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Pathogenesis, Virulence and Population Genetics of *Puccinia emaculata*, the Causal Agent of Switchgrass Rust

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To the Graduate Council:

I am submitting herewith a dissertation written by Qunkang Cheng entitled "Pathogenesis, Virulence and Population Genetics of *Puccinia emaculata*, the Causal Agent of Switchgrass Rust." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Entomology and Plant Pathology.

Mark T. Windham, Major Professor

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Pathogenesis, Virulence and Population Genetics of *Puccinia emaculata*, the Causal Agent of Switchgrass Rust

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ABSTRACT

The infection process of *Puccinia emaculata* on *Panicum virgatum* leaves was studied histologically. Appressoria were formed approximately 10 hr after inoculation and penetrations were observed over stomatal openings. Substomatal vesicles developed 3 days after inoculation (DAI) and at 5 DAI, both haustorial mother cells and haustoria were observed. At 11 DAI, uredinia were developing, but urediniospores were immature. Until 14 DAI, matured uredinia ruptured host epidermis and urediniospores were released for secondary infection. This clearly studied infection process will help understand resistance mechanisms of a resistant cultivar in future research.

Twelve agronomic and ornamental switchgrass cultivars were inoculated with 40 switchgrass rust isolates to study the different host resistance levels, rust virulence and their interactions by measuring germination percentage, latent period, the number of uredia and urediniospores produced per cm^2 of leaf surface. Ornamental switchgrass cultivars had longer latent periods and fewer numbers of uredia and urediniospores produced per cm^2. Furthermore, virulence variations were observed among 40 switchgrass rust. The results of this study will provide more information in durable horizontal resistance of switchgrass cultivars and find new resources for breeding more sustainable rust resistant switchgrass.

Six switchgrass rust isolates were sequenced. The 29 million paired-end sequence reads of length 100bp for a switchgrass rust isolate were *de novo* assembled. Due to the high contamination of the reads, only 622 rust contigs were confidently provided, which would be random samples of nucleotide pieces from the whole genome with GC content 47.33%. Variant detection was conducted by mapping sequence reads of the other five isolates on these rust contigs. The

average single nucleotide variants and insertion/deletions density are 1.62 and 0.057 per kb, respectively.

The genetic relationship among switchgrass rust isolates collected from seven locations across southeastern US were analyzed using 22 SSRs. Linkages among loci were observed among all seven subpopulations with the standardized index of association significantly different from 0 ($p< 0.01$), indicating no sexual recombination in these locations. Moderate to high genetic differentiation was discovered among these subpopulations which demonstrated multiple introductions of genotypes of *P. emaculata* introduced to these areas.

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Introduction

Switchgrass (*Panicum virgatum* L) is a warm-season perennial grass native to North America. The native range of switchgrass includes most of the continental United States (exceptions California, Oregon and Washington). Due to its C4 photosynthetic pathway, switchgrass has been designated as a sustainable bioenergy crop for ethanol and butanol. Many fungal diseases have been reported for switchgrass including rust, smuts, leaf spots, crown and root rots. Although eighty one species of fungi have been reported to cause disease on switchgrass in the United States, most of them have little economic impact, except anthracnose (*Colletotrichum navitas*) and switchgrass rust (*Puccinia emaculata*).

Understanding the characteristics of *P. emaculata* is vital for developing an effective and sustainable approach to the management of switchgrass rust. Although the switchgrass rust can significantly reduce switchgrass yields, the early stages of the pathogenesis are obscure. Understanding the pathogenesis will aid in understanding resistance mechanisms and virulence of the rust isolates. In addition, demonstrating variations in rust resistance among switchgrass cultivars (both agronomic and ornamental cultivars) and different virulence level of switchgrass rust isolates can reveal the interaction between switchgrass rust and its host. Furthermore, insight into population structure of this fungus will assist in understanding genetic variation for virulence within rust populations, developing resistant switchgrass cultivars that where resistance is stable across locations with different endemic rust strains and eventually reduce crop losses.

Chapter 1. Infection process of switchgrass rust (*Puccinia emaculata*) urediniospores on leaves of a susceptible switchgrass (*Panicum virgatum* L.)

Abstract

The infection process of *Puccinia emaculata* on *Panicum virgatum* leaves was studied histologically. Appressoria were formed approximately 10 hr after inoculation (HAI) and penetrations were observed over stomatal openings. Substomatal vesicles developed 3 days after inoculation (DAI) and at 5 DAI, both haustorial mother cells and haustoria were observed. At 11 DAI, uredinia were developing, but urediniospores were immature. Until 14 DAI, matured uredinia ruptured host epidermis and urediniospores were released for secondary infection. This clearly studied infection process will help understand resistance mechanisms of a resistant cultivar in future research.

Introduction

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial grass native to North America. The native range of switchgrass includes most of the continental United States (USDA 2005). Traditionally, switchgrass has been used for bank stabilization, and as ornamental and forage crop (Lemus et al 2002). Due to its C4 photosynthetic pathway, switchgrass has been designated as a sustainable bioenergy crop for ethanol and butanol (Bolton et al. 2008).

Switchgrass can be affected by a variety of fungal diseases including rust, smuts, leaf spots, crown and root rots (Gravert and Munkvold 2002). Although eighty one species of fungi have been reported to cause diseases on switchgrass in the United States (Farr and Rossman 2009), most of them have little economic impact. The fungi that have been reported to reduce switchgrass biomass are head smut (*Tilletia maclaganii*) (Thomsen et al. 2008), anthracnose

(*Colletotrichum navitas*) (Crouch et al. 2009), and switchgrass rust (*Puccinia emaculata*) (Hirsch et al. 2010; Zale et al. 2008).

Switchgrass rust has been reported in Arkansas (Hirsch et al. 2010) and Tennessee (Zale et al. 2008). The first disease symptom and sign observed were leaf chlorosis and uredia formed underneath adaxial epidermal cells. Urediniospores were globose or oval in shape and approximately 27 x 25 µm in size and were the primary source of inoculum for secondary disease cycle. In late summer to fall, dark brown telia were observed on infected leaves. Teliospores were two-celled, approximately 33.6 µm ± 4.8 in length and the width of apical and basal cells were 17.5 µm ± 1.2 and 15.9 µm ± 2.5, respectively (Zale et al. 2008).

Switchgrass varieties have been found to be differential for rust resistance populations (Gustafson et al. 2003). The varieties ‘Alamo’ and ‘Kanlow’ showed moderate resistance to *P. emaculata*, while ‘Summer’ was highly susceptible and the lowlands varieties maintained a higher level of resistance than upland varieties (Uppalapati et al. 2012). However, the sequence of infection events of switchgrass rust is obscure. In order to understanding the mechanisms of resistance to switchgrass rust in switchgrass, it is essential to understand the disease pathogenesis. The objective of this research was to describe the sequence of events in the infection process of *P. emaculata* in switchgrass.

Materials and Methods

Plant material

Fully expanded healthy leaves were collected from plants of the switchgrass cultivar 'Thundercloud' which is susceptible to switchgrass rust. Plants were grown in a greenhouse at the University of Tennessee at Knoxville. Leaves were washed with distilled water, cut into 4 cm long leaf segments and placed adaxial surface up on two layers of moistened P8-creped filter paper (Fisher Scientific, Pittsburgh, PA) in 10 cm diameter Petri dishes (Fisher Scientific, Pittsburgh, PA).

Isolate collection and isolation

An isolate of *Puccinia emaculata* named -'TN-03' was obtained from a diseased leaf of an ornamental switchgrass planting of 'Dewey Blue' in Knoxville, TN in 2008. This isolate was separated, identified and confirmed as *P. emaculata* by both spore morphology and internal transcribed spacer (ITS) region. The isolate had been maintained by inoculating 'Thundercloud' segments in Petri dishes in the lab every two weeks. Rust urediniospores were then collected from the segments two weeks after inoculation and stored at 4°C refrigerator for later use.

Inoculum and inoculation

Inoculum was prepared by solute urediniospores collected above in a solution of diH_2O and 1ppm Tween20. The final concentration was adjusted to about 6×10^5 urediniospores /mL with a hemocytometer. Urediniospore suspension was applied to leaf surfaces of fresh switchgrass leaf segments of 'Thundercloud' in Petri dishes mentioned above with a mist fingertip. After

inoculation, the Petri dishes were sealed with parafilm (Pechiney Plastic Packaging, Menasha, WI) and incubated at 20°C with a diurnal 12 hour light/ dark cycle.

Germination and growth of urediniospore

Early stages of pathogenesis of the disease were examined at 1, 3, 6 and 10 hours after inoculation (HAI). Leaf segments were cut to 1 cm long and placed into a clearing solution (0.15% trichloroacetic acid [w/v, Sigma-Aldrich, St. Louis, MO] in ethyl alcohol [Decon Labs, Inc., King of Prussia, PA]/chloroform [Sigma-Aldrich, St. Louis, MO], 4:1 [v/v]) to stop the growth of the urediniospores and to remove chlorophyll. The solution was exchanged once after 24 h during the 48 h clearing process. Leaf segments were stained with a freshly prepared Coomassie blue solution (0.6% Coomassie brilliant blue R 250 [w/v, Sigma-Aldrich, St. Louis, MO] in methanol [Decon Labs, Inc., King of Prussia, PA] /15% trichloroacetic acid [w/v] in H_2O, 1:1 [v/v]) for 15 s, washed in water and mounted in 50% glycerol [v/v, Mallinckrodt Paker, Inc., Phillipsburg, NJ]. Sections were then examined with an Olympus BH-2 microscope connected to a color camera (Nikon Instruments, Melville, NY).

Damaged cell observation

Switchgrass cell death was examined after staining leaf disks with trypan blue 5 DAI as described by Sillero and Rubiales (Sillero and Rubiales 2002). Inoculated leaf segments were placed on two layers of filter paper moistened with acetic acid:ethanol (1:3, vol/vol) fixation solution for 30 min. Fixed segments were then stained with 0.05% trypan blue in lactophenol:ethanol (1:2, vol/vol) in an autoclave at 121°C for 3 min. Cooled stained leaf segments were cleared on filter papers soaked with saturated chloral hydrate (5:2, wt/vol) for 48

h at room temperature by replacing filter papers and chloral hydrate once after 24 h. The segments were mounted in 50% glycerol and examined under a compound microscope with bright-field microscopy.

Tissue preparation for histology

Ten random leaf segments were chosen per sampling period at 1, 2, 3, 5, 7, 11 and 14 DAI. Leaf segments were trimmed into smaller rectangular pieces (ca. 10×5 mm), then fixed in 50% FAA solution (Fisher Scientific, Pittsburgh, PA) in glass tubes for 2 days, dehydrated in a series of 50, 75, 85, 95% and absolute alcohol (Decon Labs, Inc., King of Prussia, PA), and embedded in paraffin (Paraplast® Oxford Labware St. Louis, MO). Serial 10 µm thick cross sections were cut using a rotary microtome. Glass slides were prepared by washing with detergent (Clorox, Clorox Professional Products Company, Oakland, CA) and rinsed with distilled water. Cross sections were expanded on a puddle of distilled water at 42°C, and affixed to glass slides, without adhesive, by draining off water and drying for 12 h at 42°C. Sections were deparafinized through three changes of Microclear™ (Micron Diagnostics, Inc., Baltimore, MD) for 5 min each and rehydrated by moving slides 5 min each through a series of absolute ethanol, 95, 85, 70, 50, and 30% ethanol, and two deionized water rinses for 5 min each. The staining series consisted of 0.01% aqueous Safranin O stain (Sciencelab.com, Inc., Houston, TX) for 24 hr, followed by two 1 min deionized water rinses, 1% crystal violet (Sciencelab.com, Inc., Houston, TX) stain for 1 min, followed by 5 min deionized water rinses. Sections were dehydrated using a graded series of ethanol in reverse order of the rehydration series previously described and then stained with fast green (Sciencelab.com, Inc., Houston, TX) for 1 min, followed by 1 and 5 min absolute ethanol rinses. Sections were then rinsed three times in Microclear™ with10 min submersion per

rinse. Coverslips were attached to slides with permount (Fisher Scientific, Pittsburgh, PA) and slides were dried for 24 hr at 42°C. Sections were examined with a compound microscope (Nikon Instruments, Melville, NY).

Tissue preparation for scanning electron microscope

Paraffin embedded samples (3 samples each from 1, 3, 7, 11 DAI) were also examined using a scanning electron microscope (SEM). These serial 10-µm-thick cross sections, which were the same as those for histology, were attached to the special 1 cm diameter thin slides, deparafinized through three changes of Microclear^{TM} for 10 min each, air dried and sputter-coated with gold palladium. These samples were then examined under SEM. Samples were examined by a FEI Quanta 200 scanning electron microscope operating at an accelerating voltage of 10 keV. Three samples for each sampling time were examined using SEM.

The rust pustules on leaf surface at 14 DAI were also examined using a SEM. The tissue preparation protocol was used as described by Cheng et al. (Cheng et al. 2011). Three random samples with rust pustules were chosen and cut into 1cm×1cm segments. These three leaf segments were then fixed, dehydrate and examined under FEI Quanta 200 SEM with 10 keV.

Tissue preparation for transmission electron microscope

Five random leaf segments were chosen at 5 and 7 DAI. Leaf segments were trimmed into 2×5 mm rectangular pieces, fixed in a fixative solution (3% glutaraldehyde in 0.1M cacodylate) at room temperature for 90 min, then rinsed with 0.1 M sodium cacodylate buffer (pH=7.2) three times for 10 min each. The samples were post-fixed with 2% OsO_4 in 0.1M cacodylate buffer at room temperature for 90 min and dehydrated in a series of 25, 50, 75, 95, 100 and 100% acetone

for 30 min each. Samples were then prepared to be embedded in Spurr mix (3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexane carboxylate 5g, diglycidyl ether of polypropylene glycol 4g, nonenyl succinic anhydride 12.5g and dimethylaminoethanol 0.15g) by sequenced immerging in 3:1 acetone/Spurr mix (v/v) overnight, 1:3 acetone/Spurr mix (v/v) for 8 hrs and 100% Spurr mix overnight. Samples were embedded in molds with Spurr mix and placed in oven at 68°C for 24-48 hrs. Serial 50 nm thin sections were cut by Leica EMFC7 ultramicrotome, placed on regular mesh grids, stained by uranyl acetate and lead citrate. Sections were finally examined with Zeiss libra 200MC transmission electron microscopy.

Results

Urediniospore germination

Urediniospores were considered germinated when the germ tubes reached at least half length of the spore. At 1 HAI, a majority of urediniospores (82%, n=200) had germinated (Fig 1.1-A). In addition, 98% (n=200) of the urediniospores germinated at 2 HAI. Only single germ tube was observed for each of the germinated urediniospores (Fig 1.1).

Growth of germ tubes

At 3 HAI, germ tubes began to form branches (Fig 1.1-B) and 6 HAI, more branches had developed but appressoria were not observed (Fig 1.1-C). In addition, the germ tube growth of *P. emaculata* neither was orientated perpendicular to the long axis of the epidermal cell nor followed and surrounded the junctions between the epidermal cells (Fig 1.1-C), rather was orientated randomly unlike most rust pathogens. Furthermore, the number of branches which a

single germ tube had was not constant, varying from just one to a dozen (Fig 1.1-C, D). The length of germ tubes varied from 60μm to 200μm.

Appressoria formation

At 10 HAI, appressoria had formed over stomata in preparation for penetration (Fig 1.1-D). All of the appressoria were observed over stomatal and none were found over epidermal cells or junctions between epidermal cells. The host cell damage experiment suggested *P. emaculata* entered through stomatal opening as stomata surrounded by a cluster of urediniospores were damaged (Fig 1.2). The appressoria were not formed over the closest stoma (Fig 1.1-D) which corresponded to the randomly orientated germ tube growth mentioned above.

Penetration

At 2 DAI, infection pegs were formed underneath appressorium and penetrated through stomatal guard cells which were stained bright red by safranin O (Fig 1.3). Substomatal vesicles were formed in substomatal chambers at 3 DAI(Fig 1.4-A, B) and penetration hyphae continued to grow towards mesophyll cells.

Colonization and reproduction

When a penetration hypha contacted a mesophyll cell at 5 DAI, a haustorial mother cell was formed at the tip of the penetration hypha and adhered tightly to the host cell wall (Fig 1.5-A). The haustorial mother cell produced a penetration peg which penetrated the host cell wall and formed a haustorium within the host cell (Fig 1.5-A, B). Additional hyphae colonized mesophyll cells intercellularly and intracellularly at 7 DAI (Fig 1.6-A, B). Uredinia were developing and

urediniospores were immature at 11 DAI (Fig 1.7-A, B). At 14 DAI, the uredinia ruptured the host epidermis and mature urediniospores were released (Fig 1.8-A, B).

Discussion

This research on infection processes of *P. emaculata* on switchgrass was focused on the asexual uredinial stage due to its obvious economic importance. The life cycle of *P. emaculata* was still obscure as the alternate host plant has not been determined. The alternate hosts of some well-studied rust pathogens are, for instance, *Berberis* species for *P. graminis tritici* (wheat stem rust)(Stakman et al. 1934), and *Ribes* species for *Cronartium ribicola* (white pine blister rust)(Hunt 1984). The alternate hosts of *Hemileia vastatrix* (coffee rust)(Schieber and Zentmyer 1984) and *Phakopsora pachyrhizi* (soybean rust)(Sinclair and Hartman 1999) are still unknown. Although there is great diversity in the life cycles of rust species, urediniospores are critical for the initial infection process. Urediniospores were used as inoculum in this study. Uredia were reformed on the host with a new generation of urediniospores produced every 14 days.

Urediniospores of *P. emaculata* germinated at 1-2 HAI while the urediniospores of some rust fungi such as *P. triticina* (wheat leaf rust), germinate at 4-8 HAI at 20°C and 100% humidity (Zhang et al. 2001). Unlike *P. graminis*, *P. triticina*, or some other rust fungi, urediniospore germ tube growth of *P. emaculata* was not responsive to the topography of the host leaf surface and was randomly orientated (Figs. 1.1-A, B, C, D) and is not perpendicularly orientated to the rectangular epidermal cells (Collins and Read 1997; Hu and Rijkenberg 1998; Leonard and Szabo 2005; Wynn and Staples 1981). The hyphae of some rust fungi follow the periphery of epidermal cells of their host plants, for example, the hyphae of *Cymodothea trifolii* on *Trifolium*

repens (white clover)(Roderick 1993), grows across the epidermal cells at right angles, even *in vitro* (Hoch et al. 1987; Kwon and Hoch 1990). Recent studies have discovered mechanisms regulating hypha orientation, including calcium signaling (Brand et al. 2007; Silverman-Gavrila and Lew 2002), Bud1-GTPase module (Casamayor and Snyder 2002; Fischer and Takeshita 2008), regulatory proteins such as polarisome, Arp2/3 complex (Machesky and Gould 1999) and exocyst complexes (Lipschutz and Mostov 2002). It might be more efficient for hypha to follow the peripheries of cortical cells or across at right angles. However, in this particular *P. emaculata* and switchgrass interaction, the germ tube was not directed to stomata by surface topography or the germ tube was randomly directed to stomata by surface topography.

Penetration of *P. emaculata* through stomatal openings was observed. This differs from *Phakopsora pachyrhizi*, which penetrates directly through the leaf cuticle (Koch et al. 1983) or more specifically through anticlinal wall junctions between epidermal cells (Allen et al. 1991). Most rust pathogens penetrate through stomatal openings (Roelfs and Bushnell 1985). Unlike *P. striiformis* which does not form appressoria on wheat (Moldenhauer et al. 2006), *P.emaculata* formed appressoria. Although there are diverse penetration locations and structures, the absence or presence of appressoria was similarly stimulated by some chemical (Grambow and Grambow 1978; Grambow and Riedel 1977) and physical (topographical) signals of the host.

Puccinia emaculata infection pegs penetrated through stomatal openings and substomatal vesicles were formed in substomatal chambers, just like the other stomata entering rust fungi. This contrasts with rust fungi that penetrate directly via a penetration peg that breaches the host cuticle, enters epidermis cells, produces hyphae which traverse the epidermal cell and enter the

intercellular space of the mesophyll cells (Adendorff and Rijkenberg 2000; Koch et al. 1983). This is similar to rusts that enter stomata, such as *P. graminis* and *P. triticina*. A haustorial mother cell is formed attaching to mesophyll tissues and a haustorium is formed inside host cells to uptake nutrients. Uredinia of *P. emaculata* ruptured host epidermis at 14 DAI on the adaxial side. None of the uredinia were found on the abaxial side, which is similar to other rust fungi.

This study revealed the pathogenesis of *P. emaculata* urediniospores on a susceptible switchgrass cultivar. These findings on *P. emaculata* infection and related *P. emaculata*-switchgrass interaction will help to further in-depth study of resistance mechanisms and the molecular mechanisms of the interaction between the switchgrass and *P. emaculata*. By understanding this detailed infection process, disease control strategies will be improved and more targeted.

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Appendix

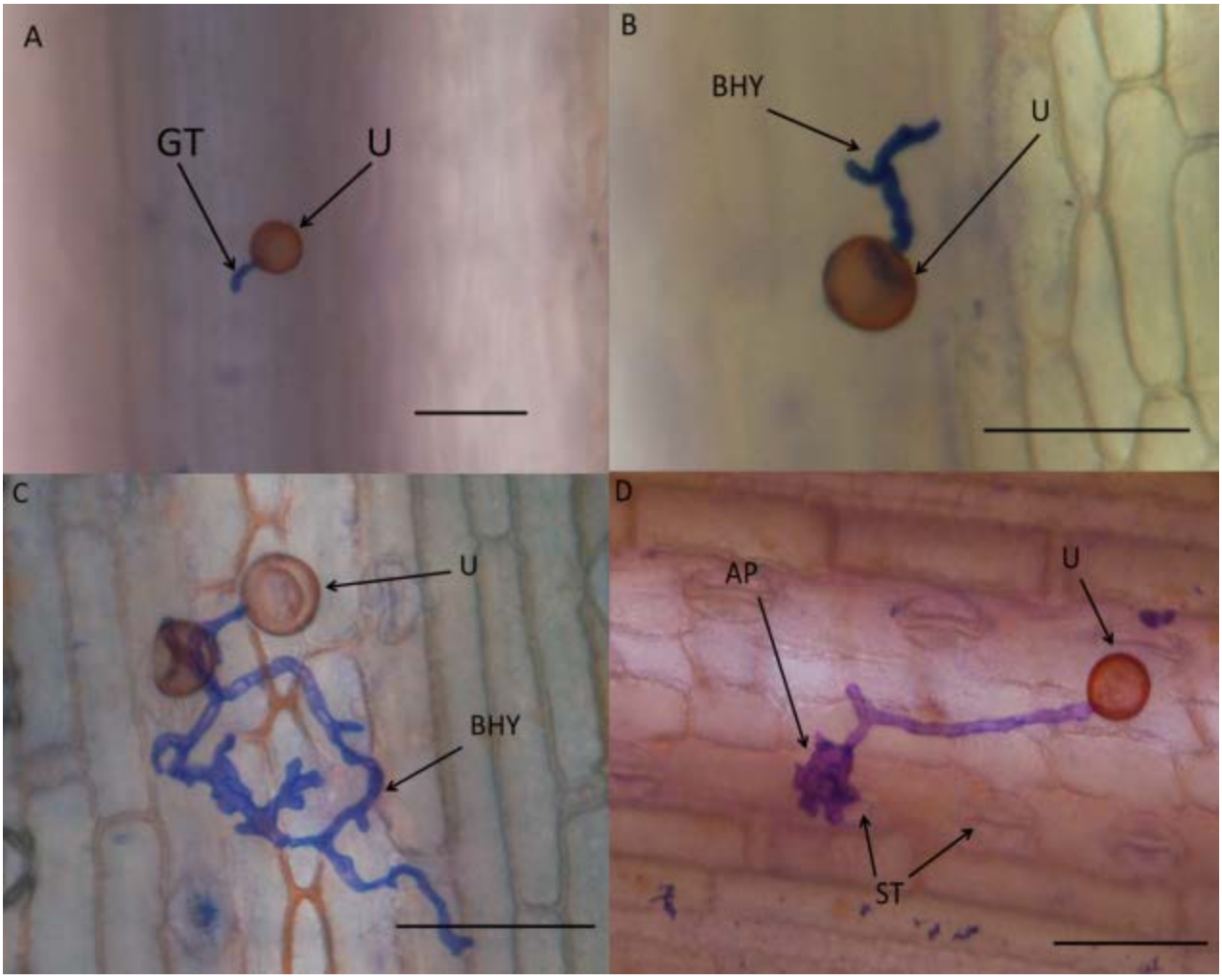

Figure 1.1 Light micrographs of *Puccinia emaculata* urediniospores on switchgrass adaxial surface. **A,** Urediniospore (U) germinated with a germ tube (GT) 1 hour after inoculation (HAI). **B,** A germinated urediniospore with a branched hypha 3 HAI. **C,** Germinated urediniospores with more branched hyphae 6 HAI. **D,** An appressoria (AP) formed at the tip of a hypha and over a stoma (ST) 10 HAI. Bar=50 μm.

