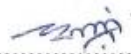Louisiana State University
Baton Rouge, La 70808

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

Dear Mr. Kularathna,

      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,

....
Churamani Khanal

APPENDIX 4: LETTER OF 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,

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

Thank you.

Sincerely,

....
Herath Kularathna

VITA

Churamani Khanal hails from Nepal. He received a B.S. in agriculture with plant pathology major from Tribhuvan University, Nepal in 2010. From 2011 to 2012, he served as an agriculture instructor as well as coordinator of Junior Technician Program in a governmental institution, Rapti Technical School, Dang, Nepal. He enrolled in the Department of Plant Pathology at the University of Arkansas, Fayetteville, Arkansas in 2012 and earned an M.S. degree in 2014 under supervision of Dr. Robert T. Robbins.