POPULATION BIOLOGY OF SWITCHGRASS RUST

(*Puccinia emaculata* Schw.)

By

GABRIELA KARINA ORQUERA DELGADO

Bachelor of Science in Biotechnology

Escuela Politécnica del Ejército (ESPE)

Quito, Ecuador

2011

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2014

POPULATION BIOLOGY OF SWITCHGRASS RUST

(*Puccinia emaculata* Schw.)

Thesis Approved:

Dr. Stephen Marek

Thesis Adviser

Dr. Carla Garzon

Dr. Robert M. Hunger

ACKNOWLEDGEMENTS

For their guidance and support, I express sincere gratitude to my supervisor, Dr. Marek, who has supported thought my thesis with his patience and knowledge whilst allowing me the room to work in my own way. One simply could not wish for a better or friendlier supervisor.

I give special thanks to M.S. Maxwell Gilley (Mississippi State University), Dr. Bing Yang (Iowa State University), Arvid Boe (South Dakota State University) and Dr. Bingyu Zhao (Virginia State), for providing switchgrass rust samples used in this study and M.S. Andrea Payne, for her assistance during my writing process.

I would like to recognize Patricia Garrido and Francisco Flores for their guidance, assistance, and friendship. To my family and friends for being always the support and energy I needed to follow my dreams.

Name: GABRIELA KARINA ORQUERA DELGADO

Date of Degree: JULY, 2014

Title of Study: POPULATION BIOLOGY OF SWITCHGRASS RUST (*Puccinia emaculata* Schw.)

Major Field: ENTOMOLOGY AND PLANT PATHOLOGY

Abstract: Switchgrass (*Panicum virgatum* L.) is a perennial warm season grass native to a large portion of North America. Because of its low input requirements, broad adaptation, and high yield potential, switchgrass has been used for forage production and soil conservation and is considered an ideal renewable biomass feedstock crop for biofuel production. However, switchgrass can be affected by several fungal diseases. One of these is rust, caused by *Puccinia emaculata*, an obligate parasitic fungus. *Puccinia emaculata* has been observed on agronomic switchgrass causing chlorosis and necrosis of leaf tissues, lodging, and plant death, reducing feedstock quality and biomass yield of switchgrass up to 60% and 50%, respectively. Currently, little is known about switchgrass rust, and its unclear etiology complicates the development of effective management strategies. In order to better understand the biology of the pathogen, this study focused on two main objectives: 1) generate a multilocus phylogeny to determine the species' phylogenetic status and to assess its genetic diversity across five US states (MS, OK, VA, IA, and SD) and 2) develop and characterize de novo simple sequence repeats (SSRs) and expressed sequence tag (EST)-SSRs to study the population biology of *P. emaculata*. Since DNA from bulked urediniospores produced mixed templates, PCR products of three phylogenetically informative genomic loci, the ribosomal internal transcribed spacer (ITS) region, and the β-tubulin (bTub) and translation elongation factor-1α (TEF1a) genes, were cloned prior to sequencing. Differences in haplotype diversity was observed among loci (ITS=13; bTub=24; TEF1a= 27), with large variation in the patterns of distribution of haplotypes across states. Distribution of bTub and TEF1a haplotypes were mostly local, while ITS haplotypes were distributed both across multiple states and locally. In order to perform multilocus phylogenetic analyses, a single spore whole genome amplification (ssWGA) protocol was standardized, which produced sufficient single cell DNA for PCR of single copy genes. Single gene and multilocus phylogenies supported the monophyletic status of *P. emaculata*. Using a *P. emaculata* di- and trinucleotide repeat-enriched library, 49 SSR loci were identified, of which 8 were informative for multispore DNA samples and 6 were informative for a collection of 25 ssWGAs from five states. To develop EST-SSRs, RNA was isolated from germinated and non-germinated urediniospores from OK and VA using a novel modified method, cDNAs were generated and submitted for RNA sequencing (RNA-Seq). Thirty three EST-SSRs were identified, 12 of which were informative for a multistate collection of 25 ssWGAs. Genetic diversity was observed across single spore collections from five states using 18 microsatellite loci. Future studies will examine genetic variation, population structure and pathogenicity variation among multistate *P. emaculata* populations.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Switchgrass (*Panicum virgatum*, L., Poaceae), is a warm-season (C4) perennial grass native to the prairies of North America. In the last 25 years or so, switchgrass has been considered an ideal candidate for development as a renewable, herbaceous biomass fuel by the US. Department of Energy (16; 22; 23; 26; 27). Switchgrass has been used for livestock pasture and forage, habitat conservation, and erosion control due to its deep roots, foliar architecture and ability to fix carbon with high water usage efficiency. Switchgrass is adapted to and established in huge ranges of North America east of the Rocky Mountains, from Quebec to Central America (20). Currently in the United States, switchgrass yields average 10-15 metric tons per hectare (4-6 dry tons/acre) per year. Common varieties include "Alamo", "Cave in Rock", and "Kanlow".

Today, switchgrass breeding programs are focused on increasing plant cellulose content and yield; however, seed yields, forage quality, and biomass of switchgrass can be negatively affected by diseases (11-14; 19; 23). Many fungal diseases have been reported to affect switchgrass. In previous studies surveying the fungal species present on switchgrass, 42 species of fungi were identified in the United States (12; 13). Fungi are present in all parts of the switchgrass plant; however, many fungi cause economically damaging diseases of foliar plant parts. Switchgrass leaf rust is the most common and important fungal disease and reported to be caused by three species: *Uromyces graminicola* Burril, *Puccinia graminis* Pers.: Pers., and *Puccinia emaculata* Schwein (1; 4; 6; 8; 13; 21). Other damaging diseases of switchgrass include

smut of the seedheads caused by *Tilletia maclagani* (Berk. G.P Clinton) and *T. pulcherrima* (Syd. & P.Syd), anthracnose and bipolaris leaf spot.

Outbreaks of switchgrass rust caused by *P. emaculata* have been reported throughout the US. During the 2000 and 2001 growing seasons, incidence of rust caused by *P. emaculata* was high in Iowa (11). In July and August 2007, *P. emaculata* pustules were found on leaf surface of switchgrass plants in eastern Tennessee, which was the first official report identifying the causal fungus using molecular methods (i.e. sequencing the nuclear ribosomal internal transcribed spacer [ITS] region) (28). Switchgrass rust caused by *P. emaculata* has also been reported from the southeastern United States, west to Texas, south into Mexico and as far north as South Dakota. Oklahoma is also one of the states that was severely impacted by switchgrass rust in 2012 (26). *Puccinia. emaculata* has been reported from many other *Panicum* species (e.g. *Panicum capillare*) from Canada to Brazil (8). Maximum yield reductions of switchgrass due to rust range from 50 to 60% (10; 24; 26)

Puccinia emaculata is an obligate parasitic basidiomycete fungus of the order *Pucciniales* (7). In nature, this pathogen is believed to be heteroecious (two hosts are necessary to complete its life cycle) and macrocyclic (five spores life cycle). Switchgrass is the uredinial-telial host and spurges (*Euphorbia* spp.) are reported to act as the aecial host (affected by *P. pammelii* (Trel.) Arth. and *P. panici* Diet., which are now considered synonymous with *P. emaculata*) (3; 27). However, attempts to inoculate *P. emaculata* on *Euphorbia corollata* were without success (2), and the alternate (aecial) host of *P. emaculata* is still unknown (28).

The polycyclic disease cycle of leaf rust is caused in the field by urediniospores (asexual spores) repeatedly infecting switchgrass plants. In many rust fungi, urediniospore germination occurs optimally at temperatures from 17°C to 27°C, 1-4 hours after deposition of pathogen spores on switchgrass leaves. Following deposition, infection occurs over a wide temperature

range, 10°C to 27°C, with at least 6 hours of free moisture from dew or rainfall (6; 18). During infection, urediniospore germ tubes form appressoria over stomata, penetrating leaves through these natural leaf openings. Seven to 10 days post inoculation, urediniospores in uredinial sori erupt through the cuticle (4; 5; 6)).

Extended periods of leaf wetness (>18 h) promote spore germination and have been reported to increase disease severity in many rust species (13; 28). New pustules, including sori (spore masses) and surrounding chlorotic or necrotic leaf tissue, become visible 7-15 days post inoculation after growing to approximately 1 mm in diameter (28). Later plant symptoms can include chlorotic or pigmented leaf spots, general chlorosis of leaf tissue, necrosis, lodging, and, if severe, plant death.

Different levels of rust disease severity have been reported among switchgrass cultivars (15) and result in biomass yield reductions from 17 to 62% in switchgrass infected plants (14; 25), which are dramatic losses for a potential biofuel crop. Switchgrass can produce an average of 75 gallons of ethanol/ton. Due to the high demand for biofuels, large acreages of switchgrass are expected to be planted in monoculture throughout the southern United States. Because of the favorable environmental conditions for fungal diseases in the southeastern United States (high humidity and warm temperatures), outbreaks of switchgrass rust will likely occur. If such outbreaks occur where the alternate host is present, the resulting genetic recombination due to sexual reproduction may cause more virulent races of *P. emaculata* to emerge (26). While other rust fungi have been reported on switchgrass, all recent reports consider *P. emaculata* to be the only rust pathogen infecting switchgrass (28).

As with most rust fungi, identification of *P. emaculata* has relied on host species (*Panicum*) and morphological characteristics, especially of the teliospores. For example, teliospores of *P. emaculata* are two-celled (27-[33-44]-49 × 15-[17-21]-24 μm), with chestnut-

brown walls and pedicels (≤80 μm) (1; 2; 6; 12). Identification of *P. emaculata* based on morphological characteristics can be ambiguous, as morphology alone fails to distinguish it from other *Puccinia* species. Two sequences of the nuclear ribosomal internal transcribed spacer (ITS) region are available in the online nucleotide database of the National Center for Biotechnology Information (NCBI) (accessions numbers: EU915294; KC515382). Uppalapati et al. (2012) reported a preliminary phylogenetic analysis of *P. emaculata*, based on ITS sequences obtained from collected urediniospores in Oklahoma and adding a selected set of other rust fungi as outgroups. Results showed that ITS sequences from *P. emaculata* were distinct from sequences from *Puccinia graminis* and instead *P. emaculata* fell into a highly supported cluster containing *Puccinia asparagi*, *Puccinia andropogonis*, and *Puccinia sorghi* (26). Uppalapati et al., described *P. emaculata* as a possible monophyletic group, based on ITS sequences only. Variations in pathogenicity of *P. emaculata* have been reported as well. Ornamental switchgrass appeared to be more virulent than agronomic switchgrass (17). Also, ten polymorphic microsatellites have been reported for *P. emaculata* and tested on urediniospores collected in Tennessee, Arkansas, Mississippi, North Carolina and Louisiana (27).

Little is known about switchgrass rust. Since identification of rusts based on morphology can be ambiguous, molecular tools should be incorporated to confirm host- and morphology-based identifications (6; 21; 27). Developing such tools will allow accurate identifications of species, timely detection, and a more accurate reporting of *P. emaculata* incidences around the states (23). All of these would facilitate the development of switchgrass rust management practices that can be incorporated into a broader integrated pest management program for switchgrass (9).

Thus, the objectives of this thesis research were: i) study the phylogeny and haplotype diversity of switchgrass rust from urediniospores collected in five different states in the United States (Iowa, Mississippi, Oklahoma, South Dakota, and Virginia) using three phylogenetically

informative loci: ITS, β-tubulin (bTub), and translation elongation factor-1α (TEF1a); and ii) develop and characterize *P. emaculata* simple sequence repeats markers (SSRs; i.e. microsatellites) *de novo* from genomic DNA and from RNA-seq gene expression data (i.e. expressed sequence tag [EST]-SSRs).

REFERENCES

1. Arthur, J. C. 1921. Origin of Potato Rust. Science 53:228-9.

2. Arthur, J. C., and Cummins, G.B., 1962. Manual of the rust in the United States and Canada. Hafner, New York. 438 p.

3. Bahri, B., Leconte, M., Vallavieille-Pope, C., and Enjalbert, J. 2009. Isolation of ten microsatellite loci in an EST library of the phytopathogenic fungus *Puccinia striiformis* f.sp. *tritici*. Conservation Genetics 10:1425-1428.

4. Bhairi, S. M., Staples, R. C., Freve, P., and Yoder, O. C. 1989. Characterization of an infection structure-specific gene from the rust fungus *Uromyces appendiculatus*. Gene 81:237-43.

5. Bonde, M. R., Berner, D. K., Nester, S. E., and Frederick, R. D. 2007. Effects of temperature on urediniospore germination, germ tube growth, and initiation of infection in soybean by *Phakopsora* isolates. Phytopathology 97:997-1003.

6. Cummins, G. B. 1971. The Rust Fungi of Cereals, Grasses and Bamboos. 2 ed. Springer-Verlag, New York.

7. de Schweinitz, L. D. 1834. Synopsis fungorum in america boreali media degentium. Secundum observationes. Transactions of the American Philosophical Society 4: 141-316.

8. Farr, D. F., and Rossman, A. Y. 2013. Fungal databases, systematic mycology and microbiology laboratory, agricultural research service, United States Department of Agriculture. Internet site: http://nt.ars-grin.gov/fungaldatabase [accessed February 2014].

9. Garland, C. D. 2008. Growing and harvesting switchgrass for ethanol production in Tennessee. University of Tennessee, cooperative extension report SP701-A, 2007. Internet site: http://economics.ag.utk.edu/publications/bioenergy/SP701-A.pdf. [accessed July 2013].

10. Gawel, N. 2009. Switchgrass production. Proceedings of Research Conference, 54 Annual report, 2010. University of Tennessee. Internet site: http://www.sna.org/resources/documents/09resconfrontpages.pdf. [accessed Jun 2013].

11. Gravert, C., and Munkvold, G. 2002. Fungi and diseases associated with cultivated switchgrass in Iowa. Journal Iowa Academy of Science 109:30-34.

12. Gravert, C. E., Tiffany, L. H., and Munkvold, G. P. 2000. Outbreak of smut caused by *Tilletia maclaganii* on cultivated switchgrass in Iowa. Plant Disease 84:596.

13. Gustafson, D. M., Boe, A., and Jin, Y. 2003. Genetic variation for *Puccinia emaculata* infection in switchgrass. Crop Science 43:755-759.

14. Hashemi, M., and Sadeghpour, A. 2013. Establishment and production of switchgrass grown for combustion. A Review. Int J Plant Biol Res 1(1): 1002.

15. Jacobs, K. A., Terrell, C. I., and Roelfs, A. P. 2003. Evaluation of ornamental switchgrass susceptibility to rust. Biological and Cultural Tests for the Control of Plant Diseases 19:O008.

16. Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H. V., Auer, M., Vogel, K. P., Simmons, B. A., and Singh, S. 2010. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. Bioresour Technol 101:4900-6.

17. Li, Y., Windham, M., Trigiano, R., Windham, A., Ownley, B., Gwinn, K., Zale, J., and Spiers, J. 2009. Cultivar-specific interactions between switchgrass and *Puccinia emaculata*. Phytopathology 99: S72.

18. Melching, J. S., Bromfield, K. R., and Kinsolver, H. H. 1979. Infection, colonization, and uredospore production on wayne soybean by four cultures of *Phakopsora pachyrhizi*, the cause of soybean rust. Phytopathology 69:1261-1265.