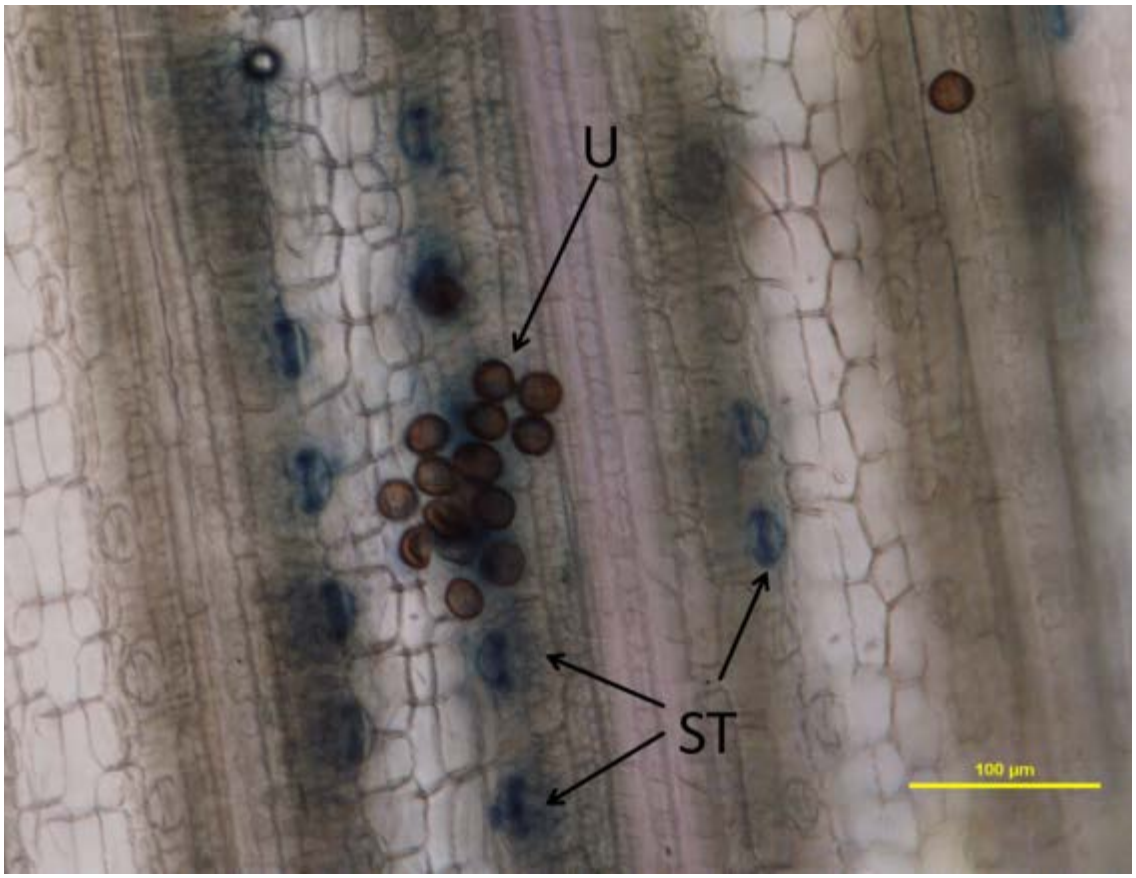

Figure 1.2 Light micrograph of a cluster of *Puccinia emaculata* urediniospores on the switchgrass adaxial surface 5 DAI. U, urediniospore; ST, stomata.

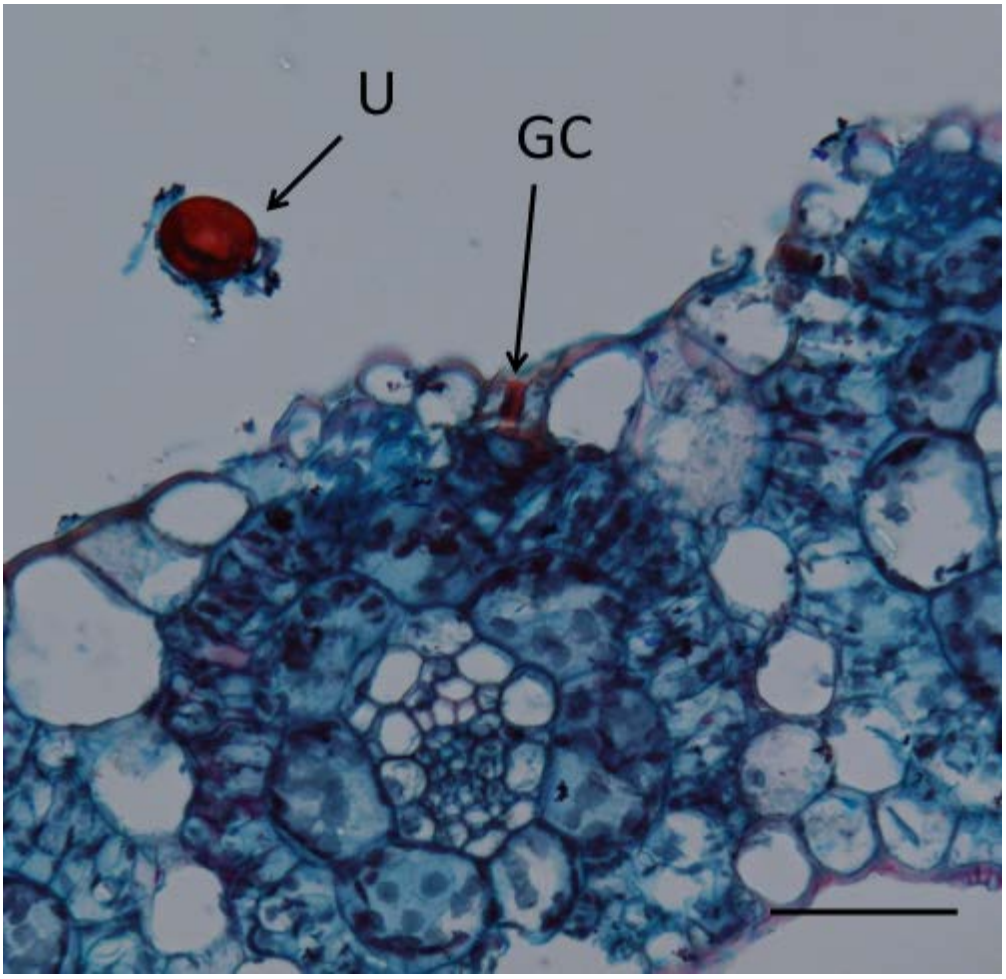

Figure 1.3 Light micrograph of switchgrass leaf adaxially inoculated with *Puccinia emaculata* urediniospore at 2 DAI. Infected host guard cells (GC) were stained bright red by safranin O. U, urediniospore. Bar=50 μm.

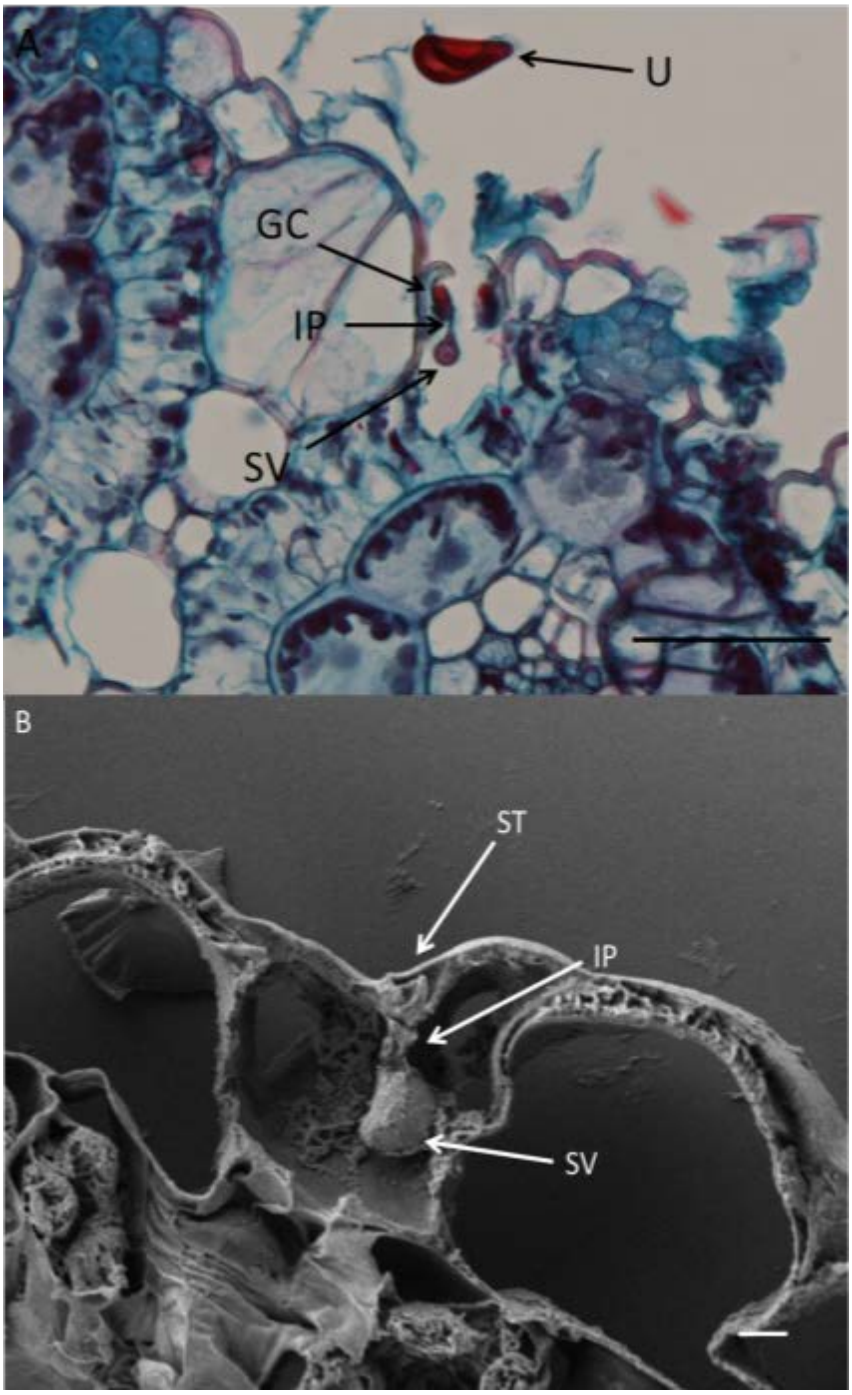

Figure 1.4 A, B, Light micrograph and scanning electron micrograph, respectively, of cross sectioned switchgrass leaves inoculated with *Puccinia emaculata* urediniospores 3 DAI. The infection peg (IP) which was formed underneath the appressorium penetrated through stoma guard cells (GC) into the substomatal chamber forming substomatal vesicle (SV). Urediniospore (U). **A,** Bar=50 μm. **B,** Bar=2 μm.

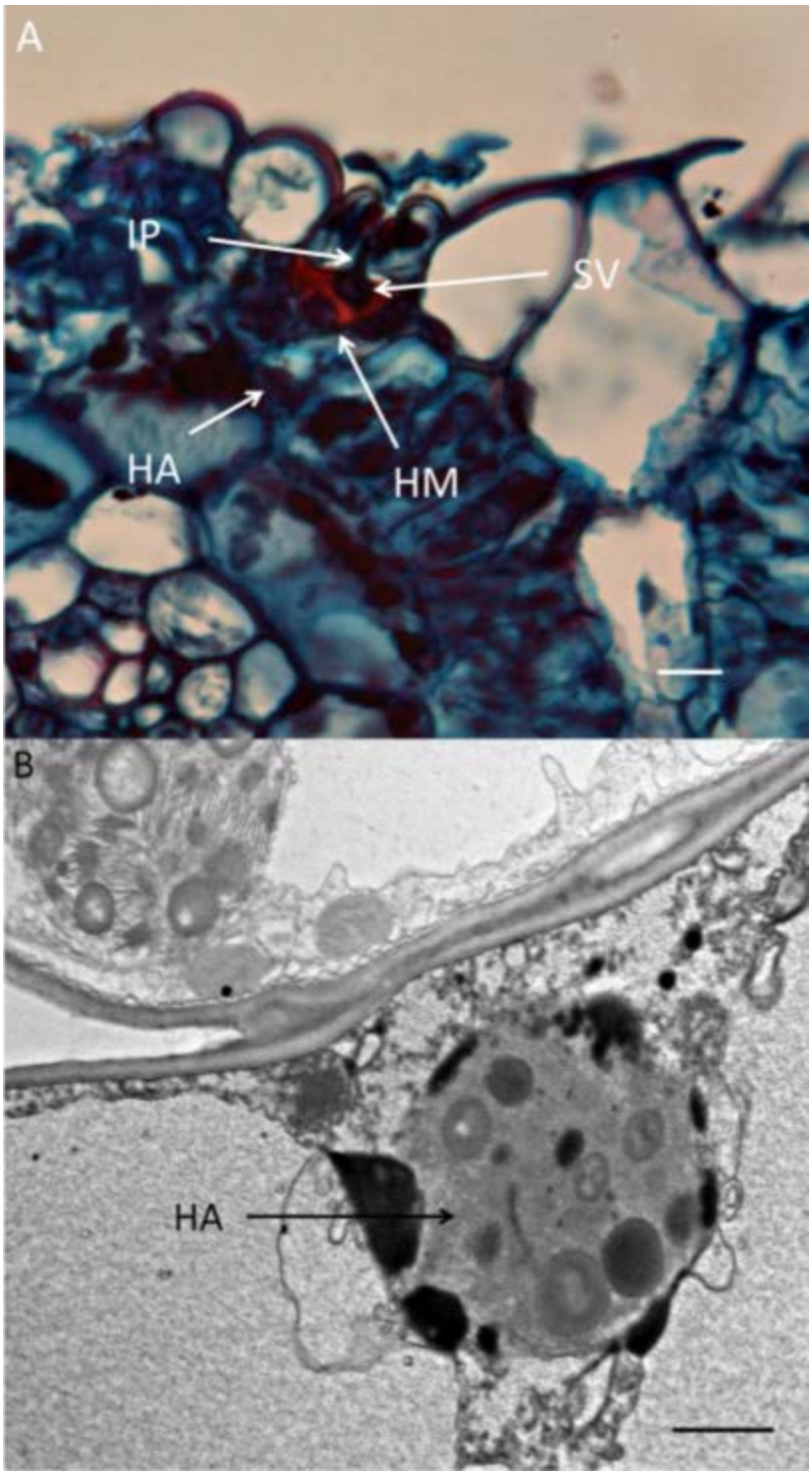

Figure 1.5 A, B, Light micrograph and transmission electron micrograph, respectively, of cross sectioned switchgrass leaves inoculated with *Puccinia emaculata* urediniospores 5 DAI. **A,** a haustorial mother cell (HM) was formed when the tip of the infection hypha contacted a host cell wall and from which the haustorium (HA) invaded into the host cell. IP, infection peg; SV, substomatal vesicle. Bar=10 μm. **B,** a haustorium (HA) in the host cell. Bar=1 μm.

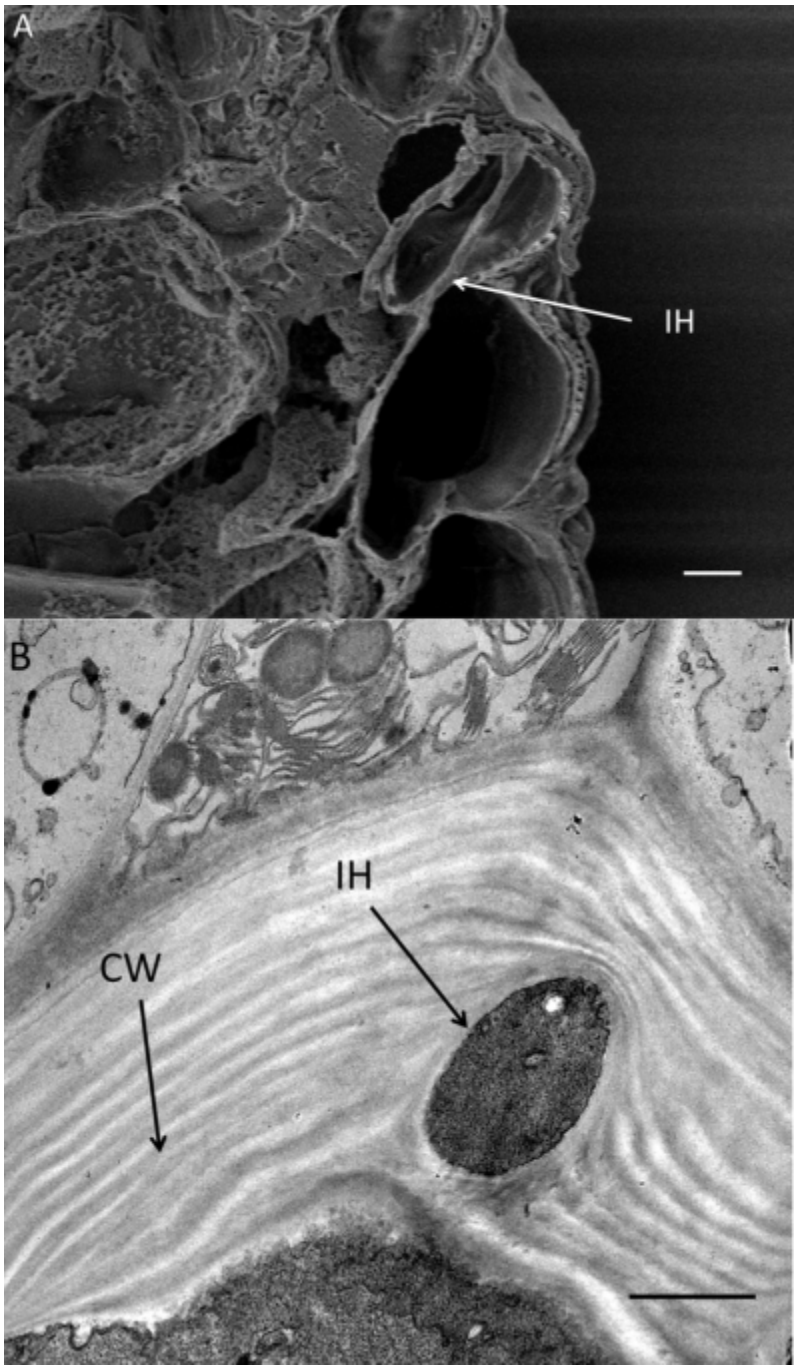

Figure 1.6 A, B, Scanning electron micrograph and transmission electron micrograph, respectively, of cross sectioned switchgrass leaves inoculated with *Puccinia emaculata* urediniospores 7 DAI. **A,** infection hypha (IH) grown intercellularly. Bar=3 μm. **B,** infection hypha (IH) penetrated through the host cell wall (CW). Bar=1 μm.

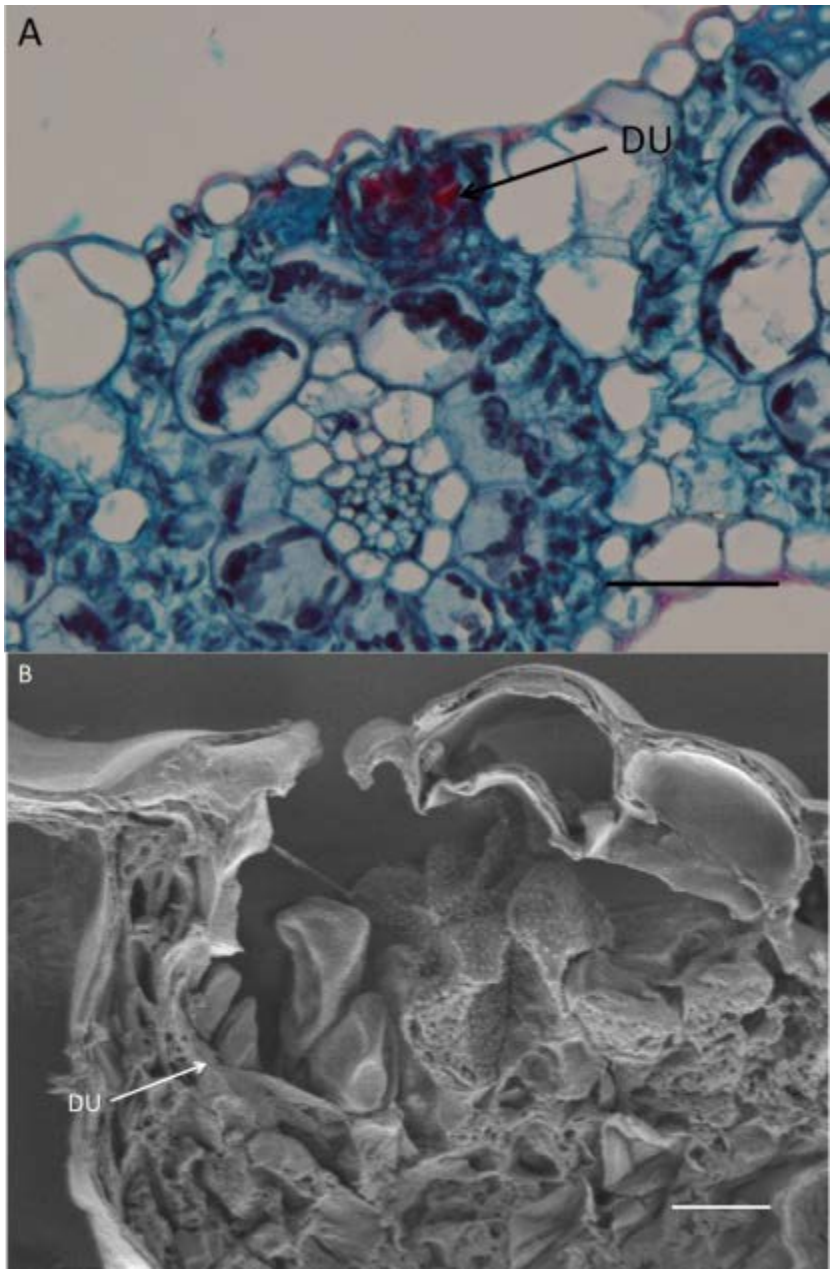

Figure 1.7 A, B, Light micrograph and scanning electron micrograph, respectively, of cross sectioned switchgrass leaves inoculated with *Puccinia emaculata* urediniospores 11 DAI. Developing uredium (DU) and urediniospores were formed underneath host epidermis. **A,** Bar=50 μm. **B,** Bar=10 μm.

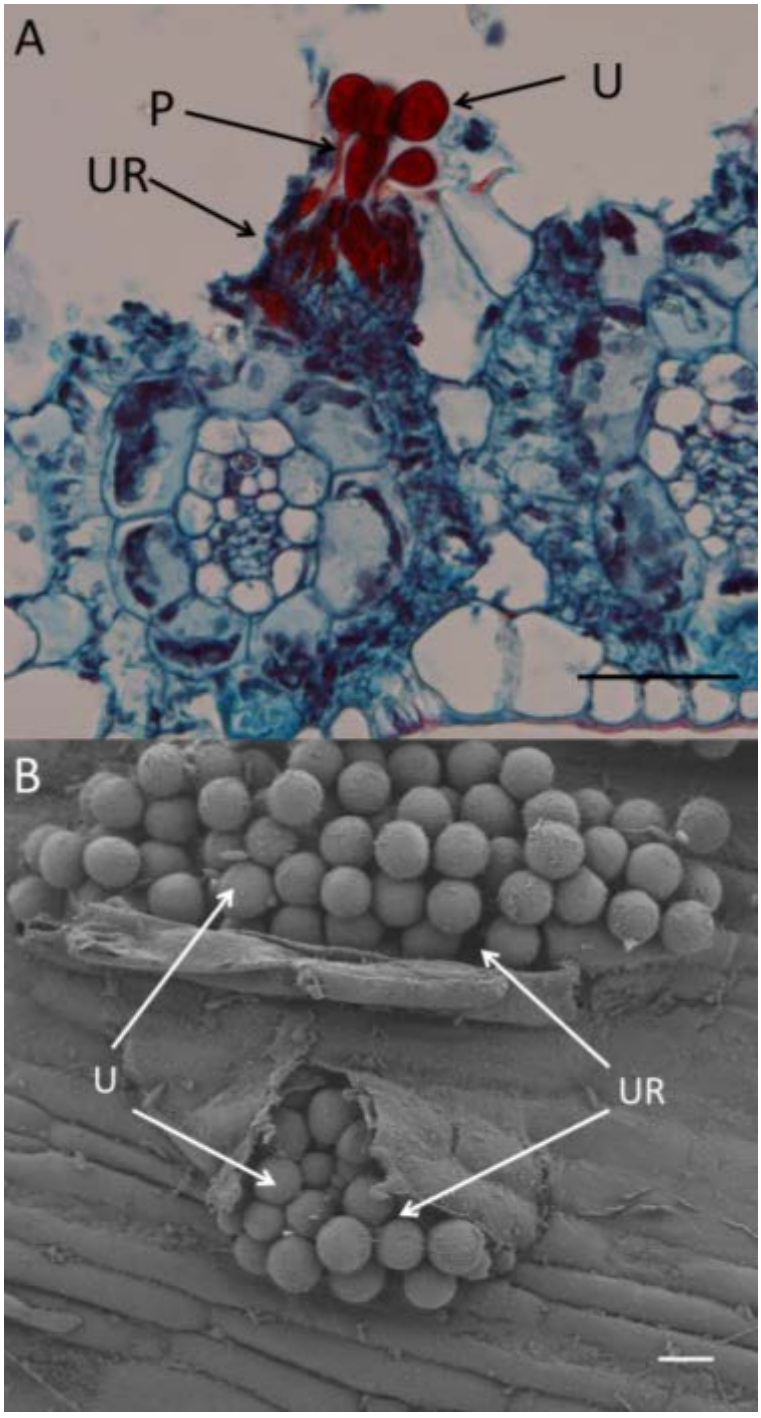

Figure 1.8 A,B, Light micrograph and scanning electron micrograph, respectively, of switchgrass leaf adaxially inoculated with *Puccinia emaculata* urediniospores at 14 DAI. Urediniospores (U) were formed in a uredium (UR) with pedicels (P). A, Bar=50 µm. B, Bar=20 µm.

Chapter 2. Interactions of switchgrass (*Panicum virgatum*) cultivars and switchgrass rust (*Puccinia emaculata*) isolates

Abstract

Twelve agronomic and ornamental switchgrass cultivars were inoculated with 40 switchgrass rust isolates collected from southeastern US in different years to study host resistance, rust virulence and their interactions by measuring germination percentage, latent period, the number of uredia and urediniospores produced per cm^2 of leaf surface. Ornamental switchgrass cultivars had significantly longer latent periods and fewer uredia and urediniospores produced per cm^2. In addition, virulence variations were observed among 40 switchgrass rust isolates. No clear pattern was found to group rusts by locations and years. The results of this study will provide more information in durable horizontal resistance of switchgrass cultivars and find new resources for breeding more sustainable rust resistant switchgrass.

Switchgrass (*Panicum virgatum* L), a warm-season perennial grass native to North America and traditionally used for hay and bank stabilization (Lemus et al., 2002), has been more recently used as a biomass crop for ethanol and butanol (Bouton, 2008; Cherney et al., 1991). With average annual biomass yield 5.2 to 11.1 Mg. ha^{-1}, switchgrass can provide approximately 60 $GJ \cdot ha^{-1}$ (Schmer et al., 2008).

Due to their different breeding method, switchgrass was divided into agronomic cultivars and ornamental cultivars. As a cross-pollinated crop, agronomy cultivars, such as ‘Alamo’ and ‘Cave-In-Rock’ (CIR), were developed with traditional breeding methods for cross-pollinated crops, which lead to genetically different individuals in a cultivar. While in the other hand, ornamental switchgrass cultivars, such as ‘Cloud nine’ and ‘Thundercloud’, were propagated vegetatively by division and are clonal. In addition, based from their habitat preference and

ploidy level, agronomic switchgrass cultivars can be grouped into two ecotypes: lowland and upland ecotypes (Porter 1966). Lowland cultivars (i.e. 'Alamo') are mostly tetraploid (2n= 4X= 36) and generally in wetter environments and well adapted to the southern USA; whereas upland cultivars (i.e. 'CIR') are tetraploid or octoploid (2n= 8X= 72), which usually found in drier locations in the central and northern USA (Barnett and Carver, 1967; Brunken and Estes, 1975; Hultquist et al., 1996; Nielsen, 1994; Zhang et al., 2011).

Many pathogens have been reported as disease-causing agents of switchgrass, including: limited amount of bacteria and nematodes (Cassida et al., 2005; Mekete et al., 2010), viruses (Sill and Pickett, 1957; Stewart et al., 2015) and primarily fungal pathogens (rust, smuts, leaf spots, crown and root rots) (Gravert and Munkvold, 2002). Among fungal pathogens, only head smut (*Tilletia maclaganii*) (Thomsen et al., 2008), anthracnose (*Colletotrichum navitas*) (Crouch et al., 2009), and rust (*Puccinia emaculata*) (Skyes et al., 2016) were reported to reduce switchgrass biomass while others have little economic impact. Although three other rust pathogens, *P. graminis, P. huberi*, and *Uromyces graminicola* were also reported to cause disease on switchgrass (Arthur, 1934; Gravert and Munkvold, 2002; Ramachar and Cummins, 1963 & 1965; Cummins, 1971), *P. emaculata* is the most widely distributed across the USA (Frazier et al., 2013; Hagan and Atridge, 2013; Hirsch et al., 2010; Lenne, 1990; Gravert and Munkvold, 2002; Zale et al., 2008) and can reduce the estimated ethanol production up to 55% due to severity infection (Skyes et al., 2016).

Variations in rust resistance among switchgrass cultivars have been reported (Gustafson et al., 2003; Jacobs et al., 2004; Li et al., 2009; Uppalapati et al., 2013). These studies focused on either

agronomic cultivars/populations (Gustafson et al., 2003; Uppalapati et al., 2013) or ornamental cultivars (Jacobs et al., 2004). Rust severity rating observations were collected on a single date. These single end-of-season ratings (from 1 to 9) were used to indicate whether cultivars were resistant or susceptible (McNeal et al., 1971). However, as a polycyclic epidemic, these ratings cannot provide any information about the latent period, which is a crucial index of host tolerance from the plant's perspective especially for the pathogen with long-distance dispersal ability. In addition, these studies did not take account the potential of different races of rust occurring in different fields in different years. Differences in rust susceptibility were considered to be a result in differing genetics in the host and no attention was given to the possibility of variable genetics within rust populations. Therefore, the objective of this study was to differentiate the variability of rust resistance / tolerance in 12 switchgrass cultivars including ornamental, lowland and upland cultivars using 40 rust isolates collected throughout southeastern US in lab settings.

Material and Methods

Plant materials

Twelve cultivars of *Panicum virgatum* were used, including 'Alamo' and 'Kanlow' (lowland switchgrass cultivars), 'Summer' and 'Cave-In-Rock' (upland switchgrass cultivars), and eight ornamental cultivars ('Badland', 'Cheyenne Sky', 'Cloud Nine', 'Dallas Blue', 'Dewey Blue', 'Prairie Sky', 'Thundercloud' and wild type *P. virgatum*). The seeds of the four agronomic switchgrass cultivars were obtained from US National Plant Germplasm System (NPGS) and 10 seedlings for each cultivar were randomly selected from germinated seeds and grown in one gallon pot. Ornamental switchgrass cultivars were ordered from Hoffman nursery (Rougemont, NC). Plants were grown in a greenhouse at 25°C with a 16-h light/8-h dark cycle. Randomly

chosen leaf segments, 4 cm long from each cultivar, were used to evaluate spore germination, latent period, and urediniospore production.

Fungal isolates collection and confirmation

Forty isolates of switchgrass rust collected from 2008-2012 across south-eastern U.S were used in the study. The detailed collection-information is listed in table 2.1. The rust isolates were confirmed to be *P. emaculata* morphologically and molecularly. The molecular confirmation was conducted by extraction rust genomic DNA with DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions, followed by amplifying ITS sequences with forward primer ITS1rustF10d (5' TGAACCTGCAGAAGGATCATTA 3') (Barnes and Szabo, 2007; Uppalapati et al., 2013) and reverse primer RUST1 (5' GCTTACTGCCTTCCTCAATC 3') (kropp et al., 1995; Uppalapati et al., 2013). PCRs were conducted in a 25 µl reaction volume with diluted DNA 2 µl (5 ng/ µl), GoTaq master mix (Promega, Madison, WI) 10 µl, each of the primers 1.5 µl, dimethyl sulfoxide (DMSO) 1.5 µl, and autoclaved double distilled water 8.5 µl. PCRs were performed in thermo cycler (Eppendorf AG, Hamburg, Germany) with the following conditions: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 120 s, and a final extension at 72 °C for 10 min. Five microliters of PCR products were electrophoresed with 1% agarose gel and visualized with a 2000 Gel Documentation System (BIO-RAD, Hercules, CA). The PCR products were directly sent to molecular cloning laboratories (MCLAB, San Francisco, CA) for cleaning and sequencing. The sequencing results were then compared and confirmed to be *P. emaculata* ITS sequences with Basic Local Alignment Search Tool (BLAST) in GenBank database from the National Center for Biotechnology Information (NCBI) website.

Inoculum and inoculation

To ensure the purity of these isolates, a single pustule from each sample was isolated, inoculated and maintained on leaf segments of susceptible 'Thundercloud' switchgrass in Petri dishes containing moistened filter paper, and cultures were incubated in a temperature controlled laboratory at 20 ± 2ºC with 10 h light per 24 h (four 40W residential fluorescent bulbs from 45 cm above leaf segments). Fresh leaf segments were inoculated and replaced around two-week intervals. Urediniospores were harvested with brush and stored in the freezer (-20 ºC) for later usage.

Leaf segments of different switchgrass cultivars were inoculated with separate suspensions of 40 rust isolates in concentration of 1×10^5 sporesml^{-1} of water-Tween 20 (0.001% v/v) with fingertip sprayers (Lehman and Shaner, 1997). Final deposition of spores on the leaf segments were approximately three spores per mm^2 by measuring the spore density on 1.5% water agar on glass slides setting along with leaf segments during inoculation. For each host cultivar and rust isolates combination, three inoculated leaf segments were placed on moistened paper towels with adaxial surface up into a 9-cm-diameter petri dish, with two layers of moistened filter paper and incubated in a temperature controlled laboratory at 20 ± 2ºC with 10 h light per 24 h as described above. Percentages of spore germination, latent period, number of uredia/cm^2 of leaf tissue, and number of urediniospores per uredium were measured.

Germination of urediniospore

A urediniospore was considered germinated when a germ-tube length was at least half the width of a urediniospore. At 12 h after inoculation (HAI), the percentage of germinated urediniospores was assessed on 10 random samples of 10 conidia for each sample on leaf segment.

Latent period

Latent period is defined as the time between when infection process begins (appressorium formation) and the uredia are first observed on inoculated leaf pieces. Uredia formation was examined on leaf pieces under a dissecting microscope daily after inoculation.

Uredium and urediniospore production

For each host cultivar and rust isolate combination, three 1 cm^2 areas on leaf segments were randomly chosen and the numbers of uredia were counted under a stereo microscope. For each of these 1 cm^2 areas, 5 uredia were randomly picked and transferred into 50µl water and 0.001% Tween 20 mixture. The urediniospore solutions were then measured using a hemacytometer under a compound microscope and the average number of urediniospore produced by a uredium was calculated. For each of the 1 cm^2 areas, the average number of spores in a uredium experiment was repeated four times. And the average number of urediniospores produced per 1 cm^2 leaf segment was calculated by multiplying the number of uredia per square centimeter by average urediniospores number per uredium.

Statistical analysis

The whole experiment mentioned above was repeated two times. The data were analyzed as a randomized complete block design with samples. The two repeated experiments were considered the block effect. The experimental unit was a leaf segment, and the measurements coming from these three 1 cm^2 areas on the leaf were considered as samples. The blocks were considered as random effects, and treatments were considered as fixed effects. After proper transformation of the data (arcsine for percentage data and common logarithm for count data), the effects of block,

switchgrass cultivars, rust isolates, and their interactions on conidia germination, latent period, conidia production were determined by analysis of variance (ANOVA), using the Mixed model procedure of SAS, version 9.3 (SAS Institute Inc. 2011). Mean separation for each variable were conducted with the Tukey's HSD test at $\alpha = 0.05$ using PDMIX800 macro (Saxton, 1998). The effects of switchgrass cultivar by rust isolate interaction were presented by heatmap using package 'gplots' in R.

Results

Forty rust isolates were all identified as *Puccinia emaculata* by blasting the ITS sequences from the NCBI website. None of these isolates were identified as *Uromyces graminicola*.

Significant differences in germination percentage of switchgrass rust were observed among switchgrass cultivars ($p < 0.0001$), which range from 99.99% (Badland) to 92.83% (Alamo) (Table 2.2). Significant differences were also found among 40 switchgrass rust isolates for their germination percentage (data not shown) with p value less than 0.0001. Isolate NC01 had the highest germination rate with 99.95% and MS0911 had the lowest with 96.73% (data not shown). Furthermore, the interactions between switchgrass cultivars and rust isolates were also significant ($p < 0.0001$) (data not shown).

Significant differences in latent period were observed among switchgrass cultivars ($p < 0.0001$). Ornamental cultivar Cloud Nine had the shortest latent period with 9.8 days and another ornamental cultivar Cheyenne Sky had the longest latent period with 14.4 days (Table 2.3). From latent period perspective, there was no difference between agronomic cultivars and ornamental cultivars. However, among agronomy cultivars, the lowland cultivars Kanlow and Alamo

significantly reduced the latent period compared for latent periods of upland cultivars Summer and Cave-In-Rock. In addition, significant differences in latent period were also found among rust isolates ($p < 0.0001$), which ranged from 12.2 day (MS0911) to 10.1 day (TN0803) (Table 2.4). Furthermore, the interactions between switchgrass cultivars and rust isolates were also significant ($p < 0.0001$) (Figure 2.1). For instance, changing from rust isolate AL-DaB to TN0924, on Prairie Sky, the latent period decreased while increased on Summer. The significant interaction effects suggested there might be some race specific resistances in these switchgrass cultivar-rust isolate combinations with longer latent period, such as Cheyenne Sky and MS0911 combination, Dallas Blue and AL-NW combination, etc. (Figure 2.1).

With the same inoculum, the average numbers of pustules per cm^2 produced on host leaf surface differed among switchgrass cultivars ($p < 0.0001$), ranging from 29.6 (Alamo) to 3.6 (Prairie Sky) (Table 2.5). Overall, ornamental cultivars produced smaller number of pustules per cm^2 comparing with agronomy cultivars. In addition, significant differences in number of pustules per cm^2 which each rust isolates produced were also found ($p < 0.0001$), ranging from 22.8 (TN0920) to 13.7 (AR01) (Table 2.6). Furthermore, the interactions between switchgrass cultivars and rust isolates were also significantly different ($p < 0.0001$) (Figure 2.2). For instance, changing from rust isolate AL-BL to TN10-4, on Cave-in-rock, the number of pustules per cm^2 decreased while increased on Dallas Blue. The significant interaction effects also suggested there might be some race specific resistances in these switchgrass cultivar-rust isolate combinations with smaller number of pustules per cm^2, such as Cheyenne Sky and MS0911 combination, Dallas Blue and AL-NW combination, etc. (Figure 2.2), which were in coincidence with the results in suggested in previous latent period experiment (Figure 2.1).

Significant differences in number of urediniospores per cm^2 were observed on leaves among switchgrass cultivars ($p < 0.0001$), which range from 44228.3 (Cloud 9) to 653.1 (Prairie Sky) (Table 2.7). Overall, ornamental cultivars produced less urediniospores per cm^2 comparing with agronomic cultivars. In addition, significant differences in number of urediniospores per cm^2 which each rust isolates produced were also found ($p < 0.0001$), ranging from 20835.3 (NC03) to 3250.9 (AL-PS) (Table 2.8). Furthermore, the interactions between switchgrass cultivars and rust isolates were also significant ($p < 0.0001$) (Figure 2.3). For instance, changing from rust isolate TN0920 to TN10-4, on Cave-in-rock, the number of urediniospores per cm^2 increased while decreased on Cheyenne Sky. The significant interaction effects suggested there might be some race specific resistances in these switchgrass cultivar-rust isolate combinations with smaller number of urediniospores per cm^2, such as Cheyenne Sky and TN10-4 combination, Cloud Nine and MS0909 combination, etc. (Figure 2.3), which were not in coincidence with the results of latent period and number of pustules per cm^2 experiments.

Discussion

Although four rust species, *P. emaculata*, *P. graminis*, *P. huberi*, and *U. graminicola*, were reported to infect switchgrass (Arthur, 1934; Gravert and Munkvold, 2002; Ramachar and Cummins, 1963 & 1965; Cummins, 1971), *P. emaculata* is the most widely distributed across USA. The urediniospores of *P. graminis* and *P. huberi* are oval while the urediniospores *P. emaculata* and *U. graminicola* of are globose in shape and are most identical, which cannot be used to differentiate these two species (Kenaley et al., 2016). Teliospore morphology can be used to separate *P. emaculata* and *U. graminis* since teliospores of *P. emaculata* have single cell while teliospores of *U. graminis* have two cells. All these 40 isolates, collected from the southeastern

US, were morphologically and molecularly confirmed as *P. emaculata* and none of them is *U. graminicola*, which corresponds with the statement that *U. graminicola* on switchgrass is usually more popular in northern states and rarely in southern states (Kenaley et al., 2016). In northern states, such as New York, Iowa, Nebraska and South Dakota, mixtures of these two species of rust fungi were detected on switchgrass.

Germination and penetration are the very first two steps in the pathogen infection process, which sounds simple and easy but has tremendous signal interactions between the pathogen and the host, especially for the obligate biotrophic fungus and their hosts. The infection process continues when fungal spores detect and recognize the biochemical, topographical and thigmotropic signals of the host surfaces (Hoch et al., 1987; Mellersh and Heath, 2003). In this rust and switchgrass interaction complex, although variations in rust germination percentage were observed among different switchgrass cultivars, the smallest germination percentage was as high as 92.83% (on Alamo). In addition, no significant differences were identified among rust isolates for their germination. Although these isolates were collected from different sources, agronomical cultivars or ornamental cultivars, they have no difference in germinating on leaf surfaces of both agronomic and ornamental cultivars.

As a polycyclic plant pathogen, wind borne urediniospores are the only rust spores that can have multiple and repeated infections on the host plant under favorable environmental conditions. Host cultivars with longer latent periods would significantly reduce the number of cycles of infection, which results in slower rates of disease development, especially for rust with the ability to wide spread for their wind-borne urediniospores. This is the first to document the latent

period of *P. emaculata* on switchgrass. Cultivars with longer latent periods, such as Cheyenne Sky and wild type *P. virgatum* could be used as genetic resource for rust resistance breeding in switchgrass.

Agronomic cultivars produced more uredia and urediniospores per cm^2 comparing with ornamental cultivars (Table 2.5; Table 2.7). Average number of uredia produced per cm^2 leaf surface could be another aspect of durable resistance of the host. In addition, a clear pattern that more uredia in the specific area would lead to relatively fewer urediniospores produced per uredium (data not shown) was observed, as an obligate biotrophic pathogen, keeping the host alive, the energy / resource that the pathogen gets from an area is limited. More uredia certainly lead to fewer urediniospore produced per uredium, which is the reason why the number of urediniospores per uredium was not directly used in analysis for indicating different resistant levels of the host cultivar.

Variations were observed among 40 rust isolates in latent period, number of uredia and urediniospores produced per cm^2. Although no clear pattern was observed to group these isolates by different collecting years or locations, the genetic variation among 40 rust isolates and cultivar-specific interactions between switchgrass and rust were observed. With some simple sequence repeats (SSRs) (Wadl et al., 2011), the population genetics of switchgrass rust isolates would have to be studied to understand the genetic diversity, gene flow, origin and epidemiology of the rust.

The present study is the first to study the germination percentage, latent period, number of uredia and urediniospores produce per cm^2 leaf surface of 40 switchgrass rust isolates on 12 agronomic and ornamental switchgrass cultivars. This continuous resistance information throughout the whole infection process on switchgrass cultivars other than the single overall end-season disease rating value provides details and new resources for developing rust resistant switchgrass cultivars to achieve sustainable control of switchgrass rust.

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Appendix

Table 2.1 Location and cultivar of switchgrass (*Panicum virgatum*) that each isolate of *Puccinia emaculata* (total 41) was collected from. Cultivars (bold) are clonal-nursery derived plants. The switchgrass cultivars in bold indicates the ornamental cultivars.

Rust Isolate	Switchgrass Cultivar	Location	Collected Year
AR-01	**Dallas Blue**	Fayetteville, AR	2008
LA-0902	**Dallas Blue**	Mobile, AL	2009
MS-09099	Alamo	MSU research plot, MS	2009
MS-0911	Cycle 2	MSU research plot, MS	2009
NC-01	**Heavy Metal**	Hoffman Nursery, NC	2008
NC-02	**Dallas Blue**	Hoffman Nursery, NC	2008
NC-03	**Northwind**	Hoffman Nursery, NC	2008
NC-05	**Cloud Nine**	Hoffman Nursery, NC	2008
TN03	**Dewey Blue**	Knoxville, TN	2008
TN 10-4	Alamo	Madisonville, TN	2010
TN 10-5	Alamo	Madisonville, TN	2010
TN 10-6	Alamo	Madisonville, TN	2010
TN-0918	**Northwind**	Knoxville, TN	2009
TN-0919	**Dallas Blue**	Knoxville, TN	2009
TN-0920	**Thundercloud**	Oak Ridge, TN	2009
TN-0921	Alamo	Knoxville, TN	2009
TN-0922	**Rotstrahlbusch**	Crossville, TN	2009
TN-0923	**Dallas Blues**	Jackson, TN	2009
TN-0924	**Rotstrahlbusch**	Jackson, TN	2009
TN-0925	**Northwind**	Knoxville, TN	2009
TN-0926	**Alamo**	Knoxville, TN	2009
GA12-01	**Dallas Blues**	Atlanta, GA	2012
GA12-02	**Dallas Blues**	Atlanta, GA	2012
GA12-03	**Dallas Blues**	Atlanta, GA	2012
GA12-04	**Dallas Blues**	Atlanta, GA	2012

Table 2.1 continued

Rust Isolate	Switchgrass Cultivar	Location	Collected Year
SC12-01	Alamo	Florence, SC	2012
SC12-02	Alamo	Florence, SC	2012
SC12-03	Alamo	Florence, SC	2012
SC12-04	Alamo	Florence, SC	2012
AL-BL	**Badland**	Brewton, AL	2012
AL-C9	**Cloud Nine**	Brewton, AL	2012
AL-CS		Brewton, AL	2012
AL-DaB	**Dallas Blue**	Brewton, AL	2012
AL-DeB	**Dewey Blue**	Brewton, AL	2012
AL-HM	**Heavy Metal**	Brewton, AL	2012
AL-NW	**Northwind**	Brewton, AL	2012
AL-PF		Brewton, AL	2012
AL-PS	**Prairie Sky**	Brewton, AL	2012
AL-PV	***Panicum virgatum***	Brewton, AL	2012
AL-SH	**Shenandoah**	Brewton, AL	2012
AL-TC	**Thundercloud**	Brewton, AL	2012

Table 2.2 The percentage germination rate of switchgrass rust (*Puccinia emaculata*) on 12 switchgrass cultivars (*Panicum virgatum*) 24 h after inoculation. Different letters in letter group indicate the effects are different.