19. Mitchell, R., Vogel, K. P., and Sarath, G. 2008. Managing and enhancing switchgrass as a bioenergy feedstock. Biofuels Bioproducts & Biorefining 2:530-539.

20. Parrish, D. J., and Fike, J. H. 2005. The biology and agronomy of switchgrass for biofuels. Critical Reviews in Plant Science 24:423-459.

21. Ramachar, P., and Cummins, G. 1963. The species of *Uromyces* on the tribe Paniceae. Mycopathologia et Mycologia Applicata 19:49-61.

22. Sarath, G., Baird, L. M., Vogel, K. P., and Mitchell, R. B. 2007. Internode structure and cell wall composition in maturing tillers of switchgrass (*Panicum virgatum*. L). Bioresour Technol 98:2985-92.

23. Saski, C. A., Li, Z., Feltus, F. A., and Luo, H. 2011. New genomic resources for switchgrass: a BAC library and comparative analysis of homoeologous genomic regions harboring bioenergy traits. BMC genomics 12:369.

24. Sykes, V., Allen, F., Mielenz, J., Stewart, N., and Windham, M. 2011. Effect of *Puccinia emaculata* infection on ethanol production potential of *Panicum virgatum*. Phytopathology, Vol. 101, No. 6, pp. S174. 3340.

25. Thomsen, P. M., Brummer, E. C., Shriver, J., & Munkvold, G. P. 2008. Biomass yield reductions in switchgrass due to smut caused by *Tilletia maclaganii*. Plant Health Progress 2008. doi.10 1094/PHP- 2008- 0317-01-RS [accessed March 2013].

26. Uppalapati, S. R., Serba, D. D., Ishiga, Y., Szabo, L. J., Mittal, S., Bhandari, H. S., Bouton, J. H., Mysore, K. S., and Saha, M. C. 2012. Characterization of the rust fungus,

Puccinia emaculata, and evaluation of genetic variability for rust resistance in switchgrass populations. BioEnergy research. 6:458-68.

27. Wadl, P. A., Dean, D., Li, Y., Vito, L. M., Scheffler, B. E., Hadziabdic, D., Windham, M. T., and Trigiano, R. N. 2011. Development and characterization of microsatellites for switchgrass rust fungus (*Puccinia emaculata*). Conservation Genetics Resources 3:185-188.

28. Zale, J., Freshour, L., Agarwal, S., Sorochan, J., Ownley, B. H., Gwinn, K. D., and Castlebury, L. A. 2008. First report of rust on switchgrass (*Panicum virgatum*) caused by *Puccinia emaculata* in Tennessee. Plant Disease 92:1710-1710.

CHAPTER II

LITERATURE REVIEW

Switchgrass

Switchgrass (*Panicum virgatum* L.) is a warm-season (C4) perennial grass in the family *Poaceae* with some genotypes growing by rhizomes (22). The native range of switchgrass includes most of North America, with the exception of west of the Rocky Mountains (47). Due to the widespread ecological adaptation of switchgrass (climate and environment range), it has been adopted as a forage and pasture crop, an ornamental plant, to control erosion while providing habitat for wildlife, and holds promise as a biofuel feedstock crop (38).

The demand for cellulosic ethanol as a supplement to conventional fossil fuels and ethanol derived from food crops (e.g. corn, sorghum, sugarcane) has increased, resulting in a need for improved varieties for use in the biofuel industry. Initially, 34 herbaceous species were screened to assess their potential for biofuel production. *Panicum virgatum* was determined to be the best adapted crop plant, with high biomass yield and quality (64). The longevity and high yielding production of switchgrass, elevated switchgrass over other grasses such as sorghum or sorghum × sudangrass hybrids (64). Switchgrass is considered an energy crop by the United States Department of Energy (DOE), because of its potential for high cellulosic content and its facility to grow without intensive fertilization or crop management (10). Due to its extensive root system, switchgrass has been planted for soil stabilization and forage for cattle. However,

switchgrass phytochemical components (flavonoids and saponins) can be toxic in sheep, horses, and goats, causing photosensitivity and liver damage (53).

Switchgrass cultivation

Switchgrass is a warm season crop and the majority of its growth occurs during the summer months of June, July and early August. It is a perennial crop that can grow in large clumps to a height of 1.8 to 2.2 m. Switchgrass is adapted to wide-ranging climatic conditions and a variety of soils, tolerating moderate soil salinity and pH levels ranging from about 4.5 to 7.6, but, prefers deep sandy loams. Thus, switchgrass is found in dry, as well as, wet habitats and can tolerate winter temperatures as low as -30°C (64).

Switchgrass genotypes are classified into two ecotypes: upland and lowland types. Genotypes within either ecotype range in growth habit from caespitose (dense tufts) to rhizomatous (sod-forming). Lowland types favor bottomland and sandy loam soils and reach heights of 3.6 meters, while upland types are better suited to well-drained soils and grow to heights of 1.8 meters and are cold tolerant. Due to its higher yield potential, the lowland switchgrass ecotype has been considered optimal for biofuel production in the United States. Lowland cultivars, such as "Alamo" and "Kanlow", have been recommended by the University of Tennessee (27) for this purpose. Switchgrass can be propagated vegetatively (clonally) and through seed. Although self-pollination is possible (~1% fertile seed), switchgrass is considered an obligate 'outcrosser', and different genotypes must be present to act as male and female plants for efficient fertilization and seed production (38). The ability to produce switchgrass clones through vegetative propagation and, more recently, selfed inbred lines (42) have been important factors for improving the yield and cellulosic content of cultivars.

Switchgrass rust caused by *Puccinia emaculata* Schw.

Classification

Puccinia emaculata Schw. is an obligate parasite and a member of the order Pucciniales (Kingdom Fungi, Phylum Basidiomycota, Class Pucciniomycetes) (38, 60). The Pucciniales are parasites of plants, insects, or other fungi. The most species-rich group in this order are important, well-known plant pathogens causing rust diseases, named after the reddish color of their urediniospores. Before the availability of DNA sequence data, the Pucciniales were placed in various positions on the fungal tree of life. For instance, based on some of their ultrastructural characters (lack of clamp connections) and parasitic life style, Pucciniales and their relatives were often thought to represent an early diverging lineage of Basidiomycota. Rusts were often classified together with the smuts (Ustilaginales) and jelly fungi (Dacryomycetales, Tremellales and Auriculariales) (44; 63) or placed with the smuts in the class Teliomycetes, subclass Teliomycetidae (Teliosporae) (7; 59). Ultrastructural studies demonstrated that rusts are not closely related to smuts (2; 23). Also, in all cases of successful axenic culture of rust fungi, mycelial-type colonies were produced. There was no sign of the yeast-like growth, characteristic of smuts in artificial culture (59).

Molecular phylogenetic studies based on ribosomal DNA (rDNA) have shown that rusts and their closest relatives in the class Pucciniomycetes are a derived group within the subphylum Pucciniomycotina (2). However, the relationships between the orders in Pucciniomycetes and even between Pucciniomycetes and other classes in Pucciniomycotina remain unresolved and additional phylogenetic studies are needed. Species of *Puccinia* and the related genus *Uromyces* are the most abundant of the rust fungi. They occur on a large number of plants and can be collected anywhere in the world.

Species concept

Rust fungi are obligate parasites of vascular plants with highly complex life cycles. A distinctive characteristic of rust fungi is a life cycle with up to five morphologically and functionally different spore states. However, many species possess fewer spore stages. While many require two unrelated host plants to complete their life cycles (heteroecious life cycle), many species complete their life cycles on a single host plant (autoecious life cycle) (20). Rust fungi (Pucciniales) cause some of the most devastating and economically important plant diseases, and therefore, have been studied in greater detail than other members of the Pucciniomycotina. Approximately 7800 species of the Pucciniales have been described. The majority of described species occur in temperate regions of North America, Europe, Australia and New Zealand. Many new genera and species are still expected to be found in tropical and subtropical regions of South America, Africa and Southeastern Asia (11). Many species of rust fungi cause internationally important plant diseases of crucial crops, such as, stem rust (*Puccinia graminis* Pers.), leaf rust (*P. triticina* Erikss.), and stripe rust (*P. striiformis* Westend.) of wheat, coffee rust (*Hemileia vastatrix* Berk. & Broome) and many others (35).

Rusts occur on a broad range of host plants from ferns to gymnosperms to angiosperms, including many dicots and monocots. As obligate parasites, rust fungi are largely host-specific and recent studies suggest close coevolutionary relationships between rusts and their host plants. Through sophisticated parasitism, rust fungi obtain nutrients from living host cells causing little or no harm, at least in the early stages of development. Host-rust fungus relationships often give useful information for the phylogeny and classification of higher plants (50). Many well-studied species of rusts have been cultured successfully on artificial media, though not permanently (e.g. *Uromyces hobsoni*) (17; 31; 55; 62). In nature, rust fungi appear to survive only as obligate parasites of living plants.

Identification

Aspects of teliospore morphology have been used as major defining traits of genera of rust fungi (48). Teliospore septation has long been regarded as a distinguishing generic character in several rust families. On *Panicum* spp., *Uromyces graminicola* forms a single-celled teliospore, while *P. emaculata* forms a two-celled teliospore (29; 56). Reports of *U. graminicola* on switchgrass have been published; however, these reports were not well documented and consequently the reported diseases may have been caused by *P. emaculata* (65).

Uredinia of *P. emaculata* were described as epiphyllous to caulicolous, adaxial to amphigenous, with oblong urediniospores possessing cell walls cinnamon brown in color, 1.5 to 2.0 µm thick, finely echinulate with three to four equatorial pores (29; 56; 65). As well, telia were described as adaxial to amphigenous, epiphyllous to caulicolous, densely crowded to scattered, oblong, and dark brown to black. Teliospores were dark brown, two-celled, ellipsoid to oblong, 33.6 ± 4.8 µm long with an apical cell width of 17.5 ± 1.2 µm and basal cell width of 15.9 ± 2.5 µm. Teliospore walls were 1.5 to 2.0 µm thick at the sides and 4 to 6 µm apically. Teliospore pedicels were brown or colorless and up to approximately one length of the teliospore, 28.5 ± 7.4 µm (65). Teliospore morphology-based identification was confirmed by sequencing of the nuclear ribosomal internal transcribed spacer (ITS) region. Results showed that ITS sequences from *P. emaculata* were distinct from sequences for *Puccinia graminis* and *Puccinia striiformis* and it fell into a highly supported cluster containing *Puccinia asparagi*, *Puccinia andropogonis*, and *Puccinia sorghi* (56; 65). Currently, two sequences are available for *P. emaculata* in GenBank (accessions numbers: EU915294; KC515382), while no sequences are available from *U. graminicola*.

The aecial state of *P. emaculata* has not been observed recently, although several putative specimens from the 1890s-1900s are housed at the US National Fungus Collections (BPI).

Teliospores have been observed on switchgrass plants in nature, but sexual reproduction through spermagonia and aecia on an alternate host has not been reported (65). *Puccinia emaculata* is probably heteroecious and macrocyclic (five-spore life cycle) (Figure. 2.1). Although *P. emaculata* has not been confirmed to infect an alternate host (29; 60), flowering spurge (*Euphorbia corollata* L.) has been reported to be an aecial host of *P. panici* and *P. pammelii*, which are now considered synonymous with *P. emaculata*. Little is known about *P. emaculata*. The historically unclear etiology of this fungus has complicated the development of effective disease management strategies (24; 60).

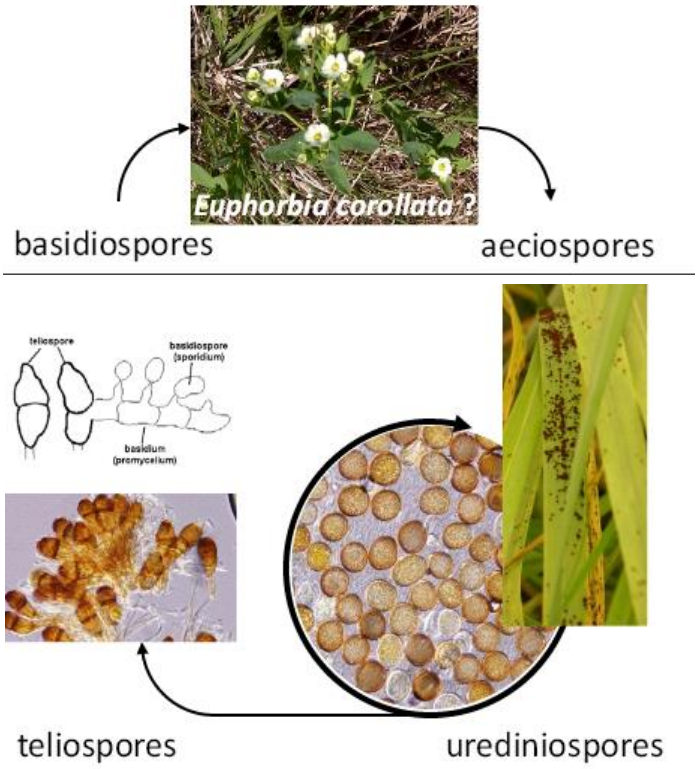

Figure 2.1. Adapted *P. emaculata* life cycle (S. Marek, unpublished)

Biology

The basic terminology of the five spore states of rust fungi was proposed by Anton de Bary and Edmond Tulasne during the 19th century and have since been modified by many mycologists (3; 21; 33; 49). However, the currently accepted spore names (spore-bearing

structure in parentheses) are as follows: basidiospores (produced on basidia), spermatia (produced in spermagonia), aeciospores (produced in aecia), urediniospores (produced in uredinia), and teliospores (produced in telia).

In heteroecious species, basidiospores and aeciospores must be disseminated between hosts and can only infect the other host. Urediniospores repetitively infect the same host, producing more uredinia, and exponentially increasing inoculum. This allows rapid asexual spread during a single growing season and also enables heteroecious species to persist on one host, even when the alternate host is not present (1; 4). This type of reproduction results in the characteristic polycyclic disease cycle of many grass rusts. As host tissues senesce at the end of a growing season, telia replace uredinia, and thick-walled, melanized teliospores are formed for survival in the absence of viable host tissue. As teliospores age, their nuclear state changes from dikaryotic to diploid. Environmental cues break teliospore dormancy resulting in the formation of basidia, meiosis, and haploid basidiospores production. Basidiospores are spread by the wind and infect the alternate host forming spermagonia and spermatia, haploid gametes. Fusion of spermatia with haploid receptive hyphae of compatible spermagonia restores the dikaryotic state of hyphae (19). Dikaryotic aecia are then formed on the underside of leaves and release aeciospores that are wind disseminated to the telial host. Aeciospores are often thicker-walled than the urediniospores.

Species of rust fungi are host-specific, being restricted to a single host species or closely related host species at particular stages of the life-cycle. When some populations within a species of a rust fungus attempt to infect closely related host species, minor morphological and/or physiological differences in the host may prevent successful infection (19; 20). Such specialized strains within a species may be designated as a "forma specialis" (plural: formae speciales) based on the host species on which it is still pathogenic, such as *Pucccinia striiformis* f. sp. *tritici*.