Cultivar	**Estimate**[a] (arcsine transformation)	**Standard Error**	**Germination Rate**[b]	**Letter Group**
Badland	1.5640	0.05517	0.9999	A
Dallas Blue	1.5457	0.05517	0.9997	A
Thundercloud	1.5273	0.05517	0.9991	AB
Cheyenne Sky	1.5166	0.05517	0.9985	ABC
Summer	1.5132	0.05517	0.9983	ABC
P. virgatum	1.5004	0.05517	0.9975	ABC
Cave-In-Rock	1.4579	0.05954	0.9936	ABCD
Kanlow	1.4303	0.05517	0.9901	BCD
Dewey Blue	1.4226	0.05517	0.9890	CD
Prairie Sky	1.4148	0.05517	0.9879	CD
Cloud Nine	1.3704	0.05517	0.9800	D
Alamo	1.1898	0.05517	0.9283	E

[a]log10 transformation for the data for SAS to analyze
[b]back transformation to better interpret the results

Table 2.3 The latent period of switchgrass rust (*Puccinia emaculata*) on 12 switchgrass cultivars (*Panicum virgatum*). Different letters in letter group indicate the effects are different.

Cultivar	Standard Error	Latent Period	Letter Group
Cheyenne Sky	0.003171	14.4	A
P. virgatum	0.003171	12.6	B
Kanlow	0.003171	12.0	C
Badland	0.003171	11.9	C
Dewey Blue	0.003171	11.9	C
Thundercloud	0.003171	11.4	D
Prairie Sky	0.003171	10.3	E
Alamo	0.003171	9.9	F
Dallas Blue	0.003171	9.8	F
Summer	0.003171	9.8	F
Cave-In-Rock	0.003171	9.4	G
Cloud Nine	0.003171	9.2	H

Table 2.4 The latent period of 40 switchgrass rust isolates (*Puccinia emaculata*) on switchgrass (*Panicum virgatum*). Different letters in letter group indicate the effects are different.

Isolate	Standard Error	Latent Period	Letter Group
MS0911	0.004281	12.2	A
AL-PV	0.004281	11.7	AB
NC05	0.004281	11.6	BC
AL-PF	0.004281	11.5	BCD
MS0909	0.004281	11.4	BCDE
LA0902	0.004281	11.4	BCDE
AL-PS	0.004281	11.4	BCDEF
AL-NW	0.004281	11.3	BCDEFG
NC01	0.004281	11.3	BCDEFGH
GA1202	0.004281	11.3	BCDEFGHI
SC1203	0.004281	11.2	BCDEFGHIJ
NC03	0.004281	11.2	BCDEFGHIJ
TN0924	0.004281	11.2	CDEFGHIJ
GA1204	0.004281	11.1	CDEFGHIJ
TN0918	0.004281	11.1	DEFGHIJK
GA1201	0.004281	11	EFGHIJKL
AL-BL	0.004281	11	EFGHIJKL
AR01	0.004281	11	EFGHIJKL
AL-HM	0.004281	10.9	FGHIJKLM
SC1202	0.004281	10.9	GHIJKLM
TN0926	0.004281	10.9	GHIJKLM
AL-CS	0.004281	10.9	GHIJKLM
TN10-6	0.004281	10.9	GHIJKLM
TN0923	0.004281	10.9	GHIJKLM
AL-TC	0.004281	10.9	HIJKLM

Table 2.4 continued

Isolate	Standard Error	Latent Period	Letter Group
AL-DaB	0.004281	10.8	HIJKLM
TN0922	0.004281	10.8	HIJKLM
TN0919	0.004281	10.8	IJKLM
AL-DeB	0.004281	10.8	JKLMN
AL-C9	0.004281	10.8	JKLMN
TN0925	0.004281	10.7	JKLMN
TN0921	0.004281	10.7	JKLMN
GA1203	0.004281	10.6	KLMNO
TN10-4	0.004281	10.5	LMNO
TN0920	0.004281	10.5	LMNO
TN10-5	0.004281	10.5	MNOP
AL-SH	0.004281	10.5	MNOP
SC1201	0.004281	10.3	NOP
SC1204	0.004281	10.3	OP
TN0803	0.004281	10.1	P

Table 2.5 Number of pustules per cm^2 of switchgrass rust (*Puccinia emaculata*) on 12 switchgrass cultivars (*Panicum virgatum*). Different letters in letter group indicate the effects are different.

Cultivar	**Estimate**	**Standard Error**	**Letter Group**
Alamo	29.6458	0.3574	A
Summer	29.4875	0.3574	A
Cave-In-Rock	24.4458	0.3574	B
Dewey Blue	22.5500	0.3574	C
Badland	21.8667	0.3574	C
Dallas Blue	21.6917	0.3574	C
Thundercloud	19.7958	0.3574	D
Kanlow	17.5792	0.3574	E
Cheyenne Sky	12.4500	0.3574	F
P. virgatum	12.2042	0.3574	F
Cloud Nine	9.2792	0.3574	G
Prairie Sky	3.5875	0.3574	H

Table 2.6 Number of pustules per cm^2 of 40 switchgrass rust isolates (*Puccinia emaculata*) on switchgrass cultivars (*Panicum virgatum*). A different letter in letter group indicate the isolates are different.

Isolate	Estimate	Standard Error	Letter Group
TN0920	22.7639	0.6525	A
GA1204	22.4861	0.6525	AB
AL-SH	22.0833	0.6525	ABC
NC01	22.0833	0.6525	ABC
AL-BL	21.6667	0.6525	ABCD
NC03	21.5417	0.6525	ABCDE
TN0921	21.4722	0.6525	ABCDEF
TN0919	21.3889	0.6525	ABCDEF
TN0922	21.3611	0.6525	ABCDEF
AL-C9	21.0417	0.6525	ABCDEFG
AL-CS	20.6528	0.6525	ABCDEFGH
GA1202	20.3889	0.6525	ABCDEFGH
TN0924	19.5000	0.6525	ABCDEFGHI
MS0911	19.3889	0.6525	ABCDEFGHIJ
NC05	19.2083	0.6525	ABCDEFGHIJ
TN0918	19.2083	0.6525	ABCDEFGHIJ
SC1201	19.0694	0.6525	BCDEFGHIJK
AL-TC	18.7361	0.6525	CDEFGHIJKL
GA1203	18.6806	0.6525	CDEFGHIJKL
TN10-5	18.5278	0.6525	CDEFGHIJKL
AL-DeB	18.4444	0.6525	DEFGHIJKL
TN10-4	18.2778	0.6525	DEFGHIJKL
SC1202	18.2500	0.6525	DEFGHIJKL
MS0909	18.1111	0.6525	DEFGHIJKL
GA1201	18.0417	0.6525	EFGHIJKL

Table 2.6 continued

Isolate	Estimate	Standard Error	Letter Group
SC1204	17.9306	0.6525	FGHIJKL
TN0925	17.8889	0.6525	FGHIJKL
AL-PF	17.6111	0.6525	GHIJKLM
TN0923	17.4583	0.6525	GHIJKLM
AL-NW	17.3194	0.6525	HIJKLM
TN10-6	17.2917	0.6525	HIJKLM
SC1203	16.7500	0.6525	IJKLMN
TN0803	16.7361	0.6525	IJKLMN
AL-HM	16.5278	0.6525	IJKLMN
AL-PV	16.3194	0.6525	IJKLMN
TN0926	15.8333	0.6525	JKLMN
AL-DaB	15.4861	0.6525	KLMN
LA0902	15.1528	0.6525	LMN
AL-PS	14.2500	0.6525	MN
AR01	13.6806	0.6525	N

Table 2.7 Number of urediniospores per cm^2 of switchgrass rust (*Puccinia emaculata*) produced on 12 switchgrass cultivars (*Panicum virgatum*). Different letters in letter group indicate the effects are different.

Cultivar	Standard Error	Spore/cm^2	Letter Group
Cloud Nine	0.02691	44228.3	A
Alamo	0.02691	31499.2	B
Summer	0.02724	24400.6	BC
Kanlow	0.02691	24165.7	BC
Dallas Blue	0.02691	20960.4	C
Dewey Blue	0.02691	9107.5	D
Thundercloud	0.02691	9099.1	D
P. virgatum	0.02691	7469.6	D
Badland	0.02691	6864.4	D
Cheyenne Sky	0.02691	2937	E
Cave-In-Rock	0.02691	2193.8	F
Prairie Sky	0.02691	653.1	G

Table 2.8 Number of urediniospores per cm^2 of 40 switchgrass rust (*Puccinia emaculata*) isolates produced on switchgrass (*Panicum virgatum*). Different letters in letter group indicate the effects are different.

Isolate	Standard Error	Spore/cm^2	Letter Group
NC03	0.04912	20835.3	A
SC1204	0.04912	18754.3	AB
SC1202	0.04912	18569.5	AB
NC01	0.04912	16734.0	ABC
TN10-5	0.04912	15925.8	ABCD
TN0921	0.04912	15268.6	ABCD
AL-CS	0.04912	14862.8	ABCD
TN0922	0.04912	14397.9	ABCD
SC1201	0.04912	14171.0	ABCDE
AL-SH	0.04912	13176.5	ABCDEF
NC05	0.04912	12661.9	ABCDEF
AL-C9	0.04912	12488.2	ABCDEF
AL-TC	0.04912	12053.1	ABCDEFG
GA1202	0.04912	12006.0	ABCDEFG
GA1201	0.04912	11587.8	ABCDEFG
AL-DeB	0.04912	11585.1	ABCDEFG
TN0924	0.04912	11574.4	ABCDEFG
AL-BL	0.04912	10122.8	BCDEFGH
GA1204	0.04912	9749.9	CDEFGHI
AL-HM	0.04912	9680.5	CDEFGHIJ
MS0911	0.04912	9458.0	CDEFGHIJK
AR01	0.04912	9447.1	CDEFGHIJK
GA1203	0.04912	9287.5	CDEFGHIJKL
TN0926	0.04912	8957.8	CDEFGHIJKL

Table 2.8 continued

Isolate	Standard Error	Spore/cm^2	Letter Group
TN0919	0.04912	8766.0	DEFGHIJKL
TN10-6	0.04912	8517.3	DEFGHIJKL
AL-NW	0.05113	7550.9	EFGHIJKLM
TN0925	0.04912	7481.7	FGHIJKLM
TN0923	0.04912	6532.8	GHIJKLMN
LA0902	0.04912	5917.0	HIJKLMNO
AL-PF	0.04912	5361.7	IJKLMNO
TN0920	0.04912	5277.2	IJKLMNO
TN0803	0.04912	5178.5	JKLMNO
TN0918	0.04912	5176.1	JKLMNO
AL-DaB	0.04912	5071.1	KLMNO
SC1203	0.04912	5047.8	LMNO
TN10-4	0.04912	4348.1	MNO
MS0909	0.04912	3934.6	NO
AL-PV	0.04912	3754.0	NO
AL-PS	0.04912	3250.9	O

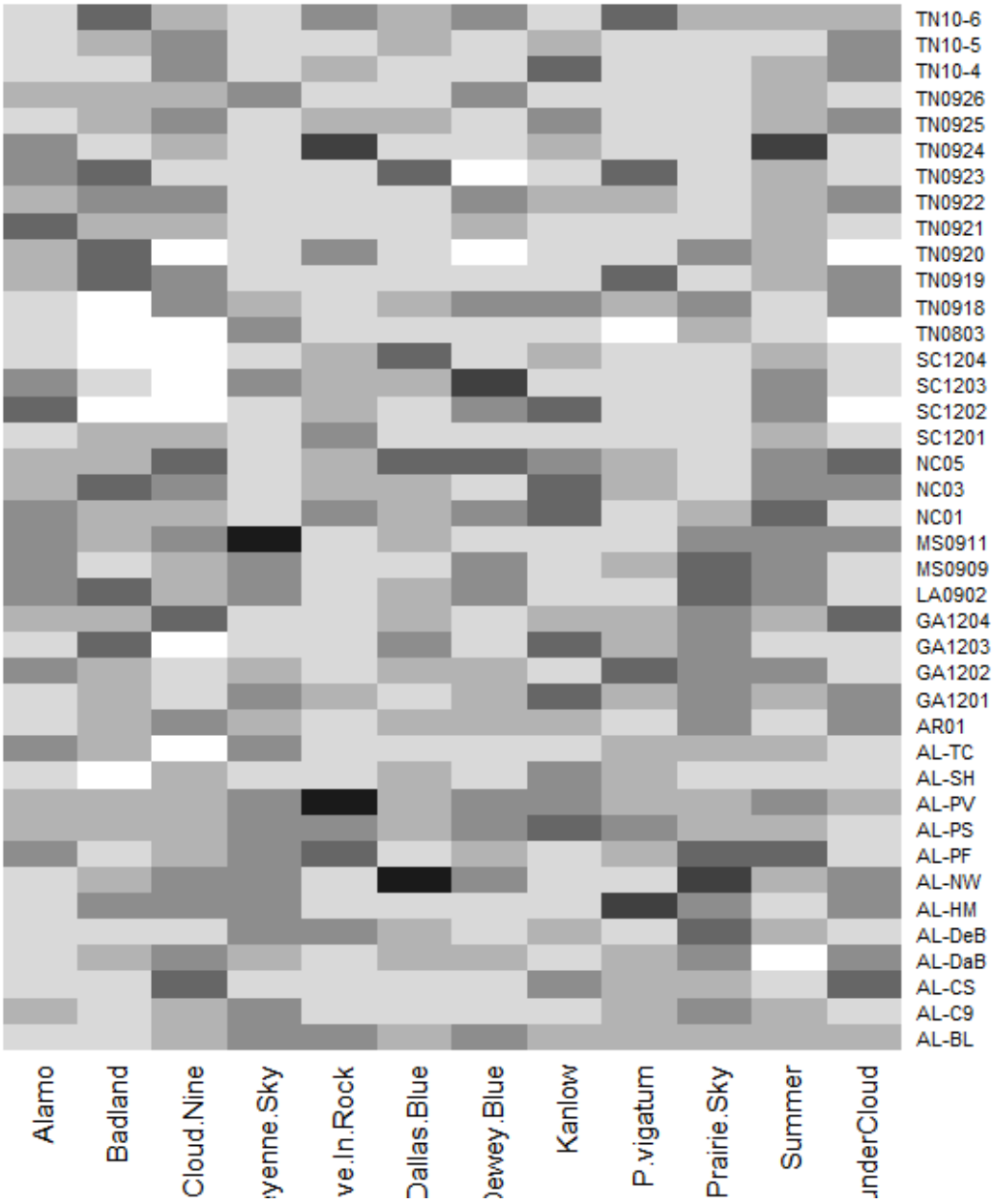

Figure 2.1 Black and white heatmap for the effects of switchgrass cultivar (x axis) and rust isolate (y axis) interaction on latent period for rust on switchgrass leaves. Darker colors indicate these switchgrass cultivar and rust isolate combinations have longer latent periods than these combinations with light colors.

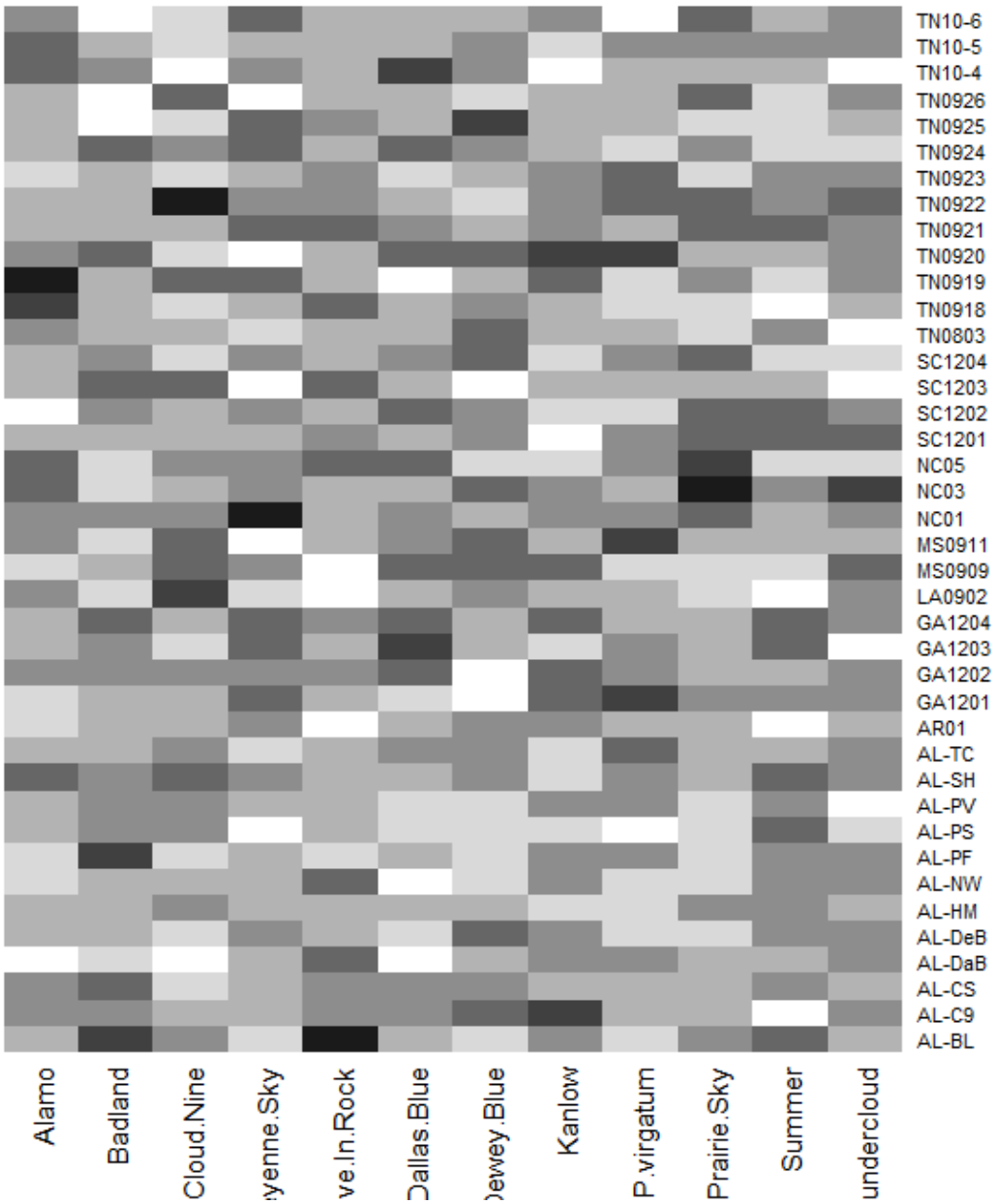

Figure 2.2 Black and white heatmap for the effects of switchgrass cultivar (x axis) and rust isolate (y axis) interaction on number of rust pustules produced per cm^2 on switchgrass leaves. Darker colors indicate these switchgrass cultivar and rust isolate combinations have more numbers of pustules per cm^2 than these combinations with light colors.

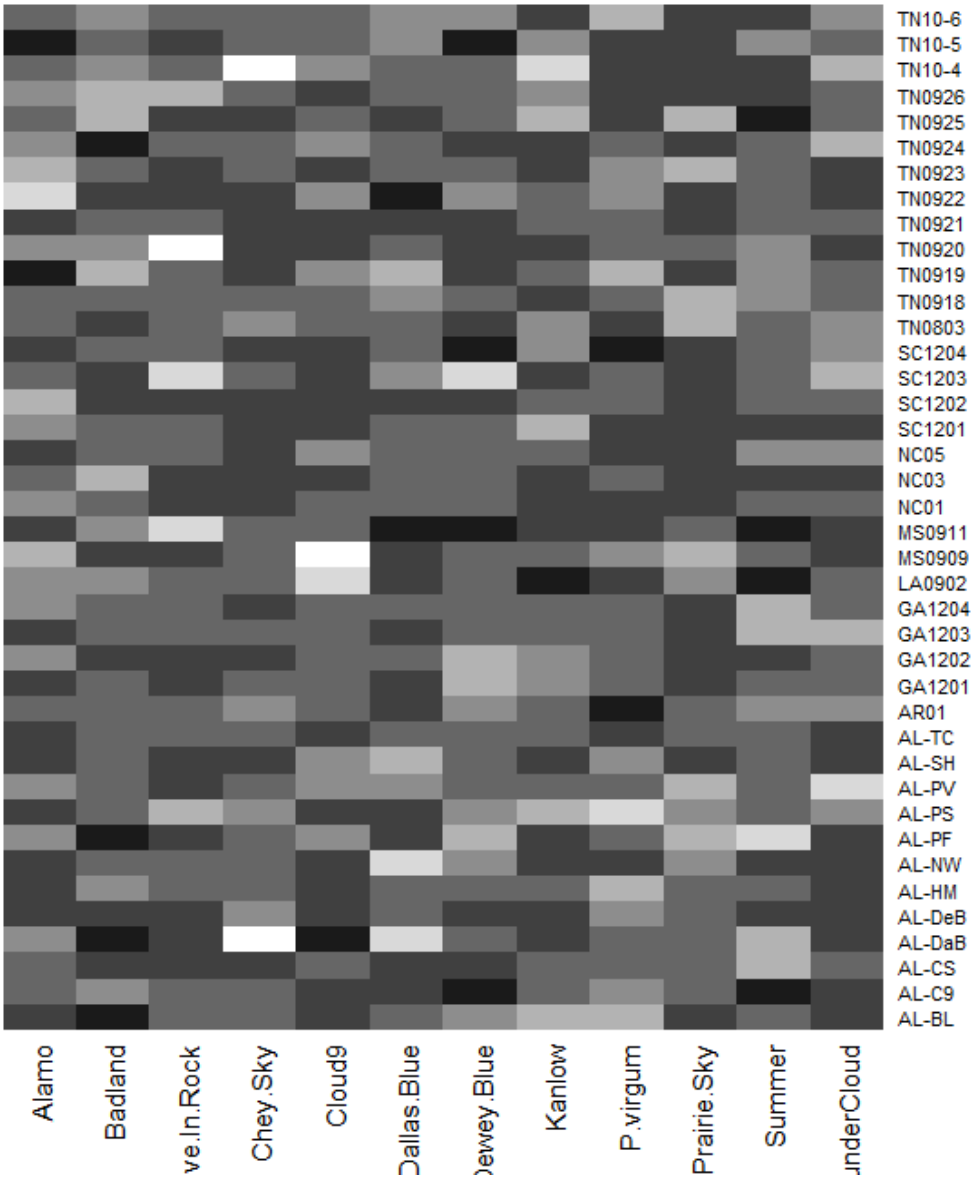

Figure 2.3 Black and white heatmap for the effects of switchgrass cultivar (x axis) and rust isolate (y axis) interaction on number of urediniospores produced per cm^2 on switchgrass leaves. Darker colors indicate these switchgrass cultivar and rust isolate combinations have more numbers of urediniospores per cm^2 than these combinations with light colors.

Chapter 3. Genome information of switchgrass rust (*Puccinia emaculata*) and its taxonomic status

Abstract

Six switchgrass rust isolates collected from Southeastern U.S. were sequenced. The 29 million paired end sequence reads of length 100bp for a switchgrass rust isolate were *de novo* assembled. Due to the high contamination of the reads, only 622 rust contigs were confidently provided, which would be random samples of nucleotide pieces from the whole genome with GC content 47.33%. Variant detection was conducted by mapping sequence reads of the other five isolates on these rust contigs. The average single nucleotide variants (SNVs) and insertion/deletions (indels) density is 1.62 and 0.057 per kb, respectively. Phylogenetic analyses using *5.8S rRNA* gene placed switchgrass rust in Pucciniaceae, separating form Melampsoraceae, Phakopsoraceae, Pucciniastreae.

Introduction

Switchgrass (*Panicum virgatum* L) is a warm-season perennial grass native to North America. The native range of switchgrass includes most of the continental United States (exceptions California, Oregon and Washington) (USDA, 2006). Traditionally, switchgrass has been used for bank stabilization as ornamental and as a forage crop (Lemus et al., 2002). Due to its C4 photosynthetic pathway, switchgrass has been designated as a sustainable bioenergy crop for ethanol and butanol (Bouton 2008). For its minimum fertilizer input requirement, switchgrass showed most potential biomass species among other perennial grasses and legumes including sorghum (Cherney et al., 1991; Wright, 2007). Average annual biomass yields from switchgrass fields 5.2 to 11.1 $Mg \cdot ha^{-1}$ which produces an average estimated net energy yield of 60 $GJ \cdot ha^{-1}$ (Schmer et al., 2008).

Many fungal diseases have been reported for switchgrass including rust, smuts, leaf spots, crown and root rots (Gravert and Munkvold, 2002). Although eighty-one species of fungi have been reported to cause disease on switchgrass in the United States (Farr and Rossman, 2009), most of them have little economic impact. Diseases reported to reduce switchgrass biomass are head smut (*Tilletia maclaganii*) (Thomsen et al., 2008), anthracnose (*Colletotrichum navitas*) (Crouch et al., 2009), and switchgrass rust (Hirsch et al., 2010; Zale et al., 2008). A new study found that the estimated ethanol production can be reduced by up to 55% due to severity infection of switchgrass rust (Sykes et al., 2016).