Phylogeny

In recent years, the taxonomy of basidiomycetes has been under major revision due to the application of molecular systematic techniques (54; 67). In the case of rust fungi, relatively few phylogenetic studies have been performed, compared to other fungi. This may be due to the obligate parasite life style, which makes isolating and maintaining pure cultures difficult. The first phylogenetic studies of rust fungi were based on 5.8S rDNA region sequences (28). Subsequently, studies were performed utilizing ITS sequences, generating information related to specific genera, such as *Puccinia*, *Uromyces*, *Cronartium* and *Peridermium* (37).

Aime et al. (2006) conducted a detailed study to determine the phylogenetic relationships among 52 rust fungi collected in Europe from the families: Pucciniaceae, Phragmidiaceae, Sphaerophragmiaceae, Uropyxidaceae, Chaconiaceae, Coleosporaceae, Cronartiaceae, Pucciniastraceae and Melampsoraceae. This work used a 535 bp region of the nuclear ribosomal large subunit (LSU) to infer relationships among rust fungi. The results confirmed the order Uredinales (now Pucciniales) is monophyletic. The genera *Puccinia, Uromyces, Cumminsiella* and *Endophyllum* have a common ancestor and separate from rust fungi that are pathogens of the Rosaceae family (*Phragmidium, Kuehneola, Triphragmium* and *Trachyspora*), which constitute another monophyletic group. Additionally, the genera *Puccinia, Uromyces, Pucciniastrum, Thekopsora* and *Uromyces* were demonstrated to be polyphyletic (2). In consequence, to clarify the polyphyletic or monophyletic nature of the genera *Puccinia* and *Uromyces*, two independent studies were completed using multilocus phylogenies comprised of the translation elongation factor 1α (TEF1a), β-tubulin, and LSU genes. These studies concluded that at least two major lineages can be inferred, which both include mixtures of *Puccinia* and *Uromyces* species. One lineage forms telia on plants of the family Poaceae, while the other forms telia on plants of the family Cyperaceae (43; 57). These studies suggest that rusts have coevolved with their hosts, and 'jumps' from one host to another have led to the evolution of new rust groups. Furthermore, some

studies suggest that host jumps could take place between unrelated hosts that occupy similar ecological niches (8; 57).

Uppalappati et al. (2012) reported the first phylogenetic study for *P. emaculata*. Switchgrass rust urediniospores were collected at several locations in Oklahoma from 2007 to 2011. Urediniospores were isolated from fields and maintained on susceptible upland switchgrass genotypes under growth chamber conditions. The ITS region was amplified from bulked urediniospores DNA, cloned and sequenced. The resulting sequences were distinct from sequences of *P. graminis.* Phylogenetic analyses were performed with similar ITS sequences from other rust fungi. This study reported that *P. emaculata* fell into a highly supported cluster with *P. asparagi, P. andropogonis,* and *P. sorghi* (56). Nonetheless, only a limited number of ITS sequences of rust fungi that infect native grasses are available and *P. emaculata* etiology is still unclear (1; 8).

DNA barcoding

Correct identification of a pathogen is essential for accurate disease diagnosis and implementation of crop management decisions (25; 26). Examination of microscopic features among similar species for identification of pathogenic fungi can be challenging due to the scarcity and/or plasticity of useful morphological features (6). Over the past 20 years, PCR and DNA sequencing have permitted phylogenetics-based classification of fungi (52). The enormous increase in the use of molecular methods to identify fungi and study their systematics has been attributed to the adoption of a rapid, cost-effective, and standardized PCR methods that allow amplification and sequencing of the ITS region of fungi (6; 25). The ITS region is now considered the ´DNA barcode´ for fungi (51), just as the mitochondrial cytochrome C oxidase I (COI) gene is considered the barcode for animals (32) and the plastid genes *rbcL* and *matK* are the barcodes for land plants (34). The term ‘DNA barcode’ refers to a locus occurring in a kingdom or phylum of organisms, which is sufficiently conserved to permit a high rate of

successful PCR amplification using 'universal' primers, but sufficiently variable to distinguish between species, facilitating identification, and with little variation within species (12; 51; 52). A comprehensive database of barcode sequences from diverse, representative species must also be available and maintained (e.g. NCBI). DNA barcodes are usually short DNA sequences (400-600 bp), just long enough to identify a particular organism to species (or at least genus) and a convenient length for optimal Sanger sequencing. Generally, the short lengths of the barcodes permit pairwise identification of species and can resolve some phylogenetic relationships at the family, genus and species levels. However, phylogenetic trees based on single barcode loci often lack sufficient support to be informative (6; 36).

In fungi, few genetic loci have been considered as barcodes. Initial phylogenetic and molecular identification studies of fungi used nuclear ribosomal genes (52). Bruns et al. (1991) described universal primers that are still used, especially for the amplification of three main components of the fungal ribosomal operon: 1) the large subunit of the ribosomal DNA (LSU; also referred to as 26S or 28S rDNA, which includes two variable subregions called D1 and D2; 2) the rDNA small subunit (SSU or 18S) (9); and 3) the rDNA ITS region, comprised of three sections: two variable transcribed spacers, ITS-1 and ITS-2, bracketing a conserved 5.8S region. Since the ITS region from some species can have a relatively large length (>1,000 bp), resulting in problems obtaining bidirectional sequences, some recent metagenomic studies have focused on either the ITS-1 or ITS-2 spacers (12; 52). The ITS barcode is an informative marker for species-level studies for most fungi, with some exceptions, such as cryptic species of genus *Debaryomyces* (14). ITS region varies in length among major taxonomic groups, due to abundant indels (insertions/deletions) among genera and species. In some species, these indels can be useful for molecular diagnostics. In some clades (e.g. species), it may be difficult to do alignments of ITS sequences, restricting their utility for phylogenetic reconstruction. This may due to non-uniform evolutionary selection pressure, influenced by the highly-conserved adjacent

rDNA (26). However, this problem has been corrected by analyzing additional loci, such as the following single-copy genes encoding proteins: β-tubulin (bTub), DNA-dependent RNA polymerase II subunits (RPB1, RPB2), chitin synthases, translation elongation factors (TEF1a), histone H3 and actin (39); as well as, two additional mitochondrial genes: cytochrome b (Cytb) and cytochrome c oxidase1 (CO1), which have been informative in numerous phylogenetic studies (11).

DNA barcodes, have the potential to resolve polyphyletic taxonomic groups and can be used to detect misidentifications due to imprecise morphological characters utilized to define species (25; 52). Thanks to years of DNA barcode studies, over 620,000 ITS sequences from fungi are available in on-line databases such as GenBank. Effectively using DNA barcodes for fungal identification requires integration of sequence analysis tools with field and laboratory data. Numerous software packages have been developed to analyze DNA barcodes, such as Geneious, ClustalX, Chromas Pro and SNAP. Such sequence analyses can be used to search world-wide fungal identification databases, create phylogenetic trees, and analyze intraspecific variation (12).

cDNA libraries, expressed sequence tags (ESTs), and RNA-seq

Messenger RNA (mRNA) isolated from cells, tissues, organisms or ecosystems, can be reverse transcribed to synthesize complementary DNA (cDNA) from the relevant biomaterials being investigated. The cDNA can then be labeled and used to probe microarrays or directly sequenced using next generation sequencing technology. The result is a catalog gene expression referred to as a transcriptome. Usually this process begins with total RNA, rather than mRNA, extracted from cells and used directly for cDNA generation. However, sometimes mRNA is selectively purified from total RNA prior to reverse transcription, so that cDNA will be enriched for protein-coding genes (41; 46). Creating cDNA is the essential first step of gene expression analysis, since all current sequencing methods require DNA and cannot sequence RNA directly.

cDNA production consists of two basic steps. First, separating and cleaning mRNA molecules from total cellular RNA. Second, mRNA molecules are reverse transcribed to cDNA using a reverse transcriptase (RNA-dependent DNA polymerase), usually purified from a retrovirus. Several methods exist for isolating and purifying mRNA from biological sources. A typical procedure would include, TRIzol® extraction of total RNA followed by purification on columns containing bound oligomeric deoxythymine nucleotides [oligo(dT)], which only binds mRNAs with poly(A) tails. The rest of the non-bound RNAs (tRNAs, rRNAs, snRNAs, etc.) are washed off the column. The bound mRNA is then eluted using a low salt elution buffer or, in some cases, heated RNAse-free water to break the hydrogen bonds between A-T base pairs. Eluted mRNA molecules are stored at -80C or used immediately for cDNA synthesis. The mRNA is reverse transcribed into cDNA using a reverse transcriptase (RT) and oligo (dT) primers, which bind to the mRNAs' poly(A) tails, providing exposed 3'-OH groups required for the initiation of cDNA synthesis by RT. The RT enzyme synthesizes complementary DNA strands resulting in a mRNA-DNA hybrid. The mRNA is removed with alkali or RNAse H treatment and single-stranded cDNAs (sscDNAs) are released. Adapter-mediated second strand synthesis then converts the sscDNA into double-stranded DNA using a DNA polymerase (13; 15). Libraries are created when double-stranded cDNAs are cloned into plasmids or phage particles and maintained in *E. coli*. By sequencing thousands or tens of thousands of these clones, a partial profile of gene expression, comprised of expressed sequence tags (ESTs), can be created.

ESTs are fragments of cDNA (mRNA) sequences derived from single sequencing reactions performed on randomly selected clones from cDNA libraries. ESTs are single-pass reads of approximately 200–800 base pairs (bp). Since they represent the expressed portion of a genome, ESTs have proven extremely useful for gene identification and verification of gene predictions. EST sequencing projects were often used as an alternative to whole genome

sequencing because of their lower costs (45). EST sequencing also has been shown to be an important genomic tool for identifying and characterizing traditionally 'anonymous' SSRs (16; 30; 40).

The concept of cDNA libraries has been extended and applied in RNA-Seq technology (18), a next generation DNA sequencing platform that sequences all of the cDNAs in a massively parallel process that can produce billions of reads simultaneously (Illumina.com). Thus, cDNA libraries of clones no longer have to be contructed. This new technology provides accurate quantitative measurements of each gene's expression and differential gene splicing, and facilitates the discovery of novel genes and gene regulation. However, individual cDNA sequence reads from RNA-Seq are often still referred to as ESTs. RNA-Seq has dramatically changed how transcriptomes are studied.

RNA-Seq technology has been used in various fungal studies, including the rust, such as *Puccinia triticina* and the closely related *P. striiformis* (41). In the case of *P. striiformis,* a full-length library was constructed from urediniospores, 196 random clones were sequenced, and the functions 51 genes were inferred to be involved in amino acid metabolism, cell defense, cell cycle, cell signaling, cell structure and growth were identified. Additionally, an EST sequencing project for *P. striiformis* identified potentially useful microsatellites and assessed their polymorphisms. Of the resulting 15 primers sets tested, 13 successfully amplified fragments of the sizes predicted from the ESTs (5). However, because the first strand of cDNAs represented in EST libraries are typically generated from the 3'-poly(A) end of mRNAs, the 5'-ends of transcripts can be missing resulting in partial cDNA sequences (41).

Simple Sequence Repeats (SSRs)

Simple sequence repeats (or microsatellites) are stretches of DNA, consisting of tandem repeats of mono, di, tri, tetra or pentanucleotide units, which are arranged throughout the

genomes of most eukaryotic species. Weber 1990 (60) developed a general approach for detecting polymorphic (variable length) microsatellites by PCR amplification from total genomic DNA using two unique primers that flank a defined microsatellite locus (61).

The standard methods for the isolation of SSRs involve the following: 1) the creation of a small insert genomic library, 2) library screening by hybridization, 3) DNA sequencing of positive clones, 4) primer design, and 5) locus-specific PCR analysis to identify polymorphisms. For the efficient generation of SSR-enriched libraries, various methods have been developed. These include a fragmentation of genomic DNA by sonication or endonuclease digestion, followed by ligation of adaptors, degenerate (nonspecific) primers and PCR analysis. Microsatellites containing fragments are enriched by hybridization to short length biotinylated SSR probes and hybridized SSRs subsequently isolated on streptavidin-conjugated magnetic beads. Bound single stranded SSRs are converted to double stranded DNA, primed by an oligonucleotide repeat, and are subsequently cloned and sequenced. Finally, locus specific primers are designed.

The two main advantages of microsatellites are the high informational content and the ease of genotyping. The ability to distinguish between closely related individuals is particularly important for many fungal species. Because of their simplicity, effectiveness, abundance, hypervariability and high reproducibility, SSRs can be used in the identification of genes responsible for special characteristics such as pathogenicity (16). SSRs have showed to be more effective than other molecular markers (e.g. Restriction fragment polymorphism [RFLP], Amplified fragment length polymorphism [AFLP]) in population genetic assays due to high levels of polymorphism. Microsatellites can be used to identify heterokaryons and have high discriminatory power for analyzing variation in the gene pools of fungi (66).

Wadl et al. 2011 (60), developed two microsatellite enriched libraries for *P. emaculata*. Microsatellite loci were characterized in the genomic DNA from bulked urediniospores collected from 20 single pustule isolates. Ten primer pairs were identified to amplify loci in the 20 *P. emaculata* isolates. All loci were polymorphic and allele numbers per locus ranged from 2 to 5. Observed heterozygosity ranged from 0.21 to 0.77. In preliminary experiments, the 10 SSR primer pairs described by Wadl et al. were tested on *P. emaculata* populations collected from five states during this investigation, and only three of the ten primer pairs produced amplifiable bands.

REFERENCES

1. Abbasi, M., Goodwin, S. B., and Scholler, M. 2005. Taxonomy, phylogeny, and distribution of *Puccinia graminis*, the black stem rust: new insights based on rDNA sequence data. Mycoscience 46:241-247.

2. Aime, M. C., Matheny, P. B., Henk, D. A., Frieders, E. M., Nilsson, R. H., Piepenbring, M., McLaughlin, D. J., Szabo, L. J., Begerow, D., Sampaio, J. P., Bauer, R., Weiss, M., Oberwinkler, F., and Hibbett, D. 2006. An overview of the higher level classification of Pucciniomycotina based on combined analyses of nuclear large and small subunit rDNA sequences. Mycoscience 98:896-905.

3. Arthur, J. C. 1905. Terminology of the spore structures in the Uredinales. Botanical Gazzete. 39:219-222.

4. Arthur, J. C., and Cummins, G.B., 1962. Manual of the rust in the United States and Canada. Hafner, New York. 438 p.

5. Bahri, B., Leconte, M., de Vallavieille-Pope, C., and Enjalbert, J. 2009. Isolation of ten microsatellite loci in an EST library of the phytopathogenic fungus *Puccinia striiformis* f. sp. *tritici*. Conservation Genetics 10:1425-1428.

6. Bergmann, T., Hadrys, H., Breves, G., and Schierwater, B. 2009. Character-based DNA barcoding: a superior tool for species classification. Berl Munch Tierarztl Wochenschr 122:446-50.

7. Bessey, E. A. 1950. Morphology and taxonomy of fungi. McGraw-Hill (Blakis-ton), New York.

8. Bhairi, S. M., Staples, R. C., Freve, P., and Yoder, O. C. 1989. Characterization of an infection structure-specific gene from the rust fungus *Uromyces appendiculatus*. Gene 81:237-43.

9. Borneman, J., and Hartin, R. J. 2000. PCR primers that amplify fungal rRNA genes from environmental samples. Applied and Environmental Microbiology 66:4356-4360.

10. Bouton, J. H. 2007. Molecular breeding of switchgrass for use as a biofuel crop. Current Opinion in Genetics & Development 17:553-558.

11. Bowman, B. H., Taylor, J. W., Brownlee, A. G., Lee, J., Lu, S. D., and White, T. J. 1992. Molecular evolution of the fungi: relationship of the basidiomycetes, ascomycetes, and chytridiomycetes. Molecular Biology and Evolution 9:285-96.

12. Bruns, T. D., White, T. J., and Taylor, J. W. 1991. Fungal molecular systematics. Annual Review of Ecology and Systematics 525-564.

13. Carninci, P., Kvam, C., Kitamura, A., Ohsumi, T., Okazaki, Y., Itoh, M., Kamiya, M., Shibata, K., Sasaki, N., Izawa, M., Muramatsu, M., Hayashizaki, Y., and Schneider, C. 1996. High-efficiency full-length cDNA cloning by biotinylated CAP Trapper. Genomics 37:327-336.

14. Casaregola, S., Weiss, S., and Morel, G. 2011. New perspectives in hemiascomycetous yeast taxonomy. Comptes rendus biologies 334:590-598.

15. Crick, F. 1970. Central dogma of molecular biology. Nature 227(5258), 561-563.

16. Chagné, D., Chaumeil, P., Ramboer, A., Collada, C., Guevara, A., Cervera, M. T., Vendramin, G. G., Garcia, V., Frigerio, J. M., Echt, C., Richardson, T., and Plomion, C. 2004. Cross-species transferability and mapping of genomic and cDNA SSRs in pines. Theoretical and Applied Genetics 109:1204-1214.

17. Coffey, M. D., and Shaw, M. 1972. Nutritional studies with axenic cultures of the flax rust *Melampsora lini* Physiol. Plant Pathology 2:37-46.

18. Costa, V., Angelini, C., and Ciccodicola, A. 2010. Uncovering the complexity of transcriptomes with RNA-Seq. BioMed Biotechnol 2010:853916.

19. Cummins, G. B. 1959. Illustrated genera of rust fungi. Burgess Publ. Co., Minneapolis.

20. Cummins, G. B., and Hiratsuka, Y. 2002. Illustrated genera of rust fungi. 3rd ed. American Phytopathological Society, 240 pp, St. Paul, Minnesota, USA.

21. Cunningham, G. H. 1930. Terminology of the spore forms and associated structures of the rust fungi. New Zealand J. Sci. Technol. 12:123-128.

22. Darke, R. 2007. Encyclopedia of grasses for livable landscapes. Portland, OR: Timber Press.

23. Donk.M.A. 1972. The heterobasidiomycetes: a reconnaissance II. Some problems connected with the restricted emendation. Pro K Ned Akad Wet Ser C, Biol Med Sci 75:376-390.

24. Duplessis, S., Cuomo, C. A., Lin, Y.-C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., Joly, D. L., Hacquard, S., Amselem, J., Cantarel, B. L., Chiu, R., Coutinho, P. M., Feau, N., Field, M., Frey, P., Gelhaye, E., Goldberg, J., Grabherr, M. G., Kodira, C. D., Kohler, A., Kües, U., Lindquist, E. A., Lucas, S. M., Mago, R., Mauceli, E., Morin, E., Murat, C., Pangilinan, J. L., Park, R., Pearson, M., Quesneville, H., Rouhier, N., Sakthikumar, S., Salamov, A. A., Schmutz, J., Selles, B., Shapiro, H., Tanguay, P., Tuskan, G. A., Henrissat, B., Van de Peer, Y., Rouzé, P., Ellis, J. G., Dodds, P. N., Schein, J. E., Zhong, S., Hamelin, R. C., Grigoriev, I. V., Szabo, L. J., and Martin, F. 2011. Obligate biotrophy features unraveled by the genomic analysis of rust fungi. Proceedings of the National Academy of Sciences, 108.22:9166-9171.

25. Feau, N., Vialle, A., Allaire, M., Maier, W., and Hamelin, R. C. 2011. DNA barcoding in the rust genus *Chrysomyxa* and its implications for the phylogeny of the genus. Mycologia 103:1250-66.

26. Feau, N., Vialle, A., Allaire, M., Tanguay, P., Joly, D. L., Frey, P., Callan, B. E., and Hamelin, R. C. 2009. Fungal pathogen (mis-) identifications: a case study with DNA barcodes on *Melampsora* rusts of aspen and white poplar. Mycol Res 113:713-24.

27. Garland, C. D. 2008. Growing and harvesting switchgrass for ethanol production in Tennessee. University of Tennessee, Cooperative extension report SP701-A, 2007. Internet site: http://economics.ag.utk.edu/publications/bioenergy/SP701-A.pdf. [accessed July 2013].

28. Gottschalk, M., and Blanz, P. A. 1984. Highly conserved 5S ribosomal RNA sequences in four rust fungi and atypical 5S rRNA secondary structure in *Microstroma juglandis*. Nucleic Acids Res 12:3951-8.

29. Gustafson, D. M., Boe, A., and Jin, Y. 2003. Genetic variation for *Puccinia emaculata* infection in switchgrass. Crop Science 43:755-759.

30. Gutierrez, M. V., Vaz Patto, M. C., Huguet, T., Cubero, J. I., Moreno, M. T., and Torres, A. M. 2005. Cross-species amplification of *Medicago truncatula* microsatellites across three major pulse crops. Theoretical and Applied Genetics 110:1210-1217.

31. Harvey, A. E., and Grasham, J. L. 1970. Growth of *Cronartium ribicola* in the absence of physical contact with host. Can.J. Bot. Rev 48:71-73.

32. Hebert, P. D., Cywinska, A., and Ball, S. L. 2003. Biological identifications through DNA barcodes. Proceedings of the royal society of London. Series B: Biological Sciences 270:313-321.

33. Hiratsuka, Y. 1973. The nuclear cycle and the terminology of spore states in Uredinales. Mycologia 65:432-443.

34. Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., Chase, M. W., Cowan, R. S., Erickson, D. L., and Fazekas, A. J. 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences 106:12794-12797.

35. Kirk, P. M., Cannon, P. F., Minter, D. W., and Stalpers, J. A. 2008. Dictionary of the fungi. 10th ed. CABI, Wallingford, UK.

36. Kress, W. J., and Erickson, D. L. 2008. DNA barcodes: genes, genomics, and bioinformatics. Proceedings of the National Academy of Sciences 105:2761-2.

37. Kropp, B. R., Hansen, D. R., Wolf, P. G., Flint, K. M., and Thomson, S. V. 1997. A Study on the phylogeny of the dyer's woad rust fungus and other species of *Puccinia* from crucifers. Phytopathology 87:565-571.

38. Lemus, R., Brummer, E. C., Moore, K. J., Molstad, N. E., Burras, C. L., and Barker, M. F. 2002. Biomass yield and quality of 20 switchgrass populations in southern Iowa, USA. Biomass and Bioenergy 23:433-442.

39. Leslie, J. F., Zeller, K. A., Logrieco, A., Mule, G., Moretti, A., and Ritieni, A. 2004. Species diversity of and toxin production by *Gibberella fujikuroi* species complex strains

isolated from native prairie grasses in Kansas. Applied and Environmental Microbiology 70:2254-2262.

40. Liewlaksaneeyanawin, C., Ritland, C., El-Kassaby, Y., and Ritland, K. 2004. Single-copy, species-transferable microsatellite markers developed from loblolly pine ESTs. Theoretical and Applied Genetics 109:361-369.

41. Ling, P., Wang, M., Chen, X., and Campbell, K. 2007. Construction and characterization of a full-length cDNA library for the wheat stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*). BMC genomics 8:1-13.

42. Liu, L., and Wu, Y. 2012. Development of a genome-wide multiple duplex-SSR protocol and its applications for the identification of selfed progeny in switchgrass. BMC genomics 13:522.

43. Maier, W., Wingfield, B. D., Mennicken, M., and Wingfield, M. J. 2007. Polyphyly and two emerging lineages in the rust genera *Puccinia* and *Uromyces*. Mycological research 111:176-185.

44. Martin, G. W. 1950. "Outline of fungi". Univ, Ia. Stud. Nat. Hist. 18:1-40.

45. Parkinson, J., and Blaxter, M. 2009. Expressed sequence tags: an overview. Methods Molecular Biology 533:1-12.

46. Peng, W., Takabayashi, H., and Ikawa, K. 2007. Whole genome amplification from single cells in preimplantation genetic diagnosis and prenatal diagnosis. European journal of obstetrics & gynecology and reproductive biology 131:13-20.

47. Pedroso, G., De Ben, C., Hutmacher, R., Orloff, S., Putnam, D., Six, J., & Linquist, B. 2011. Switchgrass is a promising, high-yielding crop for California biofuel. California agriculture, 65(3), 168-173.48.

48. Ramachar, P., and Cummins, G. 1963. The species of *Uromyces* on the tribe Paniceae. Mycopathologia et Mycologia Applicata 19:49-61.

49. Savile, D. B. O. 1968. The case against "Uredinium". Mycologia 60:459-464.

50. Savile, D. B. O. 1979. Fungi as aids in higher plant classification. Bot. Rev 45:377-503.

51. Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Bolchacova, E., Voigt, K., and Crous, P. W. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proceedings of the National Academy of Sciences 109:6241-6246.

52. Seifert, K. A. 2009. Progress towards DNA barcoding of fungi. Molecular Ecology Resources. Suppl s1:83-9.

53. Stegelmeier, B., Elmore, S., Lee, S., James, L., Gardner, D., Panter, K., Ralphs, M., Pfister, J., and Wierenga, T. 2007. Switchgrass (*Panicum virgatum*) toxicity in rodents, sheep, goats and horses. Poisonous plants: global research and solutions 113-117.

54. Swann, E. C., and Taylor, J. W. 1995. Phylogenetic perspectives on basidiomycete systematics: evidence from the 18S rRNA gene. Canadian Journal of Botany 73:862-868.

55. Turel, F. L. 1969. Saprophytic development of flax rusts, *Melampsora lini*, race No. 3. Can.J. Bot. Rev 47:821-823.

56. Uppalapati, S. R., Serba, D. D., Ishiga, Y., Szabo, L. J., Mittal, S., Bhandari, H. S., Bouton, J. H., Mysore, K. S., and Saha, M. C. 2012. Characterization of the rust fungus, *Puccinia emaculata*, and evaluation of genetic variability for rust resistance in switchgrass populations. BioEnergy Research. 6:458-68.

57. van der Merwe, M., Ericson, L., Walker, J., Thrall, P. H., and Burdon, J. J. 2007. Evolutionary relationships among species of *Puccinia* and *Uromyces* (Pucciniaceae, Uredinales) inferred from partial protein coding gene phylogenies. Mycol Res 111:163-75.

58. Vánky, K. 1999. The new classificatory system for smut fungi, and two new genera. Mycotaxon 70:35-49.

59. Von Arx, J. A. 1970. The genera of fungi sporulating in pure culture. 2nd Ed. Lehre: J. Cramer.

60. Wadl, P. A., Dean, D., Li, Y., Vito, L. M., Scheffler, B. E., Hadziabdic, D., Windham, M. T., and Trigiano, R. N. 2011. Development and characterization of microsatellites for switchgrass rust fungus (*Puccinia emaculata*). Conservation Genetics Resources 3:185-188.

61. Weber, J. L. 1990. Human DNA polymorphisms and methods of analysis. Current opinion in biotechnology 1:166-171.

62. Williams, P. G., Scott, K. J., and Kuhl, J. L. 1966. Vegetative growth of *Puccinia graminis* f.sp.*tritici* in vitro. Phytopathology 56:1418-1419.

63. Wolf, F. A., and Wolf, F. T. 1949. The Fungi. Wiley, New York, Vol. 2, p. 538.

64. Wright, L. 2007. Historical perspective on how and why switchgrass was selected as a "model" high-potential energy crop. ORNL/TM-2007/109 Oak Ridge, TN: Bioenergy resources and engineering systems.

65. Zale, J., Freshour, L., Agarwal, S., Sorochan, J., Ownley, B. H., Gwinn, K. D., and Castlebury, L. A. 2008. First report of rust on switchgrass (*Panicum virgatum*) caused by *Puccinia emaculata* in Tennessee. Plant Disease 92:1710-1710.

66. Zane, L., Bargelloni, L., and Patarnello, T. 2002. Strategies for microsatellite isolation: a review. Mol Ecol 11:1-16.

67. Zuluaga, C. M., and Cespedes, J. C. 2009. Generalidades de los uredinales (Fungi: Basidiomycota) y de sus relaciones filogenéticas fundamentales. Acta biologica colombiana. 14:41-56.

CHAPTER III

MULTILOCUS PHYLOGENY AND HAPLOTYPE DIVERSITY OF DNA BARCODES IN *Puccinia emaculata*

ABSTRACT

Rust disease caused by *Puccinia emaculata* can significantly reduce the biomass yield and biofuel feedstock quality of switchgrass. Four other *Puccinia* species have been reported as causing switchgrass rust, but two are now considered synonyms of *P. emaculata*. The purpose of this study was to use three "DNA barcodes" loci, ITS, TEF1a, and bTub, to assess the phylogenetic status, genetic diversity and haplotype distribution of *P. emaculata* urediniospores collected from cultivated switchgrass grown in Iowa, Mississippi, Oklahoma, South Dakota, and Virginia. Barcodes were amplified and the PCR products cloned and sequenced. At least five clones per spore collection were sequenced. Phylogenetic analyses based on single spore multilocus sequences strongly supported the monophyletic status of *P. emaculata*. Intraspecific variation among and within populations was observed. Numerous haplotypes of each barcode were present in each state population (ITS=14; bTub=24; TEF1a= 27), which differed in geographic distribution. The bTub and TEF1a haplotypes displayed mostly local distributions; while ITS haplotypes were distributed both in multiple states and locally. Prolonged propagation of urediniospores on plants under growth chamber conditions appeared to reduce barcode haplotype diversity. Future studies will examine the phylogeography, population structure, and pathogenicity variation within *P. emaculata*.

INTRODUCTION

The switchgrass rust fungus, *Puccinia emaculata* Schw., is an obligate parasite and a member of the order Pucciniales (3; 5). Switchgrass (*Panicum virgatum* L.) is a perennial warm-season grass native to North America. Switchgrass is used for forage production, erosion control, and as a renewable biomass feedstock source for cellulosic biofuel production. Epidemics of rust on switchgrass can reduce feedstock quality, biomass quantity, and seed production, resulting in economic losses (16)

The uredinial stage of *P. emaculata* is commonly observed on switchgrass and urediniospores can act as primary inoculum after long distance dissemination, and as secondary (repeating) inoculum within fields and regions. A first report of *P. emaculata* rust pustules on switchgrass in Tennessee was published in December 2008 (39). Subsequently, rust has been reported in Arkansas on 'Alamo' where 25% to 100% of switchgrass leaves were infected with *P. emaculata* (20). Rust has also been observed in numerous states throughout the southeastern United States, west into Texas and as far north as South Dakota. Oklahoma, a state with major cellulosic bioenergy research, has been impacted severely by switchgrass rust in the past five years (35).