Switchgrass rust (*Puccinia emaculata*) infection of switchgrass has been reported in Alabama (Hagan and Atridge, 2013), Arkansas (Hirsch et al., 2010), Tennessee (Zale et al., 2008) and Virginia (Frazier et al., 2013). The symptom of disease was leaf chlorosis. Uredia were formed underneath adaxial epidermal cells. Urediniospores were globose or oval in shape and approximately 27 x 25 µm and were the primary source of inoculum for secondary disease cycle. On water agar, urediospores can germinate one hour after inoculation (HAI) and after 2 or 3 HAI, hyphae elongate, branch, and form appressoria (Li et al., 2009). On switchgrass leaves, appressoria formed over stomata and penetrated through stomatal openings. In later summer to fall, dark brown telia can be observed on infected leaves. Teliospores are two-celled and approximately 33.6 µm ± 4.8 in length, and the width of apical and basal cells are 17.5 µm ± 1.2 and 15.9 µm ± 2.5, respectively (Zale et al., 2008).

Understanding the characteristics of *P. emaculata* is vital for developing an effective and sustainable approach to the management of switchgrass rust. It is imperative to gain more insight

into genome structure of this fungus, which will assist in developing resistant switchgrass cultivars and eventually reduce crop losses. The completion of the *P. graminis, P. triticina,* and *P. striiformis* genome sequences (Broad Institute) would provide development in the study of rust pathogens and provide reference genomes to which molecular markers can be physically mapped.

Other than the development of microsatellite markers (Wadl et al., 2011), no literature exists concerning the genetic diversity or population structure of *P. emaculata*. The objective of this study was to obtain a brief overview of the switchgrass rust genome using high throughput sequencing technology. The study also compared the sequences of six isolates collected from different locations and years throughout the Southeastern U.S. to develop single nucleotide polymorphism (SNP) markers, which would help determine the genetic diversity and understanding the population structure of switchgrass rust.

Materials and methods

Fungal isolates

Individual isolates of six *P. emaculata* isolates AL-SH, AR01, MS0909, NC01, TN0803, and TN0926 were used in the study. The collected places and host switchgrass cultivars are listed in Table 3.1. To ensure the purity of these isolates, a single pustule from each sample was isolated, inoculated and maintained on leaf segments of susceptible ‘Thundercloud’ switchgrass in Petri dishes containing moistened filter paper, and cultures were incubated in a temperature controlled laboratory at 20 ± 2ºC with continuous light (four 40W residential fluorescent bulbs from 45 cm above leaf segments). The temperature was monitored using a thermometer sensor on the bench

where the leaf segments are incubated. Fresh leaf segments were inoculated and replaced at 14-day intervals. Urediniospores were harvested and stored in the freezer (-20 ºC) for later usage.

Genomic DNA extraction and sequencing

Urediniospores were collected from above six single-pustule derived isolates and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions with minor modifications. The modifications included adding 1-mm glass beads to the sample and homogenizing with a Mixer Mill 300 at 6.0 for 20 sec three times. The extracted genomic DNA was stored at -20°C until it was shipped for sequencing.

Sequence libraries were prepared for these six isolates by the commercial sequence company (Genewiz Inc., South Plainfield, NJ), and genomic DNA were then sequenced using Illumina HiSeq 2500 system (100bp paired-end reads). The sequence (FASTQ format) outputs were used for further analysis.

Genome assembly and analysis

Unless otherwise specified, CLC Genome Workbench (Version 9.0, CLC bio Qiagen, Prismet) was used for quality reads trimming, de novo assembly and variant detection among rust isolates. The analysis workflow is depicted in Figure 3.1. The sequences from MS0909 were trimmed to remove low-quality sequences with a limit quality value of 0.05, a maximum number of two ambiguous nucleotides at the sequence ends and minimum length of 40nt. When using the *P. graminis* (wheat stem rust) or *P. triticina* (wheat leaf rust) as reference genomes, the mapping coverage was low. Therefore, the trimmed sequence data for MS0909 was preliminary de novo

assembly into contigs by default settings. These contigs were analyzed by the Blastn program on the NCBI site (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) (e-value 1e-05 cut-off) against the default database. The first organism of each significant alignment was saved and its genome was acquired from Ensembl Genome (http://useast.ensembl.org/index.html), European Nucleotide Archive (ENA), National Center for Biotechnology Information (NCBI) or The Department of Energy Joint Genome Institute (DOE JGI). These genomes with detailed information in Table 3.2 were used as a reference genome (contaminant genome) to which the original quality trimmed reads were mapped (length fraction 0.5, similarity fraction 0.8). The unmapped reads were collected and de novo assembly (minimum contig length 500) into contigs. Contigs were then blasted (blastn or blastx for the no-significant-similarity-found contigs; e-value 1e-05 cut-off) using the program on the NCBI site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Only these contigs having significant hit with fungi were kept and the rust contigs which only had significant hits with the fungi in order Pucciniales were used for further analysis.

SNP prediction and inter-isolate comparisons

The rust contigs were combined and used as a reference for SNP detection by mapping the other five isolates' trimmed sequence data on this reference to detect any variants. Length fraction 0.95 and similarity fraction 0.95 were used in mapping, and other parameters were set as default. For the quality-based variant detection, all the parameters were set as default, with neighborhood radius 5, maximum gap and mismatch count 2, minimum neighborhood quality 15, minimum central quality 20, minimum coverage 10, minimum variant frequency (100%) 35.0, and maximum expected alleles 2. Variant comparison tables were produced and exported as CSV

files for further analysis. To infer phylogenetic relationships among these sequenced 6 isolates, the alignments with only homozygous SNPs (insertion/deletions were ignored) were filtered and created by using custom R scripts and the phylogenetic trees were drawn using MEGA7 (Tamura et al., 2013).

Phylogenetic analysis

The rust contigs were blasted, mapped and annotated using the Blast2Go software (Conesa et al., 2005). From the Blast2Go results, the 5.8S *rRNA* gene of switchgrass rust was used to determine the phylogenetic position in the order of Pucciniales. The 5.8S *rRNA* gene sequences of some common rust fungi were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/). The detailed information of these rusts is listed in Table 3.3. In addition, the 5.8S *rRNA* gene sequences of *Sclerotinia sclerotiorum* (Ascomycota) was also downloaded and used as an out-group. These DNA sequences were aligned with Clustal Omega (Sievers and Higgins, 2014) and phylogenetic analysis of DNA sequences was performed using Maximum Likelihood methods in MEGA7 (Tamura et al., 2013). One thousand bootstrap replicates were used to estimate the distance criteria for individual clades reported next to each branch.

Results

Next generation sequencing (NGS) of whole genomes of 6 switchgrass rust isolates were performed. In total, 394,165,854 100bp short sequence reads were generated (Table 3.4). The number of sequencing reads for each isolate varies from 57 Million to 78 Million. Totally, about 2% of the raw reads were trimmed to remove low quality reads (Table 3.4). After mapping all

trimmed reads to the contaminant genomes (Table 3.2), only about 33% to 47% of the raw reads of these 6 isolates were left which ranged from 19 Million to 32 Million.

High quality trimmed sequences were *de nono* assembled by CLC Genomic Workbench 9.0. From about 29 Million reads, 22.5 Million reads were matched and assembled into contigs while 6.5 Million reads were not matched. Totally 51,287 contigs were assembled, which have the N50 value of 2865 bp and a maximum contig size of 46,489 bp (Table 3.5). The average coverage of these contigs ranges from 1.56 to 1228.41, and the average coverage of all contigs is 27.08 (data not shown).

All these 51,287 contigs were blasted (blastn or blastx for blastn nonhits) through NCBI website and 9,359 contigs had significant hits with fungi. In these 9359 fungal contigs, 622 contigs (N50=1547; minimum length 506; maximum length 13312; total 768218 bp) had significant hits with rust fungi in order Pucciniales, with GC content 0.4733 (data not shown).

The sequence variation among the six switchgrass rust isolates was examined by aligning the trimmed sequence reads of the other five isolates to the 622 rust contigs of MS0909. Comparing with MS0909, the isolate NC01 varies the most with 671 variations, while isolate TN0926 has the smallest variation comparing with MS0909 with 33 variations (Table 3.6). Majorities of variation came from single nucleotide variants (SNVs), which range from approximately 91% to 93% of total variations among these isolates, while multiple nucleotide variants (MNVs) and insertion/deletions (indels) are small proportion of variation (Table 3.6).

In SNVs, the mutation changing from Adenine (A) to Guanine (G) accounts for 19% of total SNVs (Figure 3.2A), followed by mutations from Cytosine (C) to Thymine (T)(18%) and from Guanine (G) to Adenine (A) (17%). The mutation from Guanine (G) to Cytosine (C) accounts for the least amount of SNVs with 2%. In addition, the majority (69%) of the SNVs came from mutations changing within purines (R) or pyrimidires (Y). The proportion of SNVs changing from R to Y or from Y to R is 31% (Figure 3.2B). Furthermore, homozygous SNVs account for 18% while heterozygous SNVs have 82% of the total SNVs (Figure 3.2C).

The SNV among the six isolates was substantial. The number of SNVs shared by six isolates is 7, only 0.56% of the total SNVs (Figure 3.3). The number of unique SNVs of NC01 comparing with MS0909, which do not share with other four isolates, is 431, which account for 66.10% of NC01's total SNVs. AR01 had 109 unique SNVs, which accounts for 34.28% of its total SNVs. In isolate TN0803, AL-BL and TN0926 unique SNVs and percentage of their corresponding total SNVs were 50 & 24.63%, 15 & 20.55% and 8 & 24.24%, respectively (Figure 3.3, Table 3.6).

The phylogenetic tree was generated using the homozygous SNP data for the six isolates (Figure 3.4). The phylogenetic tree showed that MS0909 and TN0926 were in a clade derived from AL-SH and TN0803. The isolate NC01 was far away from the other isolates in the group.

The phylogenetic tree of 15 common rust species and 1 out-group specie was constructed using 5.8S *rRNA* gene. The out-group specie, *Sclerotinia sclerotiorum*, representing Ascomycota, was

grouped into different clades for 16 rust species. *Puccinia emaculata* and *Uromyces graminicola*, grouped in a clade derived from *P. sorghi*, were placed in Pucciniaceae.

Discussion

In our study, lots of sequence reads (more than 60%) were removed due to much unexpected contaminations from all kinds of different organisms. Mycoparasite, such as *Sphaerellopsis filum* (Driessen et al, 2004; Black, 2012), and host switchgrass tissues were expected to be in the raw sequence reads. However, this obligate biotrophic fungus is harder to deal with than necrotrophic fungi since it cannot reproduce on culture media.

Reference genome was first used rather than de novo assembly for the trimmed sequenced reads since some of the genomes were already sequenced in the same genera. However, only 20-30% of reads were mapped using *P. graminis* or *P. striiformis* as reference genomes to do the assembly (data not shown). In addition, using *P. striiformis* as the reference genome, raw *P. graminis* raw sequence reads, downloaded from ENA, were assembled. Not surprisingly, only 35% of the reads were mapped in the *P. striiformis* genome. This result suggests that only approximately 30% of the genome was shared in rust species, which probably used to maintain their complex five-spore stages life cycle, differentiate complicated infection structures (appressorium, infection hyphae, haustorial mother cell, haustoria, etc.), translate small secreted proteins, etc. The other parts of genome, which were not shared with other rusts, might be associated with some function such as their specific host-recognize system.

Up to date, 14 species in Pucciniales were sequenced and the genome assemblies were uploaded in NCBI genome database, which include 7 species from Puccinia, 3 species from Cronartium, 2 from Melampsora, 1 from Endocronartium and 1 from Uromyces genus (Table 3.7). The annotated genome size ranges from 22.13Mb (pine-oak rust) to 135.29Mb (wheat leaf rust). The average genome size of Basidiomycota fungi is 46.48 Mb while the average genome size of Ascomycota fungi is 36.91 Mb (Mohanta and Bae, 2015).

The GC contents of these 14 species in Pucciniales range from 31.2% (myrtle rust) to 52.4% (peanut rust). In our study, 622 contigs of rust were obtained, which can be considered random samples from the whole genome and the GC content (47.33%) of these contigs can be a good estimate of the GC content of switchgrass rust. In bacteria, GC content is positively correlated with genome/chromosome size (Nishida 2012). This relationship is also observed in some prokaryotes, such as aerobic, facultative species. For these 14 species in Pucciniales, the correlation (r) between genome size and GC content is -0.325 (p-value=0.257, data not shown).

The average density of SNVs in our 622 rust contigs is about 1.62 per kb and 0.057 per kb for indels. However, number of SNVs across six isolates varied significantly. About half of the SNVs came from isolate NC01, in which half of them were unique to NC01. Considering just the other four isolates, average SNV density is just 0.77 per kb and indels is 0.04 per kb. Compared with other rust species, average density of SNPs and indels of *P. triticina* (wheat leaf rust) is 2.4 and 0.32 kb, respectively (Kiran et al., 2016). For *P. graminis* (wheat stem rust), the average density of SNPs and indels is 12.3 and 1.9 per kb, respectively (Upadhyaya et al., 2015). For *P. striiformis* (wheat yellow rust), the average density of SNPs is 5.29 per kb (Cantu et al., 2013).

The SNPs density of *P. emaculata* in this study is significantly lower than the other rust species, and this prevented us from developing SNP markers to study the population genetics.

The phylogenetic relationship in rust species has been studied using several different genes, such as *5.8s rRNA* (Kenaley et al., 2016), *18S rRNA* (Swann and Taylor, 1995), *28S rRNA* (Maier et al., 2003), and *COX* gene (Liu and Hambleton 2012; Tan et al., 2014). Phylogeny analysis using the *5.8s rRNA* gene can clearly separate Melampsoraceae, Phakopsoraceae, Pucciniastreae, and Pucciniaceae in Pucciniales into four different clades (Figure 3.5) and place *P. emaculata* in the pucciniaceae clade alone with *Uromyces graminicola*. *Uromyces graminicola* can also cause rust disease on switchgrass, and the urediniospores of *U. graminicola* and *P. emaculata* are identical (Gravert and Munkvold, 2002). The only difference between these two species are their different teliospores. The teliospores of *U. graminicola* are one-celled while the teliospores of *P. emaculata* are two-celled (Kenaley et al., 2016). *P. emaculata* and *U. graminicola* were clustered together 100% of the 1000 bootstraps, which suggest that these two species are revolutionized more closely than other species in their corresponding genera.

The study was the first to explore the genome of *P. emaculata*. The sequences of random pieces of its gemone were provided and some genome divergence has been observed for these six isolates. However, as mentioned above, due to contaminations in sequence reads, limited resources were gained to provide more detailed genome infromation of *P. emaculata*. Further researches would be foucused on developing efficient methods to get clean genomic DNA, such as more gentlely brush the rust urediniospores from the host tissue, clean the urediniospores and

filter out microparasite spores etc. The sequences of clean genomic DNA can give us more information about the pathogen at the genome level.

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Appendix

Table 3.1 Host cultivars and collected locations of six switchgrass rust isolates which were used in the study.

Rust Isolate	Switchgrass Cultivar	Location
AL-BL	Badland	Brewton, AL
AR01	Dallas blue	Fayetteville, AR
MS0909	Alamo	Stoneville, MS
NC01	Heavy metal	Rougemont, NC
TN0803	Dewey blue	Knoxville, TN
TN0926	Alamo	Knoxville, TN

Table 3.2 List of significant-hit organisms and the resource where the genomes were acquired.

Organisms list	**Databases**
Acaryochloris marina	Ensembl Genomes
Achromobacter xylosoxidans	Ensembl Genomes
Acidiphilium multivorum	Ensembl Genomes
Acidovorax avenae	Ensembl Genomes
Actinosynnema mirum	Ensembl Genomes
Aeromicrobium erythreum	European Nucleotide Archive
Agrobacterium tumefaciens	Ensembl Genomes
Agromyces sp. AR33	European Nucleotide Archive
Alicycliphilus denitrificans	Ensembl Genomes
Alloactinosynnema sp.	Ensembl Genomes
Altererythrobacter atlanticus	European Nucleotide Archive
Aminobacter aminovorans	European Nucleotide Archive
Amycolatopsis lurida	European Nucleotide Archive
Anaeromyxobacter dehalogenans	Ensembl Genomes
Archangium gephyra	Ensembl Genomes
Azospirillum sp.	Ensembl Genomes
Blastochloris viridis	Ensembl Genomes
Blastococcus saxobsidens	Ensembl Genomes
Bordetella petrii	Ensembl Genomes
Bosea sp.	Ensembl Genomes
Bradyrhizobium sp.	Ensembl Genomes
Brevundimonas sp.	Ensembl Genomes
Brugia timori	Ensembl Genomes
Burkholderia ambifaria	Ensembl Genomes
Castellaniella defragrans	Ensembl Genomes
Caulobacter segnis	Ensembl Genomes
Cellulomonas fimi ATCC 484	Ensembl Genomes
Chelatococcus sp. GW1	European Nucleotide Archive
Chlorella variabilis	Ensembl Genomes
Chryseobacterium sp.	Ensembl Genomes
Citromicrobium sp. JL1351	Ensembl Genomes
Clavibacter michiganensis	Ensembl Genomes
Clostridium botulinum	European Nucleotide Archive
Collimonas fungivorans	Ensembl Genomes
Comamonas testosteroni	Ensembl Genomes
Conexibacter woesei	Ensembl Genomes
Corynebacterium epidermidicanis	Ensembl Genomes
Croceicoccus naphthovorans	Ensembl Genomes
Cupriavidus nantongensis	European Nucleotide Archive

Table 3.2 Continued

Organisms list	Databases
Curtobacterium flaccumfaciens UCD-AKU	Ensembl Genomes
Cyanobium gracile	Ensembl Genomes
Cyprinus carpio	European Nucleotide Archive
Desulfosporosinus acidiphilus	Ensembl Genomes
Devosia sp. H5989	Ensembl Genomes
Diphyllobothrium latum	European Nucleotide Archive
Dyadobacter fermentans	Ensembl Genomes
Dyella thiooxydans	Ensembl Genomes
Eimeria acervulina	Ensembl Genomes
Ensifer adhaerens strain Casida A	European Nucleotide Archive
Flavisolibacter sp.	Ensembl Genomes
Frankia sp. EuI1c	Ensembl Genomes
Haliangium ochraceum DSM 14365	Ensembl Genomes
Isoptericola variabilis 225	Ensembl Genomes
Kineococcus radiotolerans SRS30216	Ensembl Genomes
Klebsiella oxytoca	Ensembl Genomes
Kribbella flavida DSM 17836	Ensembl Genomes
Leifsonia xyli	Ensembl Genomes
Lysobacter gummosus	Ensembl Genomes
Marichromatium purpuratum 984	Ensembl Genomes
Massilia sp.	Ensembl Genomes
Mesorhizobium huakuii	Ensembl Genomes
Methylibium petroleiphilum	Ensembl Genomes
Methylobacterium nodulans	Ensembl Genomes
Microbacterium mangrovi	Ensembl Genomes
Microbacterium testaceum StLB037	Ensembl Genomes
Microbacterium trichothecenolyticum	Ensembl Genomes
Microlunatus phosphovorus NM-1	Ensembl Genomes
Microterricola viridarii	European Nucleotide Archive
Mitsuaria sp.	Ensembl Genomes
Modestobacter marinus	Ensembl Genomes
Mycobacterium bovis	Ensembl Genomes
Mycobacterium intracellulare 1956	Ensembl Genomes
Myxococcus hansupus	Ensembl Genomes
Neorhizobium galegae	Ensembl Genomes
Niabella soli DSM 19437	Ensembl Genomes
Nocardia thailandica NBRC 100428	Ensembl Genomes
Nocardioides dokdonensis FR1436	Ensembl Genomes
Nostoc sp.	Ensembl Genomes

Table 3.2 Continued

Organisms list	Databases
Novosphingobium aromaticivorans	Ensembl Genomes
Paenibacillus durus	Ensembl Genomes
Panicum virgatum	DOE JGI
Paracoccus denitrificans	Ensembl Genomes
Phenylobacterium zucineum	Ensembl Genomes
Physarum polycephalum	Ensembl Genomes
Planctomyces sp	Ensembl Genomes
Porphyrobacter neustonensis	Ensembl Genomes
Pseudomonas aeruginosa	Ensembl Genomes
Pseudomonas brassicacearum	Ensembl Genomes
Pseudomonas oryzihabitans	Ensembl Genomes
Pseudoxanthomonas suwonensis	Ensembl Genomes
Ralstonia eutropha H16	Ensembl Genomes
Ramlibacter tataouinensis TTB310	Ensembl Genomes
Rathayibacter tritici strain NCPPB 1953	Ensembl Genomes
Rhizobium leguminosarum bv. trifolii WSM1325	Ensembl Genomes
Rhizobium tropici	Ensembl Genomes
Rhodobacter sphaeroides	Ensembl Genomes
Rhodopseudomonas palustris	Ensembl Genomes
Rubrivivax gelatinosus	Ensembl Genomes
Saccharothrix esanaensis	Ensembl Genomes
Sanguibacter keddieii DSM 10542	Ensembl Genomes
Shinella sp. DD12	Ensembl Genomes
Sinorhizobium fredii USDA 257	Ensembl Genomes
Solirubrobacter soli DSM 22325	European Nucleotide Archive
Sorangium cellulosum	Ensembl Genomes
Sphingobium sp. YBL2	Ensembl Genomes
Sphingomonas taxi	Ensembl Genomes
Sphingopyxis macrogoltabida	European Nucleotide Archive
Sphingopyxis terrae	Ensembl Genomes
Stenotrophomonas acidaminiphila	Ensembl Genomes
Stenotrophomonas maltophilia	Ensembl Genomes
Streptomyces albulus	Ensembl Genomes
Streptomyces fulvissimus	Ensembl Genomes
Streptomyces laurentii	European Nucleotide Archive
Streptomyces parvulus strain 2297	European Nucleotide Archive
Strongyloides stercoralis	Ensembl Genomes
Thermobispora bispora	Ensembl Genomes
Tistrella mobilis	Ensembl Genomes

Table 3.2 Continued

Organisms list	**Databases**
Variovorax paradoxus	Ensembl Genomes
Xanthomonas campestris	Ensembl Genomes
Xanthomonas sacchari	Ensembl Genomes
Xanthomonas translucens	Ensembl Genomes

Table 3.3 List of species, the common names and the gene bank accession numbers of their 5.8*S rRNA* sequences from which phylogenetic analysis was generated.

Organisms list	Common name	Gene bank accession
Cronartium ribicola	White pine blister rust	KF387533
Gymnosporangium Juniperi-virginianae	Cedar-apple rust	KR815879
Hemileia vastarix	Coffee leaf rust	EF394132
Melampsora larici-populina	Popular rust	EU014067
Phakopsora pachyrhizi	Soybean rust	EU436723
Puccinia coronata	Barley crown rust	AY573916
Puccinia graminis	Wheat stem rust	HQ317578
Puccinia hemerocallidis	Daylily rust	AF479739
Puccinia hordei	Barley leaf rust	HQ317527
Puccinia kuehnii	Sugarcane orange rust	GU443947
Puccinia melanocephala	Sugarcane rust	FJ009327
Puccinia polysora	American corn rust	HM452902
Puccinia sorghi	Maize common rust	HQ154038
Puccinia striiformis	Wheat stripe rust	JN575605
Puccinia triticina	Wheat leaf rust	HQ317544
Sclerotinia sclerotiorum	Soybean white mold	KT970793
Uromyces appendiculatus	Bean rust	AB115741
Uromyces graminicola	None	KR264701

Table 3.4 statistics for raw sequencing, trimming and mapping contaminant genomes.

Isolates	# of Raw reads	#of Reads after trim and % of raw reads	#of Reads not mapping contaminant genomes and % of raw reads
AL-SH	71,320,412	70,153,231 (98.36%)	24,166,616 (33.88%)
AR01	67,597,628	66,449,119 (98.30%)	30,778,562 (45.53%)
MS0909	61,197,856	60,184,652 (98.34%)	29,070,083 (47.50%)
NC01	57,813,288	57,044,421 (98.67%)	20,306,893 (35.13%)
TN0803	78,692,338	75,724,398 (96.23%)	32,463,604 (41.25%)
TN0926	57,544,332	56,709,656 (98.55%)	19,955,393 (34.68%)
Total	394,165,854	386,265,477 (98.00%)	156,741,151 (39.77%)

Table 3.5 Genome *de nono* assembly results for switchgrass rust isolate MS0909 using CLC Genomic Workbench.