Infection of switchgrass (primary or uredinial-telial host) by *P. emaculata* occurs when wind-blown aeciospores or urediniospores land on switchgrass leaves. Following deposition on switchgrass leaves, a spore forms a dikaryotic germ tube, which penetrates through stomatal openings (24). Uredinia usually form 7-10 days after inoculation. Telia form on senescing leaves in the late summer and fall. In telia, teliospores form and mature and nuclei within teliospores undergo karyogamy. In the spring, overwintered teliospores germinate, undergo meiosis, forming basidia, and air borne, haploid basidiospores that infect the alternate host. The alternate host is believed to be a species within the family Euphorbiacae (spurges), which, presumably, is infected

by the basidiospores resulting in formation of spermatia in spermagonia. Fusion of compatible spermatia and receptive hyphae re-establishes dikaryotic hyphae, completing the sexual life cycle of *P. emaculata*, resulting in aeciospores. Later in the spring or summer, windborne aeciospores apparently re-infect switchgrass plants, resulting in more urediniospores (29; 39). Two other species of rust fungi, *P. pammelii* (Trel.) Arthur and *P. panici* Dietel & Holw., also have been reported to infect switchgrass and the alternate hosts, *Euphorbia corollata* L. and *E. marginata* Pursh. Both *P. pammelii* and *P. panici* are now considered synonyms of *P. emaculata* (4). However, attempts to inoculate *E. corollata* with *P. emaculata* were not successful (4).

Host species and aspects of teliospore morphology have been used to define species of rust fungi. However, teliospore morphology can vary within species and overlap across species of rust fungi, making identification ambiguous for many species. Teliospores of *P. emaculata* are described as dark brown, two-celled, ellipsoid to oblong, 33.6 ± 4.8 μm long with an apical cell width of 17.5 ± 1.2 μm and basal cell width of 15.9 ± 2.5 μm. Teliospore walls are 1.5 to 2.0 μm wide at the sides and 4 to 6 μm apically. Teliospore pedicels are brown or colorless and up to approximately one length of the teliospore, 28.5 ± 7.4 μm (17; 29).

Due to the difficulty of identifying rust fungi to species, molecular techniques have been developed to accurately identify rust fungi. Two nuclear ribosomal internal transcribed spacer (ITS) region sequences are available for *P. emaculata* at NCBI (EU915294; KC515382). Uppalapati et al. (2012) reported a phylogenetic analysis of *P. emaculata* based on ITS sequences from urediniospores collected from Oklahoma and included other *Puccinia* spp. with similar ITS sequences as outgroups. Results showed *P. emaculata* is distinct from *P. graminis*, *P. triticina* and *P. striiformis* (cereal rusts) and grouped in a well-supported clade with *P. asparagi*, *P. andropogonis*, and *P. sorghi* (35). More molecular information is required to understand *P. emaculata* evolution, population structure and geographic distribution. Thus, the objectives of this study were to use sequences of three "DNA barcode" loci (ITS, β-tubulin [bTub], and translation

elongation factor-1α [TEF1a]) from urediniospores collected from cultivated switchgrass plants grown in five of the United States: Iowa, Mississippi, Oklahoma, South Dakota, and Virginia to assess the phylogenetic status, genetic diversity, and haplotype distribution of *P. emaculata*.

MATERIALS AND METHODS

Collection and maintenance of rust fungi

Puccinia emaculata urediniospores were collected from symptomatic switchgrass leaves from fields in Iowa, Mississippi, Oklahoma, South Dakota, and Virginia, in 2011 (Table 3.1) using vacuum spore collectors. Urediniospores were collected into 2 ml screw cap microfuge tubes attached to the spore collectors with tygon tubing. Collected urediniospores were dried for 2 days at room temperature over silica gel for long term storage at -80°C. Oklahoma urediniospores were collected in 2009, and since then urediniospores have been propagated under growth chamber conditions as described below. In this study, Oklahoma urediniospores harvested from symptomatic switchgrass plants under growth chambers in 2011 were used.

To increase *P. emaculata* urediniospores of collected populations, urediniospores were inoculated onto switchgrass seedlings, as follows. Urediniospores were suspended in inoculation solution (spreader sticker 0.05% [Hi-Yield, VPG, Bonham, TX], 10 mg/L Benlate SP [E.I. du Pont de Nemours and Co. Wilmington, DE], 1 mM nonanol) and sprayed onto switchgrass (cv. Dacotah) plants (5 weeks old) using a spore suspension atomizer (7). Inoculated plants were covered by porous plastic bags (measuring 15x25x25 cm; microperforated polypropylene bread bags) (Figure 3.1, A), then incubated in a dew chamber at 22-27°C overnight and transferred to a growth chamber at 27°C with a 12-h photoperiod, for symptom development.

After 15 days, single pustules were isolated from each population and re-inoculated under axenic conditions on switchgrass seedlings (a month old; cv. Dacotah) planted in autoclaved soil and magenta boxes (two coupled GA-7 Magenta boxes) (Figure 3.1, B, C). Inoculated axenic

seedlings in magenta boxes were incubated in a dew chamber and then in a growth chamber, as described previously. Urediniospores produced on axenic seedlings were then inoculated onto another set of switchgrass seedlings (cv. Dacotah) under the same conditions. The resulting increased urediniospores from each population were collected and stored dry over silica gel at −80°C. Urediniospores of *P. andropogonis* (PA), *P. sorghi* (PS) *P. striiformis* f. sp. *tritici* (PST), and *P. triticina* (PT) were collected in Oklahoma and stored, as above. These rust fungi were used as outgroups in phylogenetic analyses (Table 3.1).

Figure 3.1. Cultural methods used in this study. A. Switchgrass seedlings (cv. Dacotah) inoculated with urediniospores and covered with porous plastic bags. B. Isolation of single pustules using a microvaccum spore collector. C. Inoculated plants growing in magenta boxes.

Table 3.1. Descriptions of urediniospore collections of *Puccinia emaculata* and other rust fungi used in this study and DNA isolation methods used.

***Puccinia emaculata* collections**	**Code**	**Switchgrass cv. or host**	**Origin**	**Collection year**	**DNA Isolation**	
					Genomic DNA[a]	**Single spore[b]**
Pe-ISU	ISU1	Cave in Rock	ISU Woodruff Farm, Story Co., IA	2011	✓	✓
	ISU9		ISU Sorenson Farm, Story Co., IA	2011	✓	
Pe-MISS	MISS1	Alamo	MSU H.H. Leveck Animal Research Center, Starkville, MS	2011	✓	✓
	MISS2	Alamo Lowland		2011	✓	
Pe-SD	SDSU	Dacotah	SDSU Experiment Station Farm (Felt Farm), Brookings, South Dakota, USA	2011	✓	✓
Pe-OK	OSU09-1	Kanlow, Cave in Rock, Blackwell, Dacotah	OSU Agronomy Farm 2009, maintained 2 years in growth chamber, Stillwater, OK	2011	✓	✓
	OK-NF	Dacotah	Noble Foundation Agricultural Farm, Ardmore , OK	2011	✓	
Pe-VT	VT1-1	Cave in Rock	VT, Kentland Farm Research Center, VA	2011	✓	✓
Outgroup rust species						
P. andropogonis	PA	*Andropogon gerardii*	Robber's Cave State Park, Wilburton, OK	2013	✓	
P. sorghi	PS	*Zea mays*	Eakley, OK	2013	✓	
P. striiformis f. sp. *tritici*	PST	*Triticum aestivum*	OSU Agronomy Farm, Stillwater, OK	2013	✓	
P. triticina	PT	*Triticum aestivum*	OSU Agronomy Farm, Stillwater, OK	2013	✓	

[a] Genomic DNA extracted from multiple bulked urediniospores

[b] Whole genome amplification of DNA from single germinated urediniospores

Isolation of genomic DNA

DNA was extracted from urediniospores according to Weising (38), with slight modifications. One mL of extraction buffer (140 mM sorbitol, 220 mM Tris-HCl, pH 8.0, 22 mM EDTA, 800 mM NaCl, 0.8% cetyltrimethylammonium bromide [CTAB], and 1% sodium dodecyl sulfate [SDS]) was added to 10 mg of collected urediniospores in 2 ml screwcapped microtubes containing 3 mm glass beads and 0.5 mm zirconium beads. Samples were then homogenized in a bead beating instrument (FastPrep®-24 Instrument, MP Biomedicals, Santa Ana, CA) for 20 sec at 4 m/s. Bead beating was repeated three times. To each tube, 0.5 mL of chloroform: isoamyl alcohol (24:1, v/v) was added and incubated in a Thermomixer (Eppendorf AG, Hamburg, Germany) at 55°C shaking at 350 RPM for 30 min. The organic phase was separated by centrifugation at 12,000×*g* at 4°C for 20 min. The upper aqueous phase was transferred to a new tube, to which 700 µL isopropanol was added, mixed gently and DNA precipitated at -20°C for 30 min. DNA was pelleted by centrifugation (12,000×*g* at 4°C for 20 min), washed with 1 ml 70% ethanol and centrifuged again. The pellet was air dried and resuspended in 100 µL of TE (10 mM Tris-HCl, pH 7.4; 1 mM EDTA) (38). DNA quantity and quality were quantified by spectrophotometry using a NanoDrop ® 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and confirmed by TAE-agarose (1.5%) gel electrophoresis.

Single spore isolation and whole genome amplification

A modified cylinder loop-needle method was adapted from Heldebrand (19) and used to isolate single urediniospores of *P. emaculata*. Briefly, micropipettes were constructed from glass capillary tubing (Globe Scientific, Inc. Paramis, NJ, USA), which was exposed to a microflame and drawn out to a very fine bore, and fitted with a pipette bulb (Globe Scientific, Inc. Paramis, NJ, USA). Collected urediniospores were spread on 2% water agar in 9 cm petri dishes. After 2 hours, the petri dish was moved to the stage of a microscope and germinating single urediniospores were identified. Five single spores from each of the five populations (*n = 25*)

were collected and individually transferred to 10 µL of whole genome amplification (WGA) sample buffer.

WGA of single spores was carried out using Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) per manufacturer's instructions with modifications. Briefly, a germinated single spore was suspended in 10 µL of sample kit buffer and heated to 95°C for 10 minutes, then cooled on ice. Reaction buffer (9 µL) and enzyme mix (1 µL) were added as described for the kit. After the WGA reaction finished, 5 µl of the product were run on a TAE 1.5% agarose gel to verify WGA had occurred. A subsample of WGA products were treated with ExoSAP-IT® reagent (USB Corporations, Cleveland, OH, USA), according to manufacturer's instructions, to remove WGA primers, and stored at -20°C.

PCR, amplicon cloning, and sequencing

Three genetic loci were amplified from genomic DNA and single spore WGA DNA. Total PCR reaction volume was 20 µL (10 µL of GoTaqGreen Master Mix [Promega, Madison, WI], 1 µL of each primer [5 µM, Table 3.2], 2 µL of DNA [25 ng/µL] and 6 µL of sterile nuclease-free water). First, a ~1,250 bp region of the rDNA (partial 5.8S-ITS-2-partial LSU) was amplified using the primer pair, Rust1 (26) and PuccF2 (B. Olson, *unpublished data*; 14) with the following 2-stage program: 95°C for 10 min, followed by 10 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 4 min, followed by 20 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. Primers used to amplify bTub (1000 bp) and TEF1a (870 bp) loci were published previously (2; 15; 22; 37). The bTub locus was amplified in a 20 µL PCR with the following 2-stage program: 95°C for 10 min, followed by 10 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 4 min, followed by 20 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The TEF1a locus was amplified in 20 µL reactions using the following program: 96°C for 7 min, followed by 31 cycles of 94°C for 40 s, 56°C for 40 s and 72°C for 1.4 min, and a final extension of 72°C for

10 min. PCR products were confirmed by electrophoresis on a 1.5% agarose TAE gel. PCR products were cleaned using ExoSAP-IT® reagent (USB Corporations, Cleveland, OH, USA) and stored at -20°C.

Table 3.2. Primer sequences and source references used to amplified barcode genes from *Puccinia* species in this study.

Gene	Primers	References
ITS2-LSU	Rust1: 5'-GCTTACTGCCTTCCTCAATC -3' PuccF2: 5'- CAATGGATCTCTAGGCTCTC -3'	(14 ; 25)
bTub	Tub1510: 5'-GGTCCGATCTGGCGCCTTCG -3' Tub2435: 5'-GAGGAGCAATGTCACAGTGGGCA -3'	(37)
TEF1a	EfBasidF1: 5'-GTGCGGTGGTATCGACAAGC -3' EfBasidR: 5'-CATGTTGTCACCGTGCCATCC -3'	(37)

In order to obtain homologous bidirectional sequences PCR products amplified from genomic DNA samples were gel purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific Biosciences, Inc., Pittsburgh, PA, USA) and TA-cloned into a pGEM®-T Easy Vector System (Promega Corporation, Madison, WI, USA), each performed according to manufacturer's instructions. Plasmids containing clones were transformed into *E. coli* DH5α competent cells (18). For each locus from each rust sample at least five independent plasmid clones were selected. Plasmid clones (n = 40) were screened by colony PCR using vector promoter primers SP6 and T7. Transformant products were confirmed by electrophoresis on 1.5% agarose TAE gel, cleaned with ExoSAP-IT® reagent (USB Corporations, Cleveland, OH, USA), and then submitted to the Recombinant DNA/Protein Resource Facility at Oklahoma State University for automated sequencing.

Haplotype and phylogenetic analyses

Sequences from cloned loci amplified from genomic DNA from spore collections were used for haplotype analyses. Multiple sequence alignments of each locus were created in MEGA

5.05 (34) and sequences were collapsed into unique haplotypes using the SNAP Map tool within SNAP Workbench 2.0 (by Price et al., 2005) and used to construct maximum likelihood (ML) phylogenies.

Sequences from cloned loci amplified from single-spore WGAs were used in multilocus phylogenetic analyses. Non-coding DNA sequences (ITS) were aligned using Muscle (12) and refined manually. Protein-coding sequences (bTub, TEF1a) were aligned using MEGA 5.05 (34), with the amino acid option of Muscle (12). Ambiguous alignments for each locus were excluded using Gblocks 0.91b with default parameters on the Gblocks server (8). ITS, bTub, and TEF1a sequences from single-spore WGAs were partitioned by locus and concatenated into a single matrix using SequenceMatrix 1.7.8 (36). Phylogenetic trees based on aligned single loci and concatenated loci were created and analyzed in MEGA 5.05 using maximum likelihood (ML) and Bayesian algorithms. A general time reversible model with gamma distributed rate heterogeneity and an inferred proportion of invariable sites (GTR+GAMMA+I) was used in all the analyses. ML analysis was performed using RAxML v 7.3.1 (32) online at the CIPRES science gateaway (27) where a fast bootstrapping analysis and the search for the best-scoring ML tree was performed in a single program run and Bayesian analysis.

RESULTS

Barcode loci amplification

All barcode loci amplified successfully using either genomic DNA of collected urediniospores or single spore WGAs as template DNA. ITS, bTub, and TEF1a PCRs produced amplicon lengths of ~1,200 bp, ~1,000 bp, and ~800 bp, respectively, and each resolved as single bands on agarose gels. However, efforts to obtain high quality bidirectional gene sequences (complementary contigs) of the three loci amplified directly from the genomic DNA of bulked urediniospores resulted in partial sequences. Single-strand sequences formed poor quality,

disrupted contigs even though PCR products formed uniform single bands on gels. It was hypothesized that heterogeneous templates, due to heterozygous alleles present in collected spore populations, created competing amplicons with frame shifting indels, which disrupted sequencing. To reduce the heterogeneity of the template DNA, the following two approaches were assessed to produce high quality homozygous sequencing templates: 1.) PCR products were TA-cloned and clones sequenced or 2.) obtain single-spore cultures of *P. emaculata* and directly amplify and sequence barcodes.

In the first approach, PCR products were subcloned into a TA-cloning plasmid. Single colonies containing the expected insert were re-amplified and sequenced. This approach resulted in high quality, bidirectional contigs that permitted analysis of *P. emaculata* barcodes. For example, alignment of twenty-five ITS contigs, covering part of the ITS region and LSU, from sample OSU09-1 (multispore DNA sample), resulted in a consensus of polymorphic sequences with 37 single nucleotide polymorphisms (SNPs) and 8 indels and showed 98.9% identity with the *P. emaculata* ITS sequence EU915294 available in GenBank (NCBI). On the other hand, alignment of five contigs of bTub and five contigs of TEF1a from sample OSU09-1 produced consensus sequences with no polymorphisms among clones (i.e. monomorphic).

The second approach was to propagate homogeneous cultures of rust fungus through repeated, axenic single-pustule transfers, which should approximate single-spore isolation. However, after amplifying and sequencing each barcode locus from several single pustule urediniospore collections from OSU09-1 and VT1-1 no bidirectionally high quality contigs at any of the loci could be obtained (data not shown). Therefore, efforts were refocused on obtaining sequences from single spore WGAs.

All barcode loci successfully amplified directly from each single spore WGA (25/25). However, upon sequencing, partial-contig coverage of loci was observed in some single-spore

barcoding products, again preventing assembly of bidirectionally high quality contigs. This result implied some single spores were heterozygous at the barcode loci, with different alleles present in each haploid nucleus comprising its dikaryotic genome (i.e. N+N per urediniospore). In these cases, PCR products were TA-cloned and sequenced as previously described.

Sequence similarity of *Puccinia emaculata* loci to rust fungi in Genbank.

All TA-cloned barcode loci were used to perform BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches of Genbank (NCBI). ITS sequences from collected switchgrass rust urediniospores identified the unique rDNA sequence of *P. emaculata* (EU915294; KC515382) as the most similar (99% identity). No sequences of bTub and TEF1a from *P. emaculata* are currently available at NCBI. However, BLAST searches using bTub sequences of *P. emaculata* identified rust fungi *Uromyces inaequialtus* (97%, host: *Silene* spp., EF570855.1), *P. sorghi* (96%, host *Zea mays*, HM452904.1), and *U. polygoni-avicularis* (95%, host: *Polygonum* spp., EF570857.1) as the most similar. BLAST searches of TEF1a from *P. emaculata* identified *U. polygoni-avicularis* (93% ID, EU982006.1) as the most similar.

Phylogenetic analyses and haplotype diversity and geographic distribution of three DNA barcodes from genomic DNA of urediniospores

Phylogenetic analyses of individual barcode loci amplified, cloned, and sequenced from genomic DNA from bulked urediniospores from switchgrass rust specimens strongly supported the monophyletic status of *P. emaculata*. As sequences originated from multiple urediniospores, barcode loci could not be concatenated for a multilocus phylogeny. A phylogenetic tree constructed from ITS sequences showed *P. emaculata* formed a well-supported (maximum likelihood bootstrap proportion [MLBP] 97%) monophyletic group among switchgrass rust collections and formed another well-supported (MLBP 88%) group with *P. sorghi* and *P. andropogonis*, rust fungi infecting C4 grass hosts and *P. asparagi*, which infects asparagus

(Figure. 3.2). The group into which *P. emaculata* fell was only distantly related to the rust fungi infecting C3 grasses (e.g. cereals). ML trees based on TEF1a and bTub sequences also strongly supported (MLBP 100%) the monophyly of *P. emaculata* (data not shown). In the TEF1a phylogeny, *P. emaculata* formed a well-supported (MLBP 100%) group with *U. polygoni-avicularis*. And, in the bTub phylogeny, *P. emaculata* formed a well-supported (MLBP 100%) group with *U. inaequialtus*, *P. sorghi*, and *U. polygoni-avicularis*.

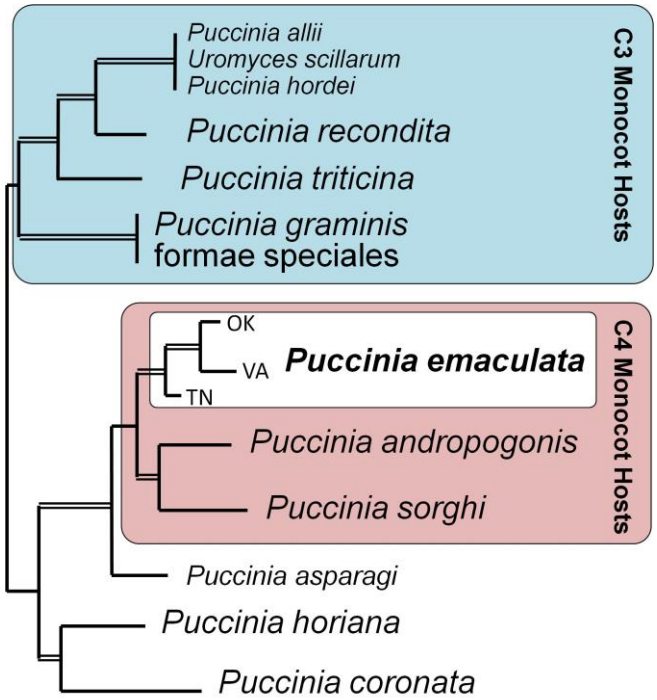

Figure 3.2. Simplified ITS-rDNA PHYML tree. General groupings of rust fungi by monocot host type are indicated. Topology of *P. emaculata* isolates from Oklahoma (OK), Virginia (VA), and Tennessee (TN, EU915294) populations also are indicated. Node support with MLBP >70% are indicated by double lines.

Haplotypes of the three barcode loci from *P. emaculata* were generated by SNAP Map (SNAP Workbench v 2.0), which collapsed sequences to informative positions, creating unique haplotypes. Relationships among haplotypes and their states of origin were analyzed using ML. The number of haplotypes observed for each locus varied: 14 of ITS (Figure 3.3A), 24 of bTub

(Figure 3.3B), and 27 of TEF1a (Figure 3.3C). Haplotype diversity by locus and state is shown in Tables 3.3, 3.4, and 3.5. The bTub and TEF1a haplotypes displayed mostly local distributions; while ITS haplotypes were distributed either in multiple states or locally. Furthermore, the TEF1a barcode showed the highest number of haplotypes followed by bTub and ITS, Fig 3.4.

Table 3.3. *Puccinia emaculata* ITS haplotypes present in 6 different states, Oklahoma (OK), South Dakota (SD), Iowa (IA), Virginia (VA), Mississippi (MS), and Tennessee (TN, EU915294)

Haplotype	IA	MS	SD	OK	VA	TN	TOTAL[b]
H1	3	4	1	1	-	-	9
H2	-	-	-	-	4	-	4
H3	-	-	-	1	-	-	1
H4	-	-	-	1	-	-	1
H5	-	-	-	-	1	-	1
H6	-	-	-	3	-	-	3
H7	-	-	-	-	-	1	1
H8	-	-	-	2	-	-	2
H9	-	-	-	1	-	-	1
H10	-	-	-	1	-	-	1
H11	1	2	-	-	-	-	3
H12	-	1	-	-	-	-	1
H13	4	3	3	-	-	-	10
H14	2	-	1	-	-	-	3
TOTAL[a]	10	10	5	10	5	1	41

[a] Total number of cloned sequences analyzed per state.
[b] Total number of times a particular haplotype was observed

Table 3.4. *Puccinia emaculata* bTub haplotypes present in 5 different states, Oklahoma (OK), South Dakota (SD), Iowa (IA), Virginia (VA), and Mississippi (MS).

Haplotype	IA	MS	SD	OK	VA	TOTAL[b]
H1	1	-	-	-	-	1
H2	2	-	-	-	-	2
H3	-	-	-	1	-	1
H4	-	-	1	-	-	1
H5	-	1	-	-	-	1
H6	-	-	-	1	-	1
H7	1	-	-	-	-	1
H8	1	-	-	-	-	1
H9	-	1	-	-	-	1
H10	-	1	-	-	-	1
H11	-		-	-	1	1
H12	-	2	-	-	-	2
H13	-	1	-	-	-	1
H14	-	1	-	-	-	1
H15	-	1	-	-	-	1
H16	5	-	-	-	-	5
H17	-	2	-	2	-	4
H18	-	-	-	1	-	1
H19	-	-	-	5	-	5
H20	-	-	-	-	1	1
H21	-	-	-	-	1	1
H22	-	-	3	-		3
H23	-	-	-	-	2	2
H24	-	-	1	-	-	1
TOTAL[a]	10	10	5	10	5	40

[a] Total number of cloned sequences analyzed per state.

[b] Total number of times a particular haplotype was observed

Table 3.5. *Puccinia emaculata* TEF1a haplotype diversity present in 5 different states, Oklahoma (OK), South Dakota (SD), Iowa (IA), Virginia (VA), and Mississippi (MS).

Haplotypes	IA	MS	SD	OK	VA	TOTAL[b]
H1	1	-	-	-	-	1
H2	-	1	-	-	-	1
H3	-	1	-	-	-	1
H4	-	-	1	-	-	1
H5	-	-	-	-	1	1
H6	-	-	-	1	-	1
H7	-	1	-	-	-	1
H8	-	2	-	-	-	2
H9	1	-	-	-	-	1
H10	1	-	1	-	-	2
H11		-	-	1	-	1
H12	1	-	-	-	-	1
H13	-	-	-	1	1	2
H14	-	-	-	-	1	1
H15	-	-	-	-	2	2
H16	-	-	-	1	-	1
H17	-	-	-	1	-	1
H18	-	1	-	1	-	2
H19	-	1	-	1	-	2
H20	2	-	-	-	-	2
H21	-	-	-	2	-	2
H22	-	-	1	-	-	1
H23	-	3	1	-	-	4
H24	1	-	-	-	-	1
H25	3	-	-	-	-	3
H26	-	-	1	-	-	1
H27	-	-	-	1	-	1
TOTAL[a]	10	10	5	10	5	40

[a] Total number of cloned sequences analyzed per state.

[b] Total number of times a particular haplotype was observed

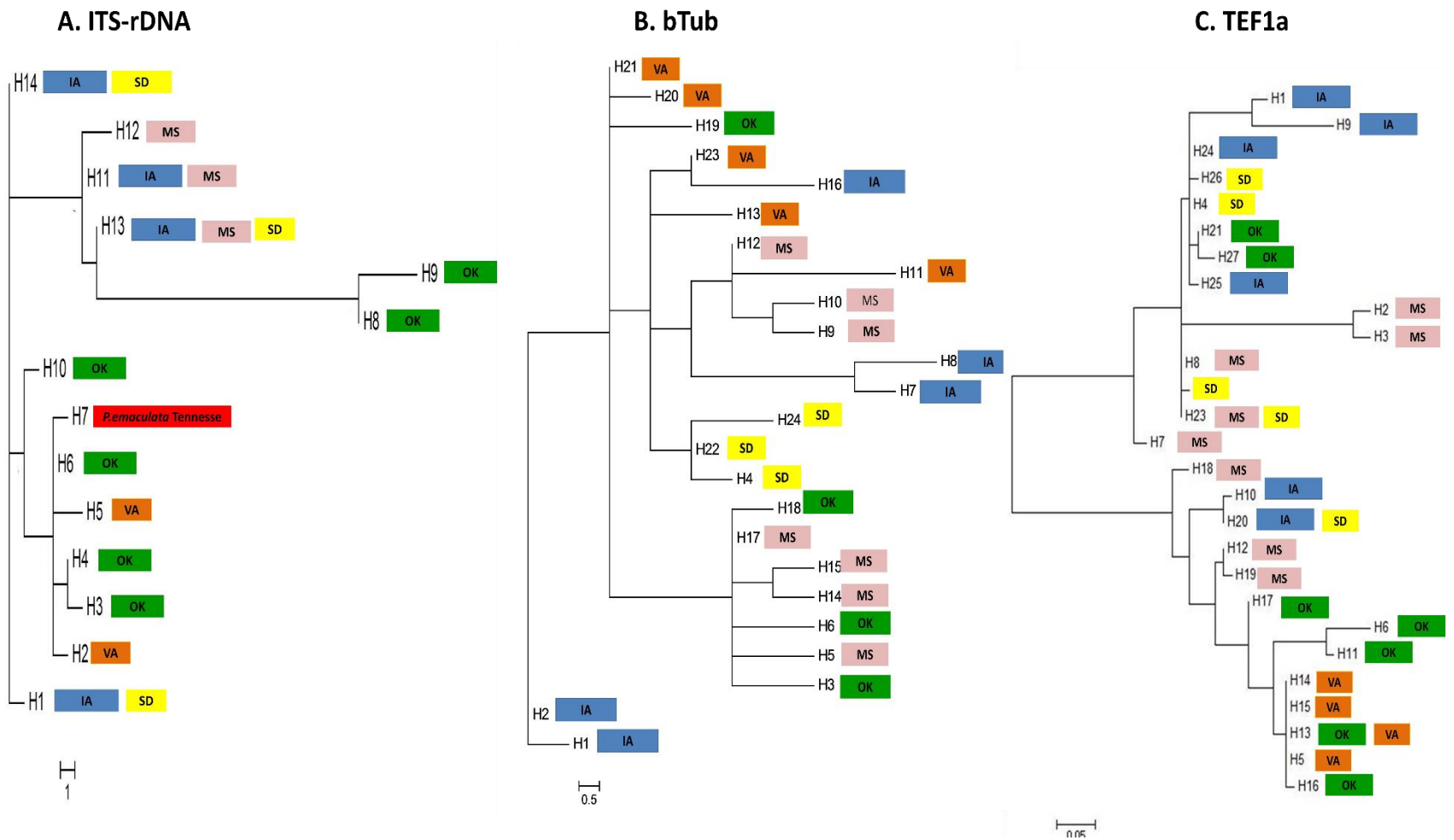

Figure 3.3. Maximum likelihood (ML) trees of haplotypes of three DNA barcode loci: **(A)** ITS (14 haplotypes), **(B)** bTub (24 haplotypes), **(C)** TEF1a (27 haplotypes), Oklahoma (OK, green), South Dakota (SD, yellow), Iowa (IA, blue), Virginia (VA, orange), and Mississippi (MS, pink). Each tree branch represents one haplotype and each color describes the five populations evaluated during this study.