Reads	29,070,083
Matched	22,533,692
Not Matched	6,536,391
Total # of Contigs	51,287
N50 (bp)	2865
Max Contig (bp)	46,489
Total Length (bp)	83,048,578

Table 3.6 Sequence variation between five switchgrass rust isolates (AL-BL, AR01, NC01, TN0803, and TN0926) and isolate MS0909, including total number of variations, the number and percentage of single nucleotide variants (SNVs), the number of multiple nucleotide variants (MNVs) and the number of insertion/deletions (indels).

VS. MS0909	Variation	SNV (% of total)	MNV	indel
AL-BL	73	67 (91.78%)	0	6
AR01	318	306 (96.23)	3	9
NC01	671	652 (97.17%)	4	15
TN0803	203	187 (92.12%)	4	12
TN0926	33	31 (93.94%)	0	2
Total	1298	1243 (95.76%)	11	44

Table 3.7 Sequenced and genome assembled species in Pucciniales in NCBI genome database with their corresponding common names, genome sizes and GC contents.

Species	Common name	Genome size (Mb)	GC content (%)
Cronartium comandrae	Comandra blister rust	68.61	40.2
Cronartium quercuum	Pine-oak rust	22.13	41.1
Cronartium ribicola	White pine blister rust	94.33	41.4
Endocronartium harknessii	Pine-pine gall rust	56.94	40.9
Melampsora larici-populina	Popular leaf rust	101.13	41
Melampsora pinitorqua	Pine twisting rust	34.03	44.9
Puccinia arachidis	Peanut rust	87.61	52.4
Puccinia graminis	Stem rust	88.72	43.3
Puccinia horiana	Chrysanthemum white rust	66.4	43.2
Puccinia psidii	Myrtle rust	129.78	31.2
Puccinia sorghi	Common maize rust	99.53	44.8
Puccinia striiformis	Wheat yellow rust	117.39	46
Puccinia triticina	Wheat leaf rust	135.29	36.8
Uromyces viciae-fabae	Faba bean rust	100.06	35.7

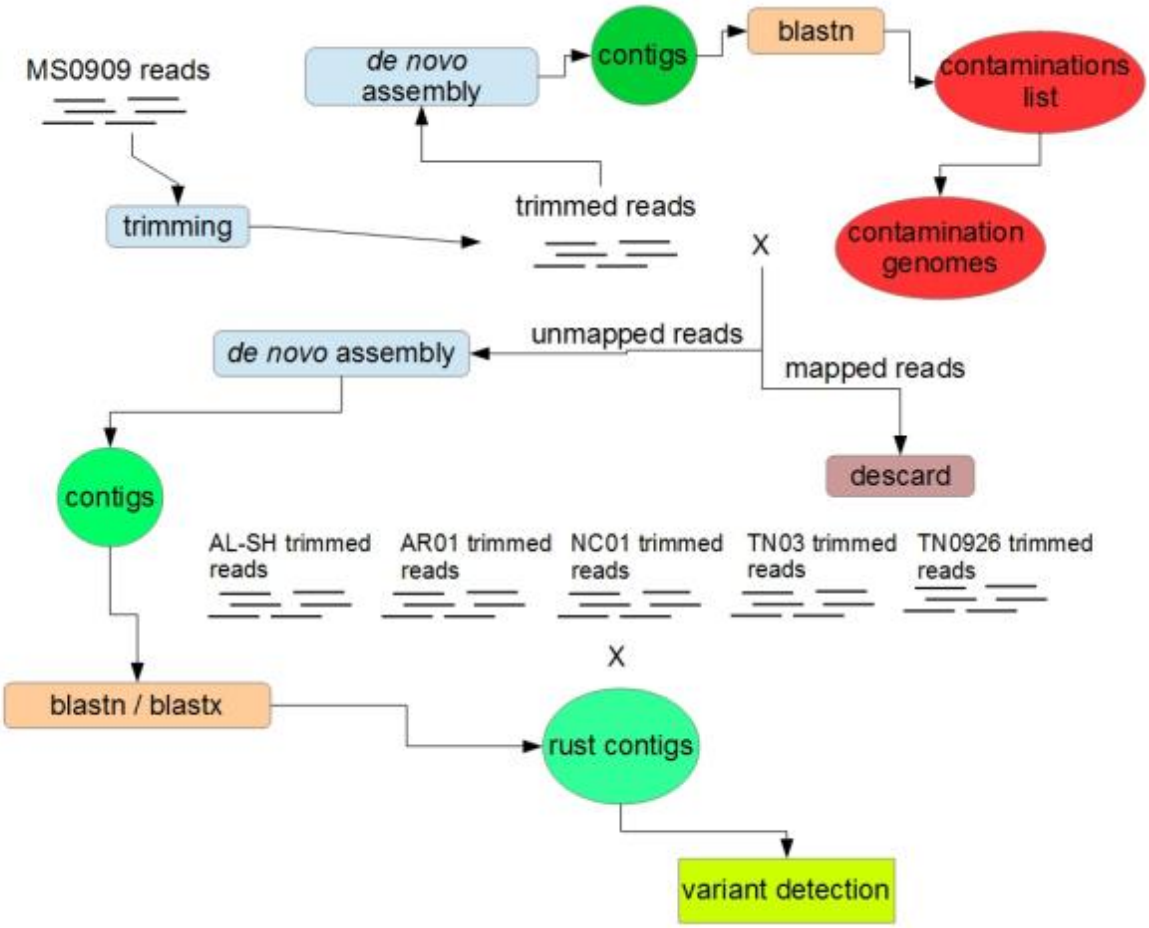

Figure 3.1 Flow chart diagrams illustrating genome assembly pipelines.

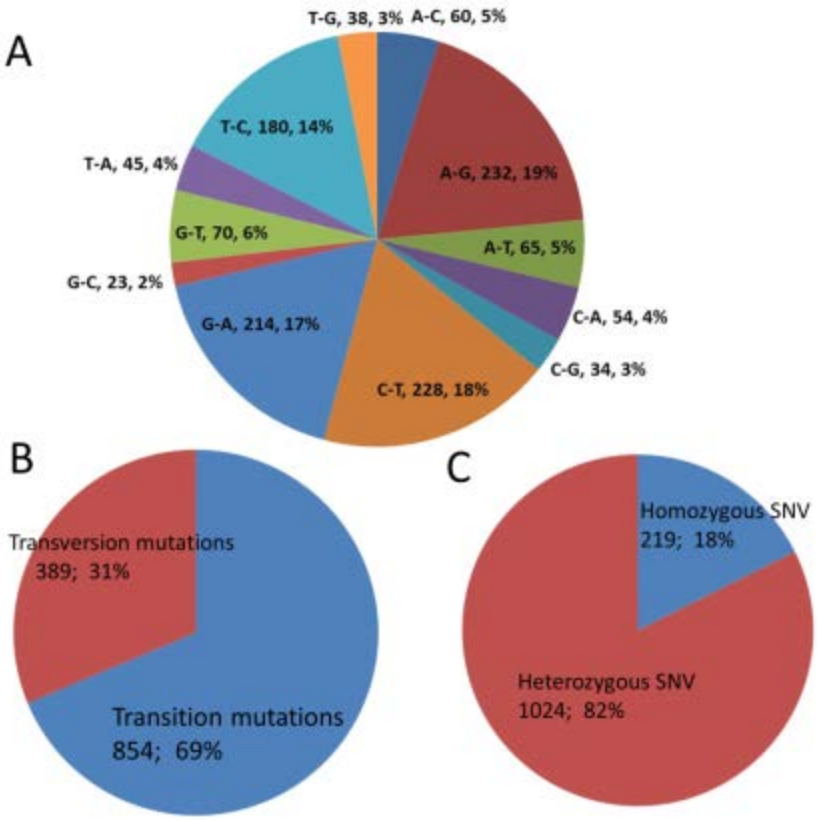

Figure 3.2 Types of single nucleotide variants (SNVs) among six switchgrass rust isolates. A, number and percentage of specific SNV of total SNV, which changes from one nucleotide (right) to another nucleotide (left). B, number and percentage of SNVs transition mutations (changing within purines (R) or pyrimidires (Y)) or transversion mutations (changing between purines and pyrimidires). C, Proportion and number of homozygous and heterozygous SNV. A: adenine; C: cytosine; G: guanine; T: thymine.

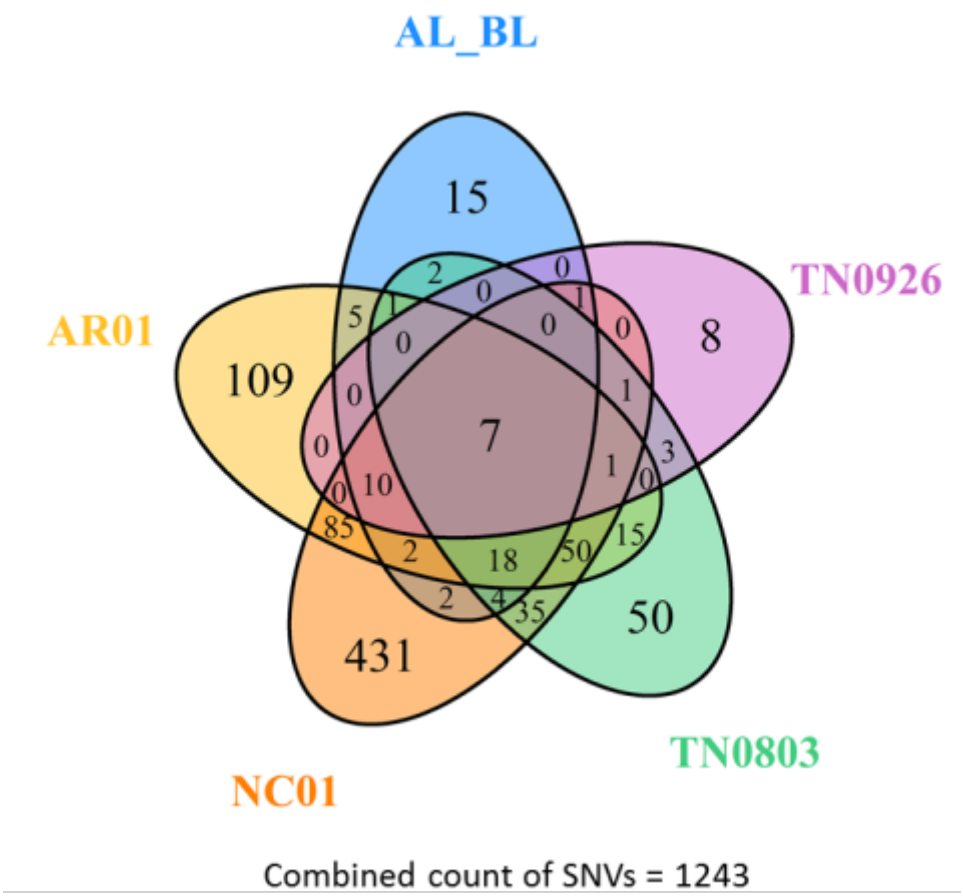

Figure 3.3 Venn diagram showing the number of shared/unique SNVs detected in six *Puccinia emaculata* isolates (AL-BL, AR01, NC01, TN0803, and TN0926 with MS0909).

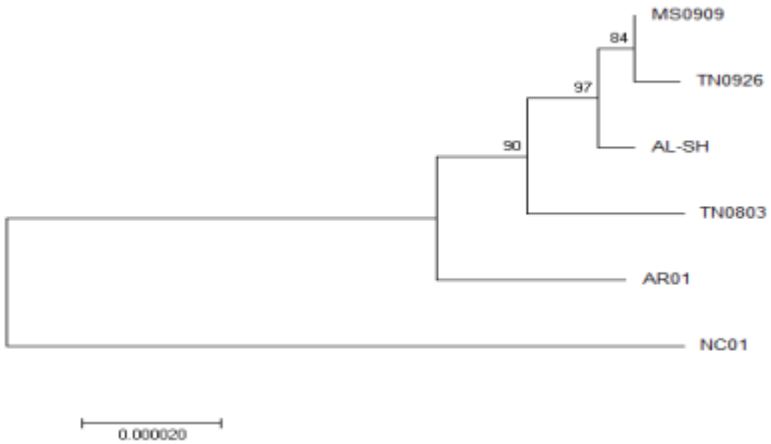

Figure 3.4 Phylogenetic tree of six *Puccinia emaculata* isolates (AL-SH, AR01, MS0909, NC01, TN0803, and TN0926). Local bootstrap support values are shown.

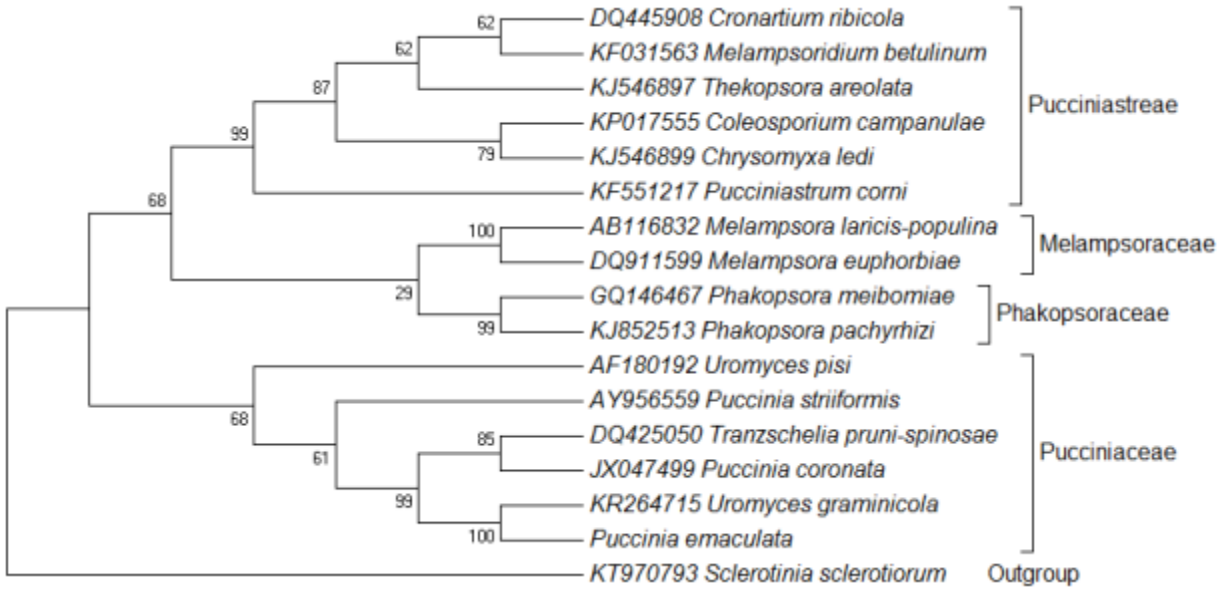

Figure 3.5 A molecular phylogenetic tree generated using maximum likelihood method based on the Tamura-Nei model with 1000 bootstrap replicates in program MEGA 7.0 for the partial 5.8S *rRNA* gene sequence from 16 different rusts including switchgrass rust and 1 out group (*Sclerotinia sclerotiorum*) with their corresponding Genebank accession numbers.

Chapter 4. Population genetic structure of *Puccinia emaculata* in Southeastern United States

Abstract

As the production of switchgrass increased as a biofuel biomass, switchgrass rust (*Puccinia emaculata*) has been drawn attention because of its widely distribution across US and reductions of biomass when disease severity is high. In this study, 22 microsatellite loci were used to analyze and determine the genetic relationship among switchgrass rust isolates collected from seven locations across southeastern US. Linkages among loci were observed among all seven subpopulations with standardized index of association significantly different from 0 ($p < 0.01$), indicating no sexual recombination in these locations. Moderate to high genetic differentiation were discovered among these subpopulations which demonstrated multiple introductions of genotypes of *P. emaculata* introduced to multiple areas.

Introduction

Switchgrass (*Panicum virgatum* L) is a warm-season perennial grass native to North America. The native range of switchgrass includes most of the continental United States (exceptions California, Oregon and Washington) (USDA, 2006). Traditionally, switchgrass has been used for bank stabilization as ornamental and as a forage crop (Lemus et al., 2002). Due to its C4 photosynthetic pathway, switchgrass has been designated as a sustainable bioenergy crop for ethanol and butanol (Bouton 2008). For its minimum fertilizer input requirement, switchgrass showed most potential biomass species among other perennial grasses and legumes including sorghum (Cherney et al., 1991; Wright, 2007). Average annual biomass yields from switchgrass fields 5.2 to 11.1 $Mg \cdot ha^{-1}$ which produces an average estimated net energy yield of 60 $GJ \cdot ha^{-1}$ (Schmer et al., 2008).

Many fungal diseases have been reported for switchgrass including rust, smuts, leaf spots, crown and root rots (Gravert and Munkvold, 2002). Although eighty-one species of fungi have been reported to cause disease on switchgrass in the United States (Farr and Rossman, 2009), most of them have little economic impact. Diseases reported to reduce switchgrass biomass are head smut (*Tilletia maclaganii*) (Thomsen et al., 2008), anthracnose (*Colletotrichum navitas*) (Crouch et al., 2009), and switchgrass rust (Hirsch et al., 2010; Zale et al., 2008). A new study found that the estimated ethanol production can be reduced by up to 55% due to severity infection of switchgrass rust (Sykes et al., 2016).

Switchgrass rust (*Puccinia emaculata*) infection of switchgrass has been reported in Alabama (Hagan and Atridge, 2013), Arkansas (Hirsch et al., 2010), Tennessee (Zale et al., 2008) and Virginia (Frazier et al., 2013). The symptom of disease was leaf chlorosis. Uredia were formed underneath adaxial epidermal cells. Urediniospores were globose or oval in shape and approximately 27 x 25 µm and were the primary source of inoculum for secondary disease cycle. On water agar, urediniospores can germinate one hour after inoculation (HAI) and after 2 or 3 HAI, hyphae elongate, branch, and form appressoria (Li et al., 2009). On switchgrass leaves, appressoria formed over stomata and penetrated through stomatal openings. In later summer to fall, dark brown telia can be observed on infected leaves. Teliospores are two-celled and approximately 33.6 µm ± 4.8 in length, and the width of apical and basal cells are 17.5 µm ± 1.2 and 15.9 µm ± 2.5, respectively (Zale et al., 2008).

Understanding the characteristics of *P. emaculata* population structure is vital for developing an effective and sustainable approach to the management of switchgrass rust. It is imperative to gain

more insight into population structure of this fungus such as population differentiation and gene flow, which will assist in developing resistant switchgrass cultivars and eventually reduce crop losses. However, up to date, other than some microsatellite markers developed by one paper (Wadl et al., 2011) and one Masters' thesis (Orquera Delgado, 2014), no study has worked on the population structures of *P. emaculata*. Therefore, the main objective of this study was to evaluate genetic structure of *P. emaculata* populations in Southeastern US in order to determine (i) the level of genetic diversity present in each examined field across different geographical locations, (ii) whether genetic recombination occurred among *P. emaculata* subpopulations, and (iii) putative origin of *P. emaculata* in Southeastern US.

Material and Methods

Fungal Isolates

Fifty-two isolates of *P. emaculata* collected from 2008-2016 across Southeastern US were used in the study (Table 4.1). To ensure the purity of these isolates, a single pustule from each sample was isolated and used to collect urediniospores to inoculate leaf pieces (cv. Thundercloud) that were laid on moist filter paper (7cm in diameter, Fisher Scientific, Hampton, NH) in a Petrie dish. Cultures were incubated in a temperature controlled laboratory at 20 ± 2ºC with continuous light (four 40W residential fluorescent bulbs from 45 cm above leaf segments). Fresh leaf segments (3 cm in length) were inoculated and replaced at 14-day intervals. Urediniospores were harvested with sterilize blades and stored in the freezer (-20 ºC) until used in population study.

Genomic DNA Extraction

Urediniospores, collected from each of the above 52 single-pustule derived isolates, were harvested for genomic DNA extraction. Genomic DNA purification kit (Thermo Scientific, Waltham, MA) was used according to manufacturer's instructions with minor modifications. The concentration and quality of extracted DNA were measured by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE) and was stored at -20°C.

Verification of *Puccinia emaculata*

Internally Transcribed Spacer Regions (ITS) of ribosomal DNA of 52 *P. emaculata* isolates were amplified using polymerase chain reaction (PCR) and commercially sequenced to species identity. The PCR reaction was conducted in 25 µl reaction volume containing: 2 µl of genomic DNA (about 8 ng), 10 µl of Go-Taq master mix (Promega, Madison, WI), 1.5 µl each of 10 µM ITS1rustF10d (5' TGAACCTGCAGAAGGATCATTA 3') (Barnes & Szabo 2007; Kenaley et al. 2016) and RUST1 (5' GCTTACTGCCTTCCTCAATC 3') (Kropp et al. 1995; Kenaley et al. 2016), 1.5 µl of dimethyl sulfoxide (DMSO), and 8.5 µl of sterile distilled water. PCR reaction was performed in thermo cycler (Eppendorf AG, Hamburg, Germany) with the following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 120 s, and a final extension at 72°C for 10 min (Kenaley et al., 2016). Resulting PCR products was visualized with 1% agarose gel using a 2000 Gel Documentation System (BIO-RAD, Hercules, CA). Following PCR amplification, samples were sent to molecular cloning laboratories (MCLAB, San Francisco, CA) for cleaning and sequencing. The sequencing results were then compared using Basic Local Alignment Search Tool (BLAST) in GenBank database from the

National Center for Biotechnology Information (NCBI) website. All samples were confirmed to as *P. emaculata*.

Microsatellite Primers Validation and Isolates Genotyping

Ten co-dominant microsatellite primers designed by Wadl et al. (2011) and twenty designed by Orquera Delgado (2014) were chosen and used to genotype *P. emaculata* isolates (Table 4.2). The primers, manufactured by Integrated DNA Technologies (IDT, Corralville, IA), were diluted to 10 µM for further analyses. Initially, four isolates were randomly chosen to validate all 30 microsatellite primers. Ten microliter PCR reactions were conducted using 1 µl of genomic DNA (4 ng), 4 µl of Go-Taq master mix (Promega, Madison, WI), 1 µl each of primers, 0.5 µl of dimethyl sulfoxide (DMSO), and 2.5 µl of sterile distilled water under the following conditions in thermo cycler: 94°C for 5 min followed by 35 cycles of 94°C for 40 s, at the locus specific annealing temperature for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were then analyzed on the QIAxcel Capillary Electrophoresis System (QIAGEN, Valencia, CA) using an internal 25 to 300 bp size standard.

Data Analysis

Raw allele length data generated by QIAxcel Capillary Electrophoresis System were statistically binned into allelic classes using Microsoft Excel Macro FLEXIBIN v2 (Amos et al., 2007). A ± 2-3 bp allelic category size was used to determine the allele size classes, which were used to in all subsequent analyses.

Clone correction of *P. emaculata* isolates were analyzed using a package "poppr" (Kamvar et al., 2015) in R (R Core Team, 2016), from which unique multilocus genotypes (MLGs) were identified. The average number of alleles per locus (N_a), the effective number of alleles (N_e), expected (H_e) and observed heterozygosity (H_o), the probability of identity (PI), polymorphic information content (PIC), tests for Hardy-Weinberg equilibrium (HWE), pairwise subpopulation genetic divergence, and geneflow were analyzed by another Microsoft Excel Macro GenAlEx v6.5 (Peakall and Smouse, 2012). To visualize the genetic distance, a principal coordinate analysis (PCoA) and an unweighted pair group method with an arithmetic mean (UPGMA) dendrogram were produced with "poppr" package in R using Bruvo's method (Bruvo et al., 2004). The standardized index of association ($\bar{r}_d$) (Agapow and Burt, 2001) was also estimated with "poppr" package in r using 10,000 permutations to test if alleles were linked across loci.

For the analysis of population structure, two Bayesian approaches were used. Structure v2.3.4 (Pritchard *et al*., 2000) program was utilized using 20 of K (1-20) with 30 independent runs and a burn-in period of 200,000 followed by 600,000 Markov Chain Monte Carlo (MCMC) repetitions. Other parameters were set as the default. Structure Harvester web v0.6.94 (Earl 2012) was used to estimate the most probable value of K (number of clusters) using Evanno's method for estimating delta K (Evanno et al., 2005). Data was visualized with Pophelper web v1.0.10 (Francis 2016).