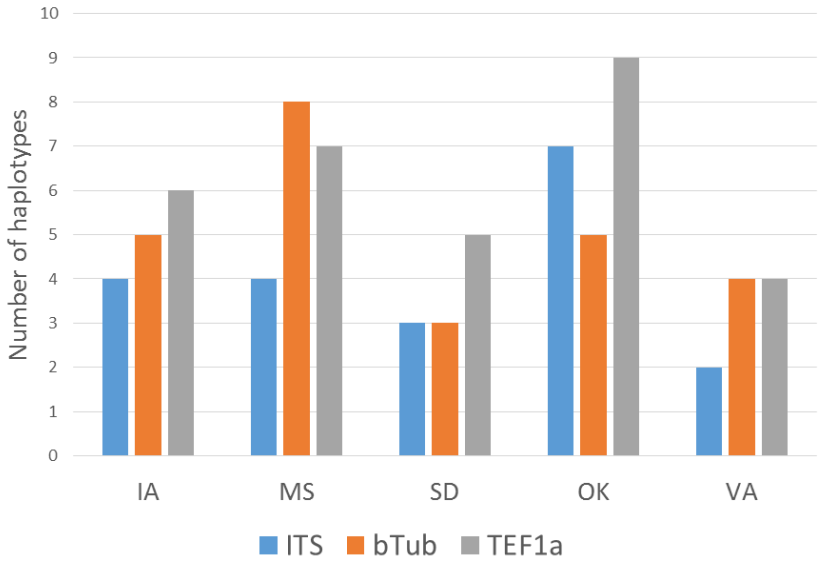

Figure 3.4. Comparison of the haplotype diversity of three barcode loci among five different states: Oklahoma (OK), South Dakota (SD), Iowa (IA), Virginia (VA), and Mississippi (MS).

In Figure 3.5, pie charts reflect individual haplotype diversity and proportions per state. This plot shows that haplotype diversity declines moving north and east along "*Puccinia Pathway*" (9; 13; 28; 30; 31). The high diversity of haplotypes present in the southern states may indicate genetic recombination is occurring, leading to new genotypes.

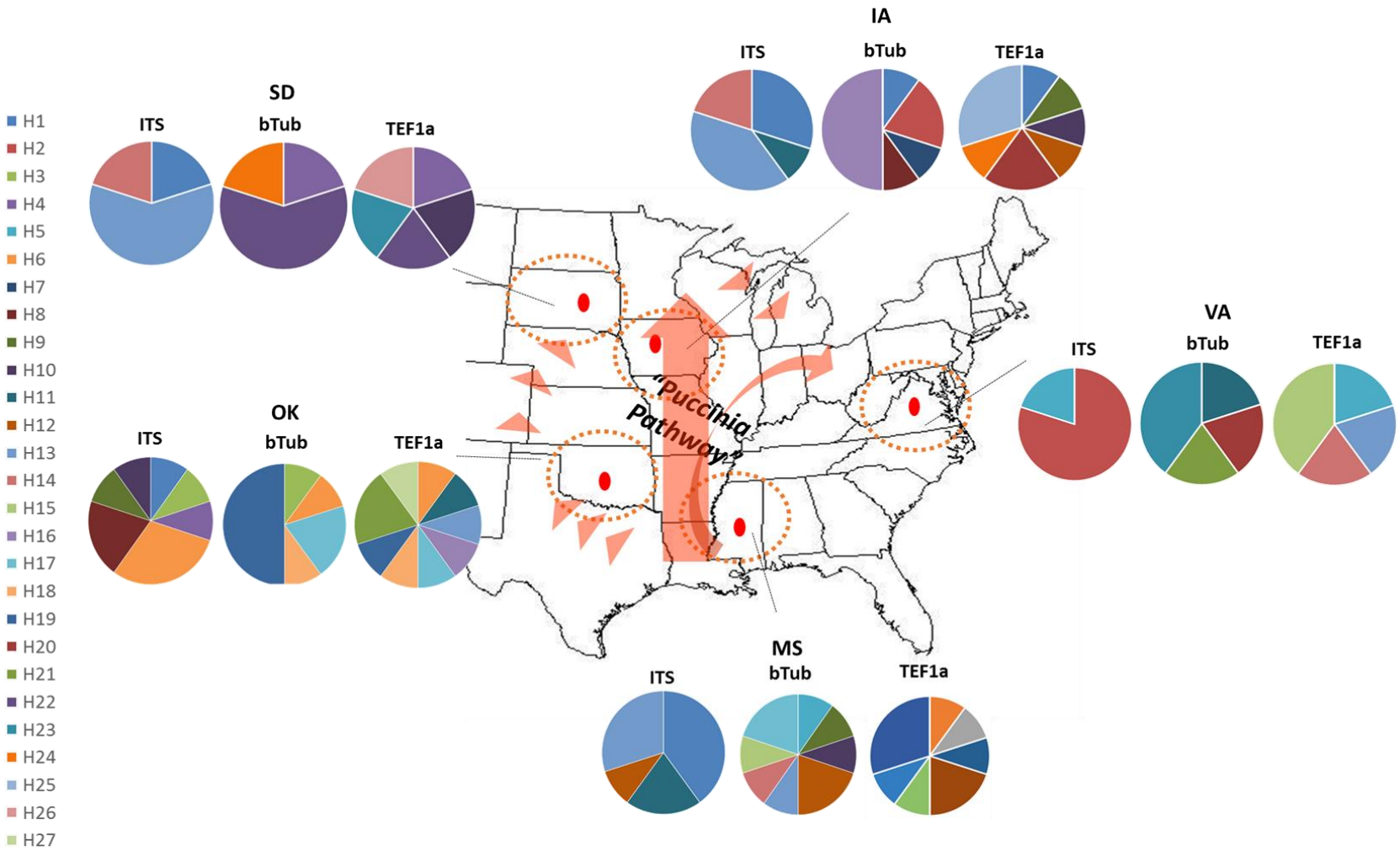

Figure 3.5. Haplotype distribution of *Puccinia emaculata* barcode loci (ITS, bTubulin, and TEFIa) in 5 states: Oklahoma (OK), South DaGFGkota (SD), Iowa (IA), Virginia (VA), and Mississippi (MS). Solid red ovals in states indicate general location of sampling sites. Pie charts depict DNA barcode loci haplotype diversity (number of wedges) and proportions of haplotypes (size of wedges) per state.

Multilocus phylogenetic analyses of three DNA barcodes from single spore whole genome amplifications

A total of 25 *P. emaculata* single urediniospore WGAs, five from each state (Table 3.1), were prepared and successfully used as templates for PCR amplification of three barcode loci (ITS, bTub, and TEF1a). PCRs and product sequencing were performed as previously described. Of a total of 25 single urediniospore WGAs attempted, 17 (68%) produced high quality bidirectional contigs for all three barcodes and were used to develop a concatenated multilocus alignment for phylogenetic tree construction. Sequences of the three barcode loci from the rust fungi uredinispores, *P. andropogonis*, *P. sorghi*, *P. striiformis* f. sp. *tritici*, and *Puccinia triticina* were included as outgroups. As individual gene trees were more or less congruent with one another, the aligned loci were concatenated and a supermatrix containing 2352 nucleotide positions (bTub- 803, TEF1a - 698, and ITS-rDNA - 851) was created. The best ML tree had a log likelihood of −21357.02. The resulting multilocus ML tree (Figure 3.6) strongly supported (MLBP/Bayesian Posterior Probability [BPP] 100/0.99) *P. emaculata* as a monophyletic species. Topology of the multilocus ML tree was congruent with the ML trees based on single loci (bTub [MLBP/BPP 100/0.99]), TEF1a [MLBP/BPP 100/0.99]), and ITS-rDNA [MLBP/BPP 100/1]), which provided strong support of *P. emaculata* as a monophyletic group. *P. emaculata* grouped strongly (MLBP/BPP 100/1) with *P. sorghi* and *P. andropogonis*, which infect C4 grass hosts (e.g. maize and big bluestem, respectively). *P. emaculata* diverged as a distinct lineage separate from *P. striiformis* f. sp. *tritici* and *P. triticina*, which infect C3 grasses (e.g. cereals).

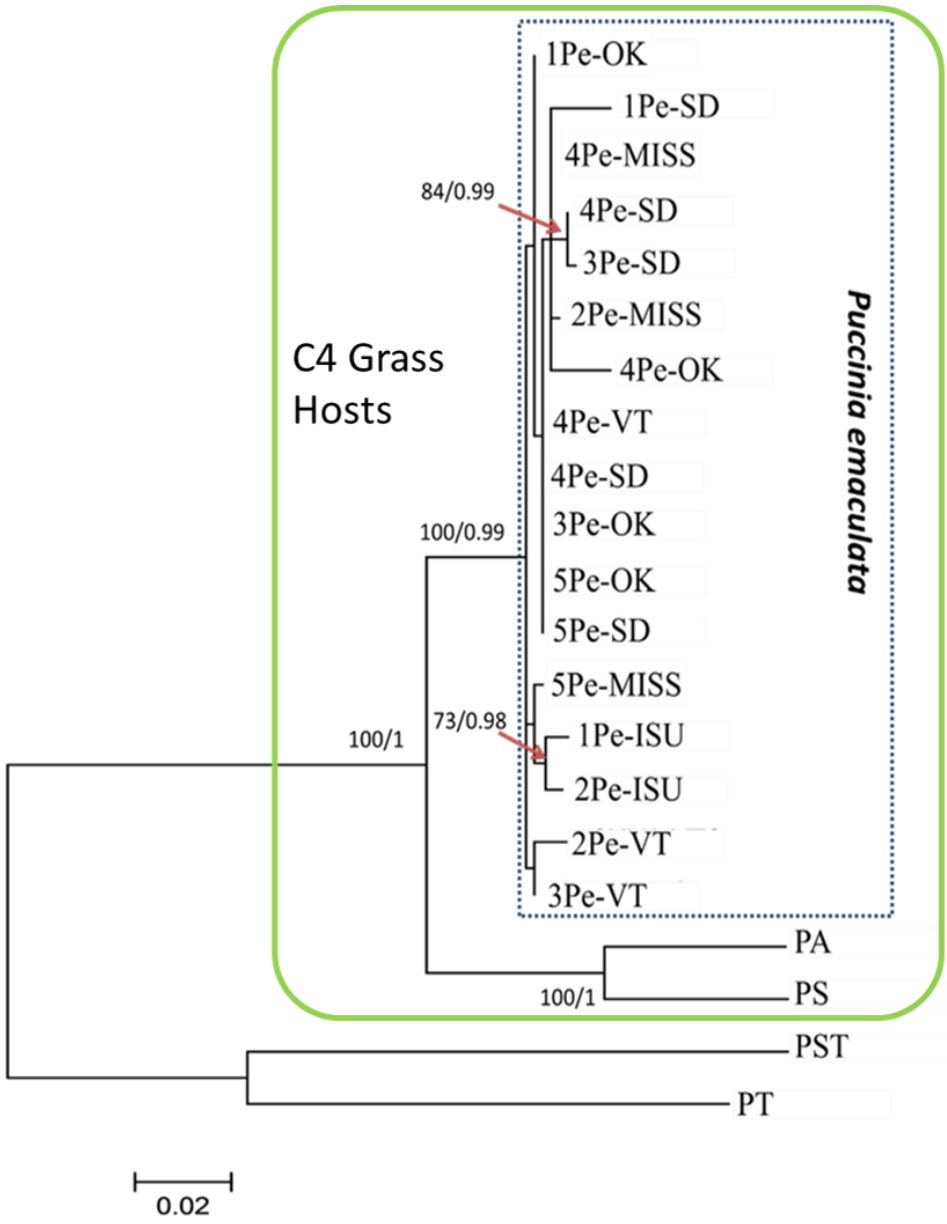

Figure 3.6. Multilocus ML tree (node supports presented MLBP/BPP; ≥75%/≥0.95) using concatenated ITS, bTub and TEF1a sequences from 17 single spores of *P. emaculata* (accession codes: Pe-ISU, Iowa; Pe-MISS, Mississippi; Pe-OK, Oklahoma; Pe-SD, South Dakota; Pe- VT, Virginia) and selected *Puccinia* spp. (*P. andropogonis*, PA; *P. sorghi*, PS; *P. striiformis* f.sp. *tritici*, PST; *P. triticina*, PT).

DISCUSSION

Phylogeny

Two of the approaches described in this study were helpful for obtaining high quality DNA sequences from *P. emaculata*. First, cloning of PCR products prior to sequencing greatly increased the quality of the sequences from multi-urediniospore samples. Second, single spore WGAs were useful for obtain single urediniospore multilocus genotypes, which allowed multilocus phylogenetic analysis of this biotrophic fungus. In all cases, the three loci, ITS, bTub, and TEF1a, were amplified without major difficulties and single bands were observed on gels. However, sequencing was challenging because of the apparent presence of heterogeneous templates in some of the samples. This was likely due to heterozygous alleles at these loci, within the two haploid nuclei of the dikaryotic urediniospores, indicating these urediniospores were heterokaryons. Either cloning amplicons prior to sequencing or single spore collection before WGA can be challenging and added significant time to these investigations. However, combining these approaches led to high quality bidirectional sequence contigs usable for multilocus phylogenetic and haplotype analyses.

The phylogenetic analyses of urediniospores collected from rust-infected leaf samples from cultivated switchgrass in five states, confirmed the conclusion of Uppalapati et al. (35) that *P. emaculata* is a monophyletic species. However, significant genetic variability of barcode loci haplotypes exists among *P. emaculata* urediniospore populations. This genetic variability and heterokaryosis has been reported in other *Puccinia* species and was shown to be a mechanism for variation (25). Also, *P. emaculata* is shown to be closely related to two species of rust fungi found on maize and big bluestem, two North American C4 grasses in the same subfamily (Panicoideae) as switchgrass. This close relationship may indicate rust species infecting different panicoid grasses coevolved with their hosts. However, if phylogenies of the rust fungi are incongruent with the phylogenies of their hosts, then host jumps may be inferred. Potential host

jump scenarios could include: 1) from a telial host to a new telial (alternative) host(s) via urediniospores, 2) from a telial host to a new aecial (alternate) host via basidiospores, or 3) from an aecial host to a new telial host via aeciospores (21; 23; 35). These different scenarios have been described for many rust pathogens (1). For example, Szabo (2006) reported that speciation of the *P. andropogonis* complex occurred via jumps involving new telial and aecial hosts (33).

Puccinia emaculata is not closely related to the rust fungi infecting the Eurasian C3 grass, wheat. Thus, recent ancestors of *P. emaculata* likely did not infect C3 grass hosts in the subfamily Pooideae. Some heteroecious rust have been suggested as not following a continuous path of coevolution, such as the case of black stem rust (1; 6; 10; 11).

The role of an aecial host in the phylogeny of *P. emaculata* remains unclear. *Puccinia emaculata* may subsist as a microcyclic rust fungus (4), as it does not need an aecial host for survival, as green switchgrass (or alternative *Panicum* spp.) may be available year-round in sub/tropical regions of North America. Although sexual recombination would not possible without aecia and an aecial host, other mechanisms of variation may be involved. Flowering spurge (*Euphorbia corollata* L.) is considered a likely aecial host. However, the life cycle and host range of *P. emaculata* must be studied further.

Distribution and spread

It was suggested that *P. emaculata* urediniospores were blown into Tennessee from gulf coast states making primary infection by aeciospores unnecessary (5). *Puccinia emaculata* Schw. was first reported in Tennessee in 2008, when the pathogen was observed on upper leaf surfaces and to a lesser extent on the undersides of switchgrass leaves (cvs. Alamo, Blackwell, Grenville, Falcon, Kanlow, and Miami) (39). The aecial stage reportedly found on species of the family Euphorbiacae (spurge) has not been observed recently.