When the overall F_{ST} is small, Structure tends to identify the number clusters of population structure incorrectly (Chen et al. 2007; Latch et al. 2006). Tess (Durand et al. 2009), which

performs well with small F_{ST} values by using spatial information to assist identify population structure (Chen et al. 2007), was then used to analysis the population structure. In TESS, 10,000 interactions with 20 replicates for each K value between 2 to 15 were done with all other parameters set to default. To estimate number of clusters, the K with minimum Deviance Information Criterion (DIC) value was selected (Caye et al., 2016). Data were then plotted and visualized in R.

In order to measure the genetic variation of individuals within and among subpopulations, analysis of molecular variance (AMOVA) was performed using Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). Two variance partitions were used for each AMOVA run: 1) all 52 isolates from the 7 subpopulations were placed in one hierarchical group, 2) *P. emaculata* isolates were grouped into clusters based on the TESS results. The genetic differentiations and the hierarchical partitions between, among and within subpopulations was generated.

Program BOTTLENECK version 1.2.02 (Cornuet and Luikart, 1996) was used to evaluate if P. emaculata subpopulations have undergone recent population bottleneck or expansion (population's size has reduced or expanded). Data was analyzed using cluster assignment based on TESS findings. Sign test was used to detect any significant excess or deficit in gene diversity (Cornuet and Luikart, 1996) under the three default evolving model: infinite allele model (IAM), stepwise mutation model (SMM), and two-phase mutation model (TPM) using default settings with 10,000 permutations.

Results

A single band of about 850 bp (expected size) was amplified using ITS1rustF10d and RUST1 primers for all of the 52 *P. emaculata* isolates. These amplicon sequences were matched with *P. emaculata* (Accession Number: EU915294) with 0.0 E-value in the NCBI database. Therefore, all 52 isolates were confirmed as *P. emaculata* using both morphological and molecular tools.

From 30 original primers, 22 primers successfully amplified across four randomly chosen *P. emaculata* isolates (Table 4.2). Selected microsatellite primers were used in further population genetics analyses. Across 22 microsatellite loci, the total number of alleles was 91, with an average of 4.14 per locus varying from 2 to 7 (Table 4.3). The effective number of alleles per locus varied from 1.0 to 3.1 (Table 4.3). Across all subpopulations for each of the 22 loci, the average observed heterozygosity (H_o) was 0.23, ranging from 0 to 0.86. The average expected heterozygosity (H_e) was 0.41, ranging from 0.04 to 0.66. The proportion of total genetic diversity attributed to different subpopulation ranged from 0.04 to 0.42 for the 22 loci, with an overall average of 0.18 (Table 4.3), which indicated moderate to high genetic differentiation across all loci with limited gene flow (N_m= 1.853) (Table 4.3).

Among 52 rust isolates in 7 subpopulations, 52 unique multilocus genotypes (MLGs) were identified (100%) (Table 4.4). The number of private alleles ranged from 0 for the GA and NC subpopulation to 10 for the AL2 subpopulation. Shannon's information index of allelic diversity (*I*) ranged from 0.44 (NC subpopulation) to 0.83 (AL1 subpopulation). For all the subpopulations, observed heterozygosity (H_o) was significantly smaller than expected heterozygosity (H_e), which indicated probable population structures among these subpopulations.

In addition, NC subpopulation and GA subpopulation were mostly genetically divergent from each other with highest F_{ST} value 0.2 among seven subpopulations; AL1 subpopulation and TN subpopulation were least genetically divergent from each other with F_{ST} value 0.03 (Table 4.5). Furthermore, the gene flow among seven subpopulations varied from 1.0 to 9.76 with gene flow between NC and GA subpopulation the least 1.0 and gene flow between AL1 and TN subpopulation the largest 9.752 (Table 4.6). These results matched the results provided by pairwise population differentiation (F_{ST}) between populations because when populations are more likely to be genetically different, the gene flow between populations tends to be smaller.

The temporal dynamics of *P. emaculata* were investigated by comparing isolates in AL1 and AL2, collected from the same location but in 2012 and 2016, respectively. Small population differentiation was observed between these two subpopulations (F_{ST} =0.09) (Table 4.5). However, the number of private alleles in AL2 (10) is significantly higher than AL1's (1) (Table 4.4).

We further tested whether the populations were in linkage disequilibrium, which would be expected due to linkage among loci, indicating clonal reproduction. Significant linkage among loci were observed among all the seven subpopulations ($p<0.01$) (Figure 4.1) with standardized index of association ($\bar{r}_d$) ranging from 0.09 to 0.47, significantly differed from zero, which indicated that there were no sexual mating in these sampled subpopulations (locations) and supporting the hypothesis that switchgrass rust reproduced asexually (could be clonally) in southeastern US.

STRUCTURE results suggested five clusters (K=5) based on ΔK criterion (Evanno et al., 2005) (Fig 4.2). These five clusters were admixed among original seven subpopulations. From K=4 to K=5, STRUCTURE defined one individual each from AL1 subpopulation (AL-PS) and TN subpopulation (TN0918) to form the fifth cluster (Fig 4.3). However, TESS indicated four clusters (K=4) based on its minimum DIC value (Fig 4.4). Based on our biological knowledge, there is no reason to simply split AL-PS and TN0918 out to form a new cluster in STRUCTURE when K=5. Therefore, four clusters (K=4) were chosen for further analysis. The genetic groupings did not correspond with the geographical sampling origin. Some isolates from AL1, AL2, GA, TN and SC subpopulations were grouped into cluster 1 (red) (Fig 4.5) with no isolates coming from NC and ALM (Arkansas-Louisiana-Mississippi) subpopulations. Some isolates from AL1, AL2, GA, TN and NC subpopulations were grouped into cluster 2 (yellow) (Fig 4.5) with no isolates coming from SC and ALM (Arkansas-Louisiana-Mississippi) subpopulations. Cluster 3 (blue) consisted from some isolates from ALM, AL2, GA, NC and SC with no AL and TN isolates. Cluster 4 (pink) consisted of isolates from ALM, AL2, SC and TN with no AL, GA and SC isolates.

The neighbor-joining UPGMA dendrogram, based on Bruvo's genetic distance among 52 isolates, indicated similar grouping patterns as TESS (Figure 4.6). However, for the clusters suggested by STRUCTURE, two isolates "TN0918" and "AL-PS", grouped in cluster 1 in TESS, formed a new cluster by themselves (Figure 4.6). All genetic clusters were supported with bootstrap value 70.

Analysis of molecular variance (AMOVA) performed on the original seven subpopulations indicated that 11.83% and 88.57% of the genetic variation was attributed to variations among and within subpopulations respectively (F_{ST}= 0.14; $p < 0.01$) (Table 4.7), which indicated that most of the genetic variation (88.57%) was individually based rather than among subpopulations. Further investigation of the differentiation among the four clusters generated by TESS reviewed that 32.82% of the variation were explained among subpopulations ($p < 0.01$), 25.65% among individuals within subpopulations ($p < 0.01$), and 41.53% individually based ($p < 0.01$) (F_{IS}= 0.38; F_{IT}= 0.58; F_{ST}= 0.33). The high values of F_{IS} and F_{IT} revealed the relative high genetic inbreeding and heterozygosity reduction within subpopulations because of non-random mating.

The BOTTLENECK was used to test whether a recent population bottleneck/founder events or population expansion occurred. During a bottleneck event, heterozygosity decline less than allelic diversity because bottleneck tends to eliminate out low-frequency alleles, which leads to heterozygosity excess (Allendorf, 1986; Nei et al., 1975). For the seven subpopulations, significant bottleneck effects were observed in GA, NC, and SC subpopulations under three mutation models with significant excess of heterozygosity with sign test (Table 4.8). AL1 and TN subpopulations didn't result in significant bottleneck while ALM and AL2 populations only have bottleneck effect under infinite allele model (Table 4.8).

Discussion

Understanding genetic variation in *P. emaculata* is fundamental and essential to develop new cultivars of switchgrass with resistance to this pathogen. As the first study to look into the population genetics of switchgrass rust, 52 *P. emaculata* isolates collected from seven locations

across Southeastern US were studied with 22 highly informative microsatellite primers to understand the genetic diversity, gene flow and epidemiology of switchgrass rust. Moderate to high genetic differentiation and limited gene flow were observed among subpopulations and high heterozygosity and admixture were found within subpopulations.

Probably due to its long-distance dispersal ability, the southeastern switchgrass rust subpopulations do not have a clear genetic structure and cannot be separated into groups based on areas or collecting years. The similar results were found for some other long-dispersal pathogens when the subpopulations were collected in a small scale such as from a few states/provinces or in a single country (Admassu et al., 2010). However, for the intercontinental collections, significant high genetic differentiations would be found among subpopulations and these subpopulations can be grouped by geographic origins, despite the long-distance dispersal ability (Ali et al., 2014; Brown and Hovmøller, 2002; Hovmøller et al., 2011; Thach et al., 2016).

Although teliospores were produced on leaves at the end of season and would germinate to form basidiospores, there is no report of basidiospores infecting an alternate host. This study revealed that no genetic recombination between molecular markers as the standardized index of association were significantly different from zero. This strongly indicated there is no sexual reproduction for *P. emaculata* in these sampling sites, from South Alabama to Tennessee. Probably like wheat leaf rust (Kolmer, 2005), *P. emaculata* overwinters in Mexico and throughout US in each spring by wind. However, this possibility should get direct experimental support by collecting and analyzing more samples from the regions across whole North America continent, especially Mexico and USA.

Moderate to high genetic differentiations (pairwise F_{ST} ranged from 0.025 to 0.199) were found among these *P. emaculata* subpopulations across Southeastern US, which suggested that multiple *P. emaculata* genotypes were introduced into multiple areas. The moderate to high genetic diversity within subpopulations is likely to be an outcome of the susceptibility of most switchgrass cultivars to *P. emaculata*. The similar pattern was also found in the high genetic diversity of *P. graminis* subpopulation in Australia (Keiper et al., 2005). In addition, the high heterozygosity within subpopulations was detected, which is like to reflect the host adaption and the genetic diversity of switchgrass cultivars in these collection sites.

In conclusion, the results of the present work illustrate the population structure of *P. emaculata* in Southeastern US. The results suggested for the first time that no sexual recombination for *P. emaculata* occurred in Southeastern US and there are multiple origins for the pathogen introducing to this area. The highly genetically diverse pathogen structure would affect the durability of resistant genes deployed in switchgrass germplasms and emphasizing the requirement of different resistant resources for switchgrass breeding.

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Appendix

Table 4.1 *Puccinia emaculata* isolates used in population genetics study. Cultivars (bold) are clonal- nursery derived plants.

Population Code	Rust Isolate	Switchgrass Cultivar	Location	Collected Year
ALM	AR-01	**Dallas Blue**	Fayetteville, AR	2008
ALM	LA-0902	**Dallas Blue**	Mobile, AL	2009
ALM	MS-09099	Alamo	MSU research plot, MS	2009
ALM	MS-0911	**Cycle 2**	MSU research plot, MS	2009
NC	NC-01	**Heavy Metal**	Hoffman Nursery, NC	2008
NC	NC-02	**Dallas Blue**	Hoffman Nursery, NC	2008
NC	NC-03	**Northwind**	Hoffman Nursery, NC	2008
NC	NC-05	**Cloud Nine**	Hoffman Nursery, NC	2008
TN	TN03	**Dewey Blue**	Knoxville, TN	2008
TN	TN 10-4	Alamo	Madisonville, TN	2010
TN	TN 10-5	Alamo	Madisonville, TN	2010
TN	TN 10-6	Alamo	Madisonville, TN	2010
TN	TN-0918	**Northwind**	Knoxville, TN	2009
TN	TN-0919	**Dallas Blue**	Knoxville, TN	2009
TN	TN-0920	**Thundercloud**	Oak Ridge, TN	2009
TN	TN-0921	Alamo	Knoxville, TN	2009
TN	TN-0922	**Rotstrahlbusch**	Crossville, TN	2009
TN	TN-0923	**Dallas Blues**	Jackson, TN	2009
TN	TN-0924	**Rotstrahlbusch**	Jackson, TN	2009
TN	TN-0925	**Northwind**	Knoxville, TN	2009
TN	TN-0926	Alamo	Knoxville, TN	2009
GA	GA12-01	**Dallas Blues**	Atlanta, GA	2012
GA	GA12-02	**Dallas Blues**	Atlanta, GA	2012
GA	GA12-03	**Dallas Blues**	Atlanta, GA	2012
GA	GA12-04	**Dallas Blues**	Atlanta, GA	2012
SC	SC12-01	Alamo	Florence, SC	2012
SC	SC12-02	Alamo	Florence, SC	2012
SC	SC12-03	Alamo	Florence, SC	2012
SC	SC12-04	Alamo	Florence, SC	2012
AL1	AL-BL	**Badland**	Brewton, AL	2012
AL1	AL-C9	**Cloud Nine**	Brewton, AL	2012
AL1	AL-CS	Unknown	Brewton, AL	2012
AL1	AL-DaB	**Dallas Blue**	Brewton, AL	2012
AL1	AL-DeB	**Dewey Blue**	Brewton, AL	2012
AL1	AL-HM	**Heavy Metal**	Brewton, AL	2012

Table 4.1 continued

Population Code	Rust Isolate	Switchgrass Cultivar	Location	Collected Year
AL1	AL-NW	**Northwind**	Brewton, AL	2012
AL1	AL-PF	Unknown	Brewton, AL	2012
AL1	AL-PS	**Prairie Sky**	Brewton, AL	2012
AL1	AL-PV	***Panicum virgatum***	Brewton, AL	2012
AL1	AL-SH	**Shenandoah**	Brewton, AL	2012
AL1	AL-TC	**Thundercloud**	Brewton, AL	2012
AL2	P1-1	Alamo	Brewton, AL	2016
AL2	P1-3	Alamo	Brewton, AL	2016
AL2	P1-4	Alamo	Brewton, AL	2016
AL2	P2-1	Alamo	Brewton, AL	2016
AL2	P2-3	Alamo	Brewton, AL	2016
AL2	P2-4	Alamo	Brewton, AL	2016
AL2	P2-5	Alamo	Brewton, AL	2016
AL2	P3-1	Alamo	Brewton, AL	2016
AL2	P3-2	Alamo	Brewton, AL	2016
AL2	P3-3	Alamo	Brewton, AL	2016
AL2	P3-3	Alamo	Brewton, AL	2016

Table 4.2 Sequences, repeat motif, annealing temperature (*T*a), and allelic size range (bp) of 30 microsatellite primers used in estimating genetic diversity of *Puccinia emaculata* isolates. The primers in bold were selected for final genotyping of *P. emaculata* isolates.

Locus	Forward (F) and Reverse (R) Primers (5' – 3')	Repeat Motif	T_a(°C)	Size (bp)
Pe2-002	F: TTCGTCTTGTCTTTGCATCG R: TGCAACACACACACACATGC	(TGT)3…(TTG)3 …(TG)6	55	199–263
Pe2-005	F: GCAACACATCATCAGCAAGG R: TGGCTATCACGATGGATTCG	(CA)8	55	181–225
Pe2-016	F: GCTCCTCTTGAAGTGTTCAGC R: GACCATGATTACGCCAAGC	(TG)5	55	209–347
Pe2-018	F: TTTCTTCTACGCACACACAGC R: GCAGTTCAAGCCTCACTTGG	(AC)5	55	202–206
Pe4-008	F: TATCCGCACTACCATCATCC R: AAGCACCTCCGTATATGTGC	(CACCATCACA)3 …(CACACCAT)2	55	240–294
Pe4-010	F: GCAGAAATCGGAACAACTCG R: TGACCATGATTACGCCAAGC	(GTGTGC)5 …(GT)15	55	225–318
Pe4-015	F: GGTCAGAGGTCTCGAATTGC R: AGCCACTACCTCCACTGAGC	(AC)15…(GA)9	55	145–151
Pe4-019	F: GGCGTCGACTATAACCAACC R: GACCATGATTACGCCAAGC	(AC)12	52	108–181
Pe4-024	F: GAATCCCTGTATGGCAAAGG R: AGGCATAGGCAAAACTCACG	(CAT)3 …(CAT)13	55	257–293
Pe4-032	F: CACTGATTGGGTGTGTTTGC R: TTGTTGCTGGAGTGTGTAGTAGC	(ACACA)3 …(ACACAAC)2	55	197–223
OPE2	F: TGATGGGGAAACAGTGAAAG R: AAGGCAAAGACGAAAGCAAA	(GA)10	59	126
OPE5	F: AAAGGTTGAGTGGTAGTGGT R: GCTAATGATGACGAAGTTGT	(TAG)10	54	147
OPE19	F: GATGGCAGATTACAAGACAGAG R: CAACTTCGTCAAAAACAGTCC	(TAC)8	57	228
OPE21	F: TGGCTGATTACAAGACAGAGTT R: GGTTGAGTGGTAGTGGTAGAGA	(TAC)10	57	390
OPE28	F: CCCGAAATGACACATCAAAA R: ACACACACACACACACAGCAC	(TG)10	59	328
OPE32	F: ACAAGCCATCCAAGGGAGT R: CAAGTTCATCGGCATCGTT	(GAT)12	60	171
OPE59	F: ATGGCAGATTACAAGACAGAGT R: CTAGCAGAATCACATCAACAAC	(TAC)7	56	378

Table 4.2 continued

Locus	Forward (F) and Reverse (R) Primers (5' – 3')	Repeat Motif	T_a(°C)	Size (bp)
OPE60	F: TTGCTTAGAGGAGGCTGAAA R: TTTTTGGTTAGCGGAGAAGG	(AC)44	58	346
OEPE6a	F: GTAATAAGAGCCAACACGGAGG R: TAGGATTAGGCATGGCGTACTT	(AAACT)12	60	381
OEPE6b	F: AACTTTTCCTGCACCCTTTT R: CTGTGATTAGCCCTTGAAACAC	(TG)7	59	244
OEPE28	F: AACGTCTCCAAAAGCTGATCTC R: GATTGCAGTAAGACAAGGGGAC	(GGT)6	60	278
OEPE68	F: TGGAGAGATAGACCCAATAGCC R: CAACTCATCAACACACAACCCT	(GTT)8	60	313
OEPE71	F: AGCGTGACAAGTGAACAAGAGA R: TACAACCCGAAACTCCTCAACT	(AG)8	60	345
OEPE92	F: TTGTTAAAGGATACGAGCCGAT R: ACCAAACCAGATTGAGCAGATT	(GA)18	60	167
OEPE128	F: TTTGCGACGACCGAGGGG R: TCACCAACCGCTGCCCTATCTT	(GAG)7	61	128
OEPE141	F: ATCGGCTTCACATCTGGTATCT R: ATCAGGTTCTGCGTCTTCTGTC	(TGA)6	61	275
OEPE156	F: GAAAGAGCAACCAAGTGAAACC R: CCTCATCAACATCCACAACAAC	(GA)7	60	271
OEPE160	F: TACCGAGAGCTTTTGAGAGACC R: TTTCCACCAAGAACCACTACAA	(TG)7	60	126
OEPE161	F: ATCAATGTAAGCACCAAAGCAG R: CAGGAACCATACAAGCCTAACC	(GA)10	60	123
OEPE162	F: CTCCTCTCGCTTCCCAGTC R: TTACCTACATCTTCATCCGCCT	(CT)8	60	263

Table 4.3 Genetic diversity indices for *Puccinia emaculata* isolates across seven different subpopulations and 22 microsatellite markers.

Locus	N_a[a]	N_e[b]	I[c]	H_o[d]	H_e[e]	F_{is}[f]	F_{it}[g]	F_{st}[h]	N_m[i]
OEPE156	6	2.3	0.89	0	0.54	1	1	0.18	1.14
OEPE160	5	3.03	1.13	0.53	0.66	0.19	0.23	0.06	4.23
OEPE161	5	2.46	0.95	0.03	0.58	0.96	0.96	0.07	3.56
OEPE162	7	1.84	0.7	0.2	0.4	0.51	0.72	0.42	0.34
OEPE6B	4	2.33	0.89	0	0.56	1	1	0.04	6.18
OEPE71	7	2.82	1.09	0.44	0.59	0.26	0.46	0.27	0.68
OEPE92	4	2.28	0.91	0.32	0.53	0.39	0.51	0.2	0.97
OPE2	3	1.07	0.1	0	0.05	1	1	0.09	2.69
PE2_005	3	2.2	0.8	0	0.49	1	1	0.14	1.53
PE2_018	7	3.08	1.17	0.59	0.62	0.05	0.23	0.19	1.09
PE4_010	5	1.86	0.63	0	0.41	1	1	0.37	0.42
PE4_015	3	2.19	0.77	0.43	0.46	0.06	0.33	0.29	0.61
PE4_032	4	1.67	0.6	0.35	0.35	0.01	0.4	0.39	0.39
OEPE128	3	1.35	0.36	0	0.22	1	1	0.13	1.68
OEPE141	3	1.82	0.62	0	0.41	1	1	0.13	1.69
OEPE28	3	1.3	0.35	0	0.19	1	1	0.1	2.27
OEPE68	5	2.53	0.98	0.75	0.58	-0.3	-0.07	0.18	1.18
OPE19	2	1.05	0.08	0	0.04	1	1	0.06	4.01
OPE21	5	2.61	1.03	0.86	0.61	-0.41	-0.28	0.09	2.55
OPE5	3	1.66	0.57	0.61	0.37	-0.64	-0.41	0.14	1.51
OPE59	2	1.43	0.38	0	0.26	1	1	0.17	1.19
OEPE6A	2	1.09	0.08	0	0.05	1	1	0.22	0.88
Average	4.14	2	0.69	0.23	0.41	0.55	0.64	0.18	1.85

[a]N_a - number of different alleles
[b]N_e - number of effective alleles
[c]I - Shannon's information index
[d]H_o - observed heterozygosity
[e]H_e - expected heterozygosity
[f]F_{is} - departure from Hardy-Weinberg proportions within subpopulations
[g]F_{it} - overall departure from Hardy-Weinberg proportions in the entire population
[h]F_{st} - genetic divergence among subpopulations
[i]N_m - gene flow

Table 4.4 Analysis of 52 *Puccinia emaculata* isolates from seven geographic regions / years using 22 Microsatellite primers.

Pop	N[a]	MLG[b]	PA[c]	N_a[d]	N_e[e]	I[f]	H_o[g]	H_e[h]	F[i]	$\bar{r}_d$[j]	p.rd[k]
AL1	12	12	1	3.23	2.2	0.83	0.21	0.46	0.61	0.24	0.001
ALM	4	4	1	2.05	1.7	0.53	0.22	0.33	0.38	0.33	0.000
GA	4	4	0	2.05	1.84	0.57	0.26	0.37	0.34	0.10	0.047
NC	4	4	0	1.77	1.62	0.44	0.27	0.29	0.18	0.47	0.000
AL2	11	11	10	3.14	2.19	0.8	0.2	0.44	0.61	0.17	0.000
SC	4	4	1	2.55	2.25	0.81	0.26	0.5	0.51	0.14	0.004
TN	13	13	2	3.14	2.2	0.82	0.21	0.46	0.64	0.20	0.000
Average			2.1	2.56	2	0.69	0.23	0.41	0.47		

[a]N - number of isolates in each subpopulation
[b]MLG - number of multilocus genotype
[c]PA - number of private alleles
[d]*N*a - number of different alleles
[e]*N*e - number of effective alleles
[f]*I* - Shannon's information index
[g]*H*o - observed heterozygosity
[h]*H*e - expected heterozygosity
[i]*F* - fixation indices
[j]$\bar{r}_d$ - standardized index of association adjusted by sample size of loci
[k]p.rd – p value to test if standardized index of association significantly differs than 0

Table 4.5 Pairwise population differentiation (F_{ST}) matrix based on Nei genetic distance for seven switchgrass rust populations using 22 microsatellite primers.

	AL1	AR_LA_MS	GA	NC	AL2	SC	TN
AL1	0						
AR_LA_MS	0.11	0					
GA	0.11	0.11	0				
NC	0.15	0.19	0.2	0			
AL2	0.09	0.09	0.1	0.13	0		
SC	0.07	0.14	0.1	0.17	0.11	0	
TN	0.03	0.11	0.13	0.19	0.1	0.09	0

Table 4.6 Pairwise gene flow (N_m) matrix for seven switchgrass rust populations using 22 microsatellite primers.

	AL1	**AR_LA_MS**	**GA**	**NC**	**AL2**	**SC**	**TN**
AL1	0						
AR_LA_MS	2.14	0					
GA	2.07	2.08	0				
NC	1.38	1.04	1.01	0			
AL2	2.62	2.41	2.26	1.63	0		
SC	3.35	1.56	2.21	1.22	2.04	0	
TN	9.75	1.94	1.74	1.05	2.17	2.70	0

Table 4.7 Analysis of molecular variance (AMOVA) for 52 switchgrass rust (*Puccinia emaculata*) isolates across 22 Microsatellite primers. Three analyses were conducted: (i) using original seven subpopulations as one hierarchical group; (ii) based on the four subpopulation-clusters suggested by TESS.

Variance Partition	df	Sum of Squares	Variance Component	% of Variation	Fixation Indices[a]	p Value
(i)						
Among populations	6	80.920	0.62 Va	11.43		
Within populations	97	463.013	4.77 Vb	88.57	$F_{ST} = 0.14$	< 0.01
Total	103	543.93	5.39			
(ii)						
Among populations	3	159.51	1.91 Va	32.82	$F_{IS} = 0.38$	< 0.01
Among individuals within populations	48	258.93	1.49 Vb	25.65	$F_{IT} = 0.58$	< 0.01
Within individuals	52	125.50	2.41 Vc	41.53	$F_{ST} = 0.33$	< 0.01
Total	103	543.94	5.81			

[a]F_{IS}: inbreeding coefficient of measuring of departure from HW proportions within subpopulation; F_{IT}: overall departure from HW proportions in the entire population; F_{ST}: genetic divergence among subpopulations

Table 4.8 Bottleneck effect determination with BOTTLENECK program using three mutation models (I.A.M, infinite allele model; S.M.M, stepwise mutation model; T.P.M, two-phase mutation model) for 52 switchgrass rust isolates using 22 Microsatellite primers. Significant estimates of the heterozygosity deficiency/excess over all loci were tested with the sign test.

	Number of loci with heterozygosity deficiency/excess		
Cluster	I.A.M	S.M.M	T.P.M
AL1	7/14 ^{ns}	9/12 ^{ns}	8/13 ^{ns}
ALM	3/12*	4/11 ^{ns}	4/14 ^{ns}
GA	1/15**	1/15**	1/15**
NC	0/13**	0/13**	0/13**
AL2	4/15*	9/10 ^{ns}	4/14 ^{ns}
SC	1/19**	1/19**	1/19**
TN	7/14 ^{ns}	8/13 ^{ns}	8/13 ^{ns}

$^{ns}p> 0.05$, $*p\leq 0.05$, $**p\leq 0.01$

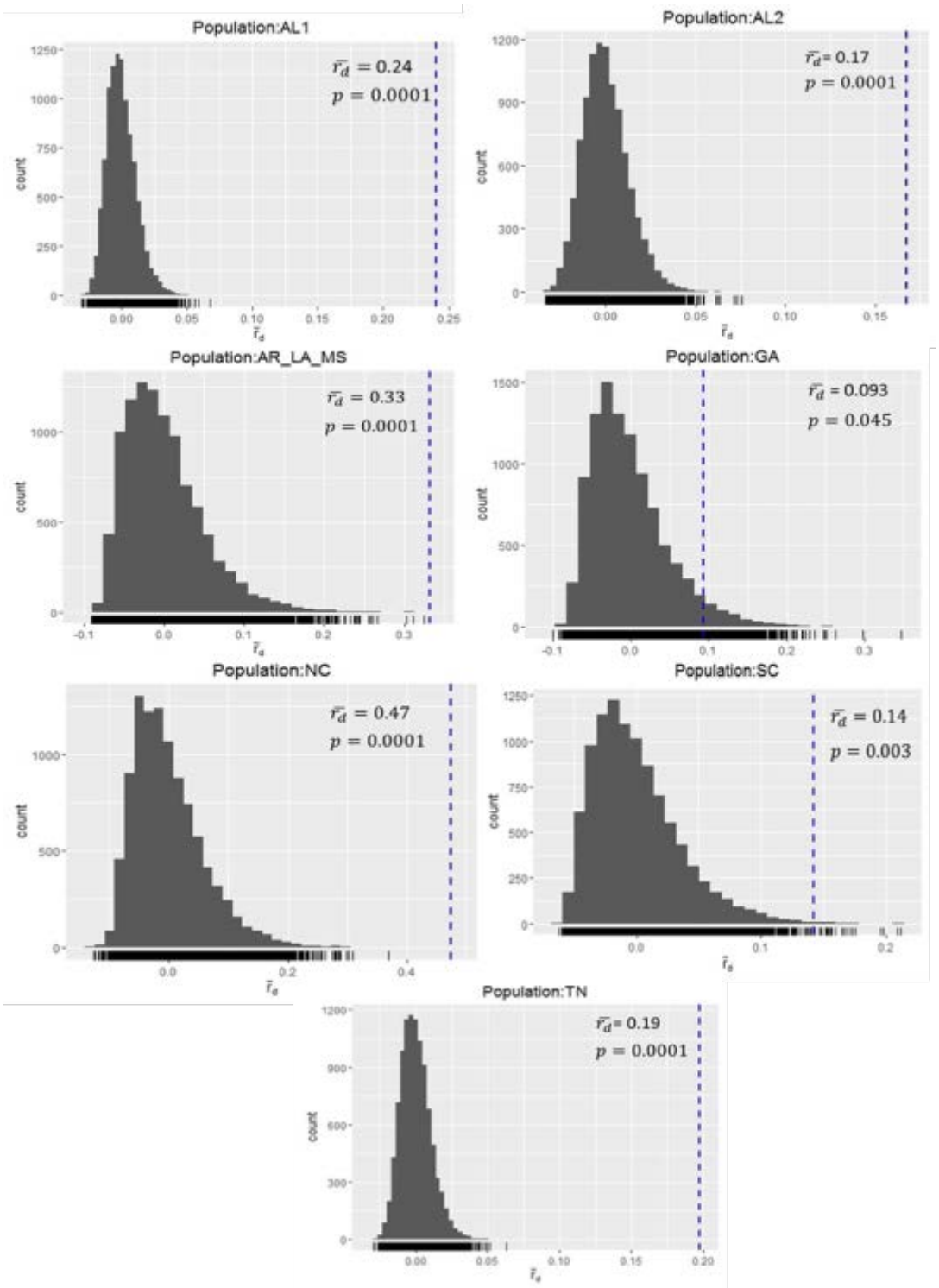

Figure 4.1 Visualization of linkage disequilibrium of seven populations of 52 switchgrass rust isolates with 22 Microsatellite primers by detecting association between alleles at different loci (Index of Association) for 10,000 permutations using Poppr package in R. $\bar{r}_d$, the standardized index of association adjusted by sample size of loci.

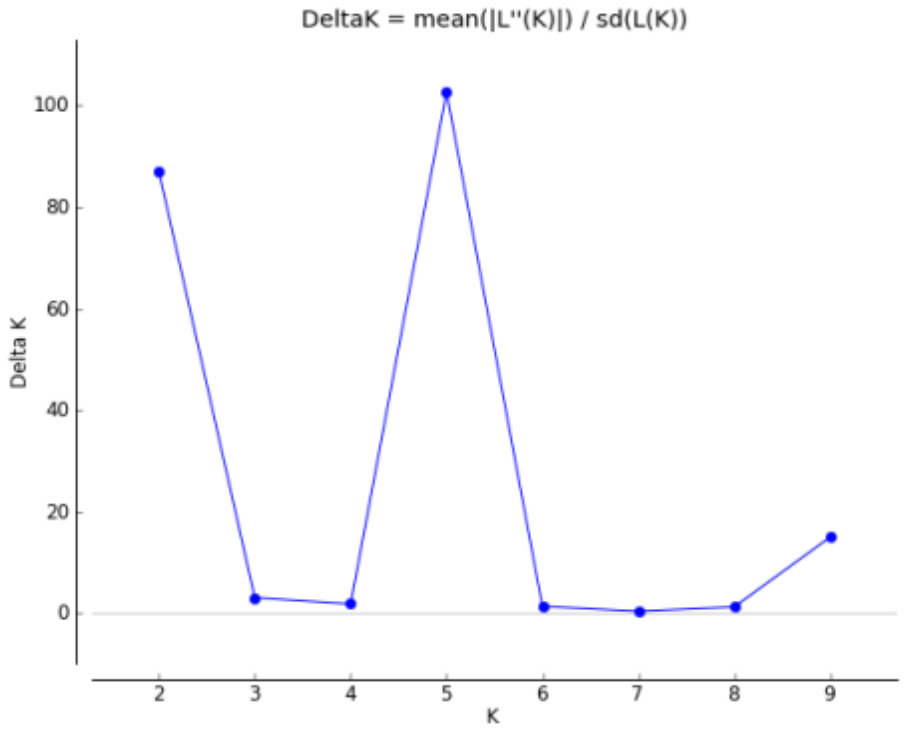

Figure 4.2 Maximum ΔK at K=5 for 52 *Puccinia emaculata* isolates using 22 Microsatellite primers.

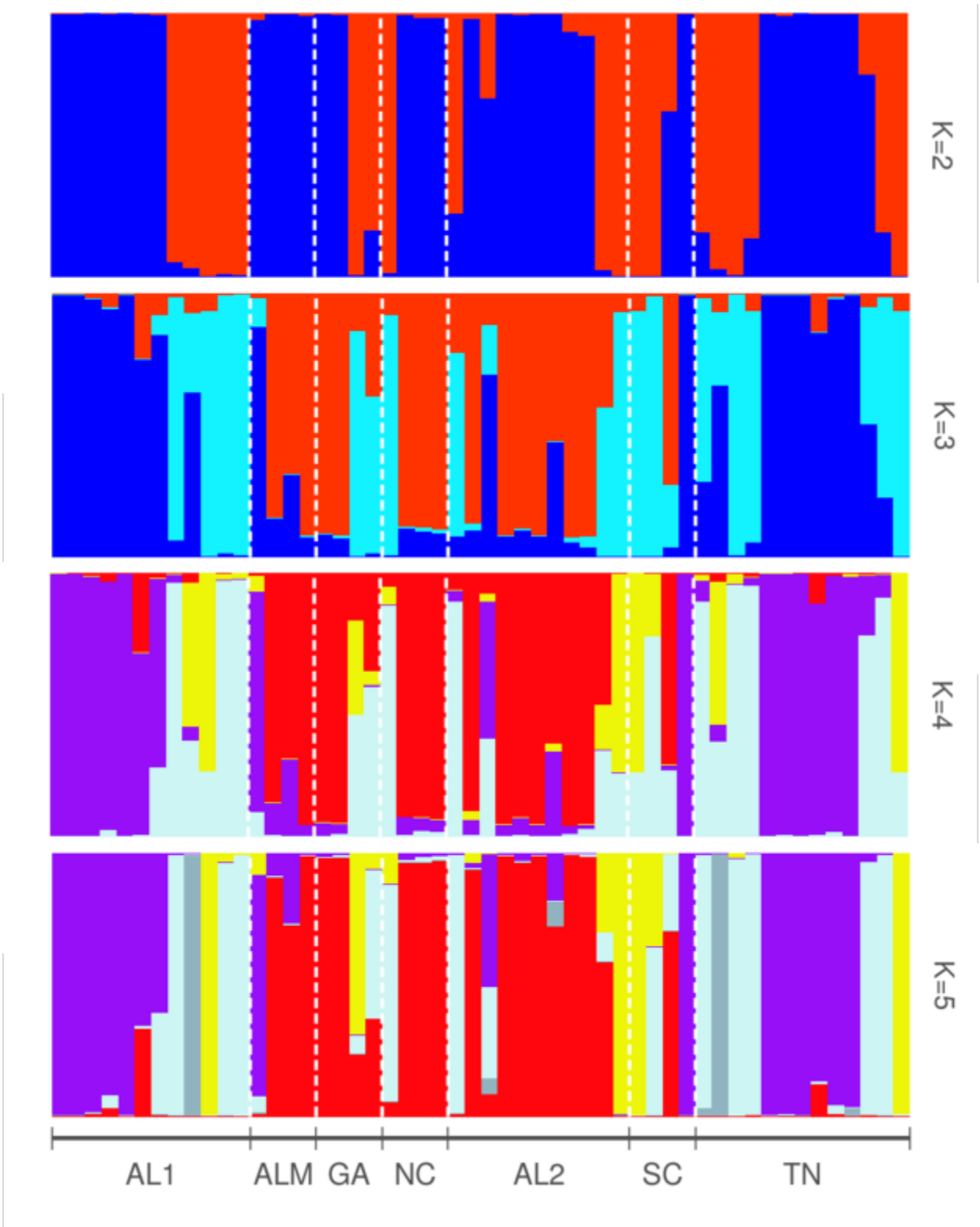

Figure 4.3 Structure graph for genetic cluster of 52 *Puccinia emaculata* isolates using 22 Microsatellite primers.

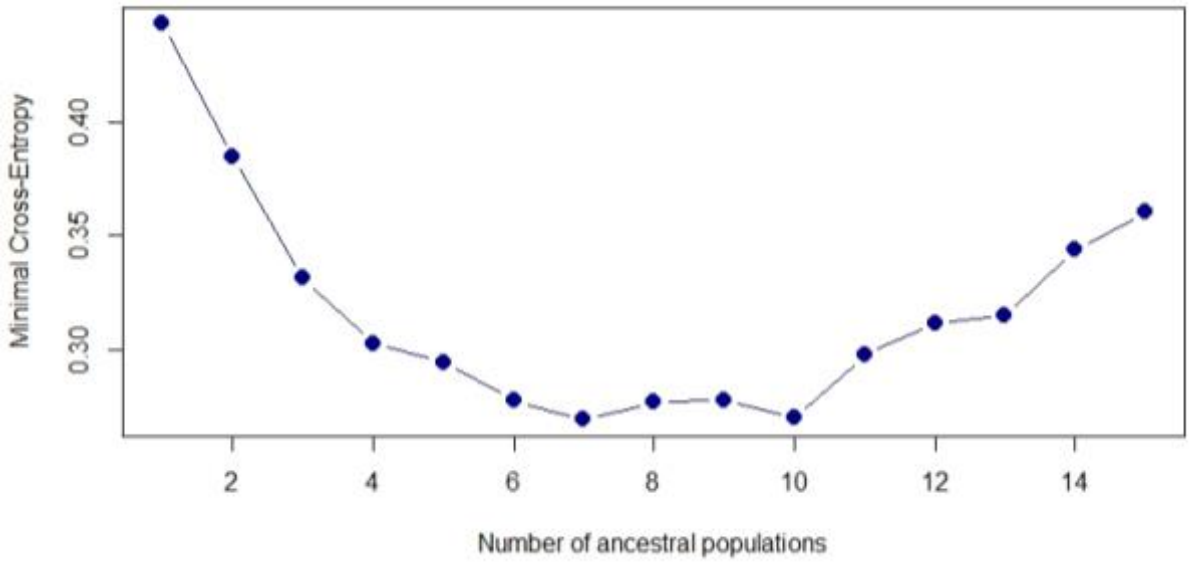

Figure 4.4 Cross-entropy plot for the switchgrass rust (*Puccinia emaculata*) SSR data when the number of cluster K ranging from 1 to 15 generated by TESS3 package in R. The retained value of K is K=4.

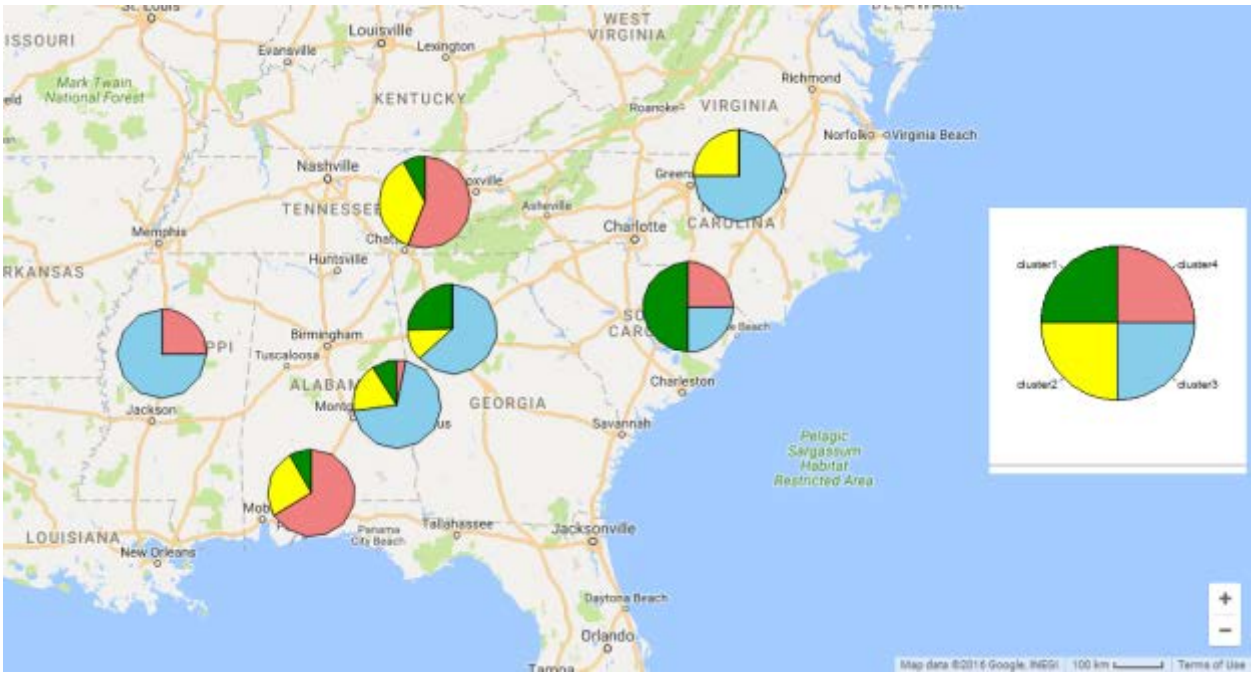

Figure 4.5 Interpolated values of admixture coefficients for sub-clustering pattern of switchgrass rust (*Puccinia emaculata*) collected from seven locations of Southeastern US. Pie charts are based on TESS results with K=4.

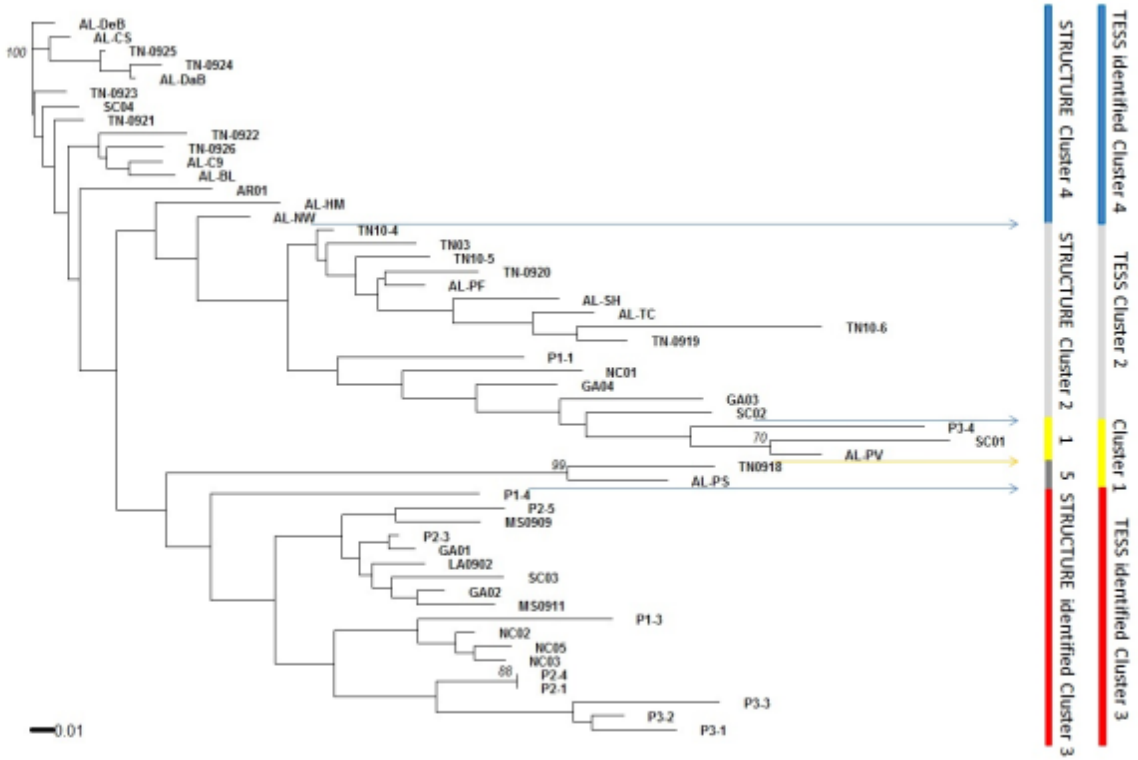

Figure4.6 Neighbor-joining tree of 52 switchgrass rust isolates based on Bruvo's distance using Arithmetic Averages (UPGMA) algorithms with 1,000 bootstraps with Poppr package in R. STRUCTURE and TESS identified clusters were overlaid right on the tree.

Conclusion

In this dissertation, the pathogenethesis, virulence, genome information, and population genetics of switchgrass rust (*Puccinia emaculata*) were investigated.

The infection process of *P. emaculata* urediniospores on a susceptible switchgrass cultivar was revealed. Appressoria were formed approximately 10 hr after inoculation (HAI) and penetrations were observed over stomatal openings. Substomatal vesicles developed 3 days after inoculation (DAI) and at 5 DAI, both haustorial mother cells and haustoria were observed. At 11 DAI, uredinia were developing, but urediniospores were immature. Until 14 DAI, matured uredinia ruptured host epidermis and urediniospores were released for secondary infection. These findings on *P. emaculata* infection and related *P. emaculata*-switchgrass interaction will help to further in-depth study of resistance mechanisms and the molecular mechanisms of the interaction between the switchgrass and *P. emaculata*. By understanding this detailed infection process, disease control strategies will be improved and more targeted.

The present study is the first to study the germination percentage, latent period, number of uredia and urediniospores produce per cm^2 leaf surface of 40 switchgrass rust isolates on 12 agronomy and ornamental switchgrass cultivars. Ornamental switchgrass cultivars averagely showed significantly in increasing latent period and reducing number of uredia and urediniospores produced per cm^2. In addition, virulence variations were observed among 40 switchgrass rust. No clear pattern was found to group rusts by their collecting locations and years. This continuous resistance information throughout the whole infection process on switchgrass cultivars other than the single overall end-season disease rating value would provide more details and new resources

for developing rust resistant switchgrass cultivars to achieve sustainable control of switchgrass rust.

The genome information of *P. emaculata* was investigated in this study. 622 rust contigs were confidently provided, which would be random samples of nucleotide pieces from the whole genome with GC content 47.33%. Variant detection was conducted by mapping sequence reads of the other five isolates on these rust contigs. The average single nucleotide variants (SNVs) and insertion/deletions (indels) density is 1.62 and 0.057 per kb, respectively. Phylogenetic analyses using *5.8S rRNA* gene placed switchgrass rust in Pucciniaceae, separating form Melampsoraceae, Phakopsoraceae, Pucciniastreae. However, due to contaminations in sequence reads, limited resources were gained to provide more detailed genome infromation of *P. emaculata*. Further researches would be foucused on developing efficient methods to get clean genomic DNA. The sequences of clean genomic DNA can give us more information about the pathogen in the genome level.

Finally, 22 microsatellite primers were used to analyze and determine the genetic relationship among switchgrass rust isolates collected from seven locations across southeastern U.S. Two to seven alleles were identified for each locus. The expected heterozygosity (*H*e) ranged from 0 to 0.86 and observed heterozygosity (*H*o) ranged from 0.04 to 0.66. Significant linkage among loci were observed among all seven subpopulations with standardized index of association significantly different from 0 ($p < 0.01$), indicating no sexual recombination in these locations. Moderate to high genetic differentiation were discovered among these subpopulations for this

long-distance disposal fungus, demonstrating multiple origins of genotypes of *P. emaculata* introduced to these areas.

Vita

Qunkang Cheng was born in Gugao, Jiangsu province, China. He graduated from Nanjing Agricultural University in 2004 with a B.S. in Plant Protection. In 2011, he received his MS in Plant Pathology and minor in statistics from the University of Tennessee at Knoxville (UTK) under the direction of Dr. Mark T. Windham. In 2016, he received a Ph.D. from UTK majoring in Entomology, Plant Pathology & Nematology and MS in Statistics. He was mentored and advised by Dr. Mark T. Windham.