The high diversity of *P. emaculata* haplotypes observed in the southern U.S compared to northern and eastern U.S. suggests a possible southern center of diversity. The high haplotype variability of urediniospores in Mississippi suggests this state is near such a center. Assuming the genetic diversity of a species is highest in its geographic origin (1), the high number of haplotypes present in Mississippi and Oklahoma suggests that the center of origin is located in the southern regions of North America, perhaps as far south as Mexico. Sexual recombination of *P. emaculata* on an alternate host might be occurring in these southern regions. *Puccinia emaculata* urediniospores may be following the *"Puccina Pathway"*, described for *P. graminis* f.sp. *tritici*, *P. triticina*, and *P. coronata*, where spores are dispersed south-to-north from northern Mexico/Texas to the US/Canadian border (9; 28; 30; 31). For example, in stem rust, the first spores infecting wheat differ based on the region in which the wheat is grown. In *warm climates*, wheat is planted in late fall and harvested in early summer. The first spores to infect the young wheat plants in fall are urediniospores. These generally come from infected volunteer wheat plants. These plants can become infected by spores produced on late-maturing wheat plants still in the field. However, in regions with *temperate climates* wheat may be planted either in the fall (winter wheat) or the spring (spring wheat). Few winter wheat varieties can survive well through the severe winters of Minnesota, North Dakota, and Manitoba, so most of the wheat grown there is spring wheat. The first rust spores to infect spring wheat in temperature regions may be aeciospores from barberry, the alternate host, or urediniospores from infected wheat in distant regions. Therefore, two disease cycles for stem rust are possible (28; 31). This mixture of disease cycles could be occurring with *P. emaculata* on switchgrass and its possible alternate and alternative host(s). However, this is difficult to determine, since *P. emaculata*'s alternate host is still unclear. The probability of viable spores reaching a target and causing infection after a long distance dispersal is in general very low, but considering the large numbers of rust spores released each year, cumulative probabilities may favor successful infections (9; 28; 30; 31). The back-

and-forth movement between source and target has been shown to promote the rapid evolution of pathogenic forms of rust.

This is the first haplotype diversity study of *P. emaculata* urediniospores around the United States. However, it is not a comprehensive study and larger numbers of samples from different states within the geographic range of the pathogen need to be examined to confirm these observations.

Phylogeny

The multilocus phylogeny shows that *P. emaculata* is a monophyletic species, in agreement with previous literature (35). The ITS sequences obtained from urediniospore collections from six different states were nearly identical to the previously published sequences of *P. emaculata* (GenBank Accession No: EU915294; KC515382) and distinct from ITS sequences of other *Puccinia* species. This is the first study of bTub and TEF1a sequences from *P.emaculata*. At present, a limited number of ITS (n = 3,795), bTub (n = 302), and TEF1a (n = 164) sequences from rust fungi are available, making it difficult to generate multilocus phylogenetic analyses (35). Furthermore, this is the first multilocus phylogenetic analysis of *P. emaculata*. However, future phylogenetic work would greatly benefit from additional urediniospore collections and sequences of bTub and TEF1a genes. Additionally, sequences of *P. emaculata* from alternate hosts would improve the understanding of the evolution and host range of this species. In order to understand the population dynamics and epidemiology of this rust fungus, future research must define the roles of asexual and sexual reproduction in the disease cycle of *P. emaculata* and their contribution to its genetic variability, host range, and pathogenicity.

REFERENCES

1. Abbasi, M., Goodwin, S. B., and Scholler, M. 2005. Taxonomy, phylogeny, and distribution of *Puccinia graminis*, the black stem rust: new insights based on rDNA sequence data. Mycoscience 46:241-247.

2. Aime, M. C., Matheny, P. B., Henk, D. A., Frieders, E. M., Nilsson, R. H., Piepenbring, M., McLaughlin, D. J., Szabo, L. J., Begerow, D., Sampaio, J. P., Bauer, R., Weiss, M., Oberwinkler, F., and Hibbett, D. 2006. An overview of the higher level classification of Pucciniomycotina based on combined analyses of nuclear large and small subunit rDNA sequences. Mycologia 98:896-905.

3. Arthur, J. C. 1905. Terminology of the spore structures in the Uredinales. Botanical Gazette. 39:219-222.

4. Arthur, J. C., and Cummins, G.B., 1962. Manual of the rust in the United States and Canada. Hafner, New York. 438 p.

5. Black, J. A. 2012. The epidemiology of *Puccinia emaculata* (rust) in switchgrass and evaluation of the mycoparasite *Sphaerellopsis filum* as a potential biological control organism for switchgrass rust. Master's Thesis, University of Tennessee. Internet site: http://trace.tennessee.edu/utk_gradthes/1251 [accessed May 2012]

6. Bowman, B. H., Taylor, J. W., Brownlee, A. G., Lee, J., Lu, S. D., and White, T. J. 1992. Molecular evolution of the fungi: relationship of the basidiomycetes, ascomycetes, and chytridiomycetes. Molecular Biology Evolution 9:285-96.

7. Browder, L. E. 1971. Pathogenic specialization in cereal rust fungi, especially *Puccinia recondita* f. sp. *tritici,* concepts, methods of study, and application. Technical bulletin (No. 1432). US agricultural research service.

8. Castresana, J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17:540-552.

9. Chester, K. S. 1946. The nature and prevention of the cereal rusts as exemplified in the leaf rust of wheat. Chronica Botanica Co.: Waltham, Massachusetts, USA).

10. Cummins, G. B., and Hiratsuka, Y. 2002. Illustrated genera of rust fungi. 3rd ed. American Phytopathological Society, 240 pp, St. Paul, Minnesota, USA

11. Duplessis, S., Cuomo, C. A., Lin, Y.-C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., Joly, D. L., Hacquard, S., Amselem, J., Cantarel, B. L., Chiu, R., Coutinho, P. M., Feau, N., Field, M., Frey, P., Gelhaye, E., Goldberg, J., Grabherr, M. G., Kodira, C. D., Kohler, A., Kües, U., Lindquist, E. A., Lucas, S. M., Mago, R., Mauceli, E., Morin, E., Murat, C., Pangilinan, J. L., Park, R., Pearson, M., Quesneville, H., Rouhier, N., Sakthikumar, S., Salamov, A. A., Schmutz, J., Selles, B., Shapiro, H., Tanguay, P., Tuskan, G. A., Henrissat, B., Van de Peer, Y., Rouzé, P., Ellis, J. G., Dodds, P. N., Schein, J. E., Zhong, S., Hamelin, R. C., Grigoriev, I. V., Szabo, L. J., and Martin, F. 2011. Obligate biotrophy features unraveled by the genomic analysis of rust fungi. Proceedings of the National Academy of Sciences, 108.22:9166-9171.

12. Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792-1797.

13. Freese, H., and Yarger, D. 1974. Movement of southern corn leaf blight spores. Iowa state journal of research. Internet site: http://www.nal.usda.gov/ [accessed Sep 2013]

14. Gilley, M., Tomaso-Peterson, M., Orquera, G., and Marek, S. 2013. First report of rust caused by *Puccinia emaculata* on cultivated switchgrass in Mississippi. The Journal of the Mississippi Academy of Science. 58:197.

15. Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., and Gisi, U. 2006. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. Pest Manag Sci 62:465-72.

16. Gravert, C., and Munkvold, G. 2002. Fungi and diseases associated with cultivated switchgrass in Iowa. Journal Iowa Academy of Science 109:30-34.

17. Gustafson, D. M., Boe, A., and Jin, Y. 2003. Genetic variation for *Puccinia emaculata* infection in switchgrass. Crop Science 43:755-759.

18. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology 166 (4), 557-558.

19. Hildebrand, E. M. 1938. Techniques for the isolation of single microorganisms. The Botanical Review 4:627-664.

20. Hirsch, R. L., TeBeest, D. O., Bluhm, B. H., and West, C. P. 2010. First report of rust caused by *Puccinia emaculata* on switchgrass in Arkansas. Plant Disease 94:381-381.

21. Hovmoller, M. S., Sorensen, C. K., Walter, S., and Justesen, A. F. 2011. Diversity of *Puccinia striiformis* on cereals and grasses. Annual Review of Phytopathology 49:197-217.

22. Kropp, B. R., Hansen, D. R., Wolf, P. G., Flint, K. M., and Thomson, S. V. 1997. A study on the phylogeny of the dyer's woad rust fungus and other species of *Puccinia* from crucifers. Phytopathology 87:565-571.

23. Leonard, K. J., and Szabo, L. J. 2005. Stem rust of small grains and grasses caused by *Puccinia graminis*. Molecular Plant Pathology 6:99-111.

24. Li, Y., Windham, M., Trigiano, R., Fare, D., Spiers, J., and Copes, W. 2007. Microscopic and macroscopic studies of the development of *Puccinia hemerocallidis* in resistant and susceptible daylily cultivars. Plant Disease 91:664-668.

25. Liu, B., Chen, X., and Kang, Z. 2012. Gene sequences reveal heterokaryotic variations and evolutionary mechanisms in *Puccinia striiformis*, the stripe rust pathogen. Open Journal of Genomics 1:1, 2012.

26. Liu, Z., Szabo, L. J., and Bushnell, W. 1993. Molecular cloning and analysis of abundant and stage-specific mRNAs for *Puccinia graminis*. Molecular plant microbe interactions 6:84-84.

27. Miller, M. A., Pfeiffer, W., and Schwartz, T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Pages 1-8. Gateway computing environments workshop (GCE), 2010.

28. Nagarajan, S., and Singh, D. 1990. Long-distance dispersion of rust pathogens. Annual Review of Phytopathology 28:139-153.

29. Ramachar, P., and Cummins, G. 1963. The species of *Uromyces* on the tribe Paniceae. Mycopathologia et Mycologia Applicata 19:49-61.

30. Simons, M. D. 1970. Crown rust of oats and grasses. American Phytopathology Society. Monographs 5, 47 pp.

31. Stakman, E. 1933. Epidemiology of cereal rusts. In Pro. Pac. Sci. cong. 5th, 1-14 June 1933, Victoria and Vancouver, B.C. University of Toronto Press, Toronto, Ont. Vol. 4. Pp. 3177-3184.

32. Stamatakis, A., Hoover, P., and Rougemont, J. 2008. A rapid bootstrap algorithm for the RAxML web servers. Systematic Biology 57:758-771.

33. Szabo, L. J. 2006. Deciphering species complexes: *Puccinia andropogonis* and *Puccinia coronata*, examples of differing modes of speciation. Mycoscience 47:130-136.

34. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution. 28 (10), 2731-2739.

35. Uppalapati, S. R., Serba, D. D., Ishiga, Y., Szabo, L. J., Mittal, S., Bhandari, H. S., Bouton, J. H., Mysore, K. S., and Saha, M. C. 2012. Characterization of the rust fungus, *Puccinia emaculata*, and evaluation of genetic variability for rust resistance in switchgrass populations. BioEnergy Research. 6:458-68.

36. Vaidya, G., Lohman, D. J., and Meier, R. 2011. SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27:171-180.

37. van der Merwe, M., Ericson, L., Walker, J., Thrall, P. H., and Burdon, J. J. 2007. Evolutionary relationships among species of *Puccinia* and *Uromyces* (Pucciniaceae,

Uredinales) inferred from partial protein coding gene phylogenies. Mycol Res 111:163-75.

38. Weising K, Nybom H, Wolff K, Meyer W (1995). DNA Fingerprinting in plants and fungi. CRC Press, Boca Raton, FL

39. Zale, J., Freshour, L., Agarwal, S., Sorochan, J., Ownley, B. H., Gwinn, K. D., and Castlebury, L. A. 2008. First report of rust on switchgrass (*Panicum virgatum*) caused by *Puccinia emaculata* in Tennessee. Plant Disease 92:1710-1710.

CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF SIMPLE SEQUENCE REPEATS (SSRs) AND EXPRESSED SEQUENCE TAG (EST) - SSRs FOR *Pucccina emaculata* AND POTENTIAL USE IN OTHER SPECIES OF *Puccinia*

ABSTRACT

Puccinia emaculata causes switchgrass rust, an important disease reducing the biomass production and biofuel feedstock quality of switchgrass. Attempts to study the population biology of *P. emaculata* using ten microsatellite markers developed previously by Wadl et al., 2011 resulted in no amplification (seven loci) or low levels of polymorphism (three loci). This study reports the development and characterization of 20 novel simple sequence repeat (SSR) microsatellite loci developed from a microsatellite-enriched library and expressed sequence tags (ESTs) from *P. emaculata*. Microsatellites were characterized using single urediniospores of *P. emaculata* from rust samples collected in Iowa (IA), Mississippi (MS), Oklahoma (OK), South Dakota (SD) and Virginia (VA). Five urediniospores per sampled state were used (n = 25). Most identified microsatellite markers were highly polymorphic (PIC average 0.7181), with a mean of 8.3 alleles per locus (range 3 to 17) and a mean expected heterozygosity of 0.1390 (range 0.04 to 0.16). Genetic diversity analysis was possible in single spore samples based on 18 polymorphic SSR loci. Analyses showed moderate genetic variation between states (F_{st} = 0.123) and frequent

gene flow (*Nm* > 1.858). Evaluation of allelic patterns across *P. emaculata* populations displayed a high number of private alleles in VA, IA, SD and lower numbers in OK and MS, which indicated that a high level of migrant exchange and gene flow occurred from these latter two states. Additionally, of the 49 SSRs initially identified in *P. emaculata*, 18 were transferable to *P. striiformis* f. sp. *tritic*i, 23 to *P. triticina*, 20 to *P. sorghi* and 31 to *P. andropogonis*. Thus, these markers should be useful for population structure assessment, QTL mapping, and ecological studies of *P. emaculata* and potentially other *Puccinia* species.

INTRODUCTION

Switchgrass (*Panicum virgutum* L.) is a perennial warm season prairie grass native to North America and is a promising biomass crop for renewable energy production (4). In 1991, switchgrass was chosen as a model species for the Bioenergy Feedstock Development Program by the United States Department of Energy (DOE) (21). However, switchgrass production can be limited by a variety of diseases caused by fungi (14; 39). Rust, caused by *Puccinia emaculata* Schw., is one of the most common switchgrass diseases that can occur at high incidence levels, causing economic losses (25). Infection by this pathogen reduces biomass yield, seed production, and forage and feedstock quality of switchgrass (32). *Puccinia emaculata* has been reported in numerous states throughout the eastern United States, west into Texas and north into South Dakota (11; 40). Switchgrass in Arkansas and Oklahoma is frequently affected by *P. emaculata* (17; 41). In Arkansas, *P. emaculata* has been reported on "Alamo" switchgrass, where 25% to nearly 100% of switchgrass leaves were infected (25), and in Tennessee, high rust severity reduced ethanol yield up to 60% (25).

Puccinia emaculata is an obligate biotrophic parasite and is likely heteroecious, requiring two different hosts to complete its life cycle. *Puccinia emaculata* reproduces asexually on switchgrass plants, repetitively infecting and forming urediniospores every 7-10 days throughout

the growing season and forming resting teliospores when leaves senesce in late summer and fall. Presumably teliospores germinate in the spring, undergo meiosis, and form basidiospores that should infect the alternate host (29). Sexual reproduction in *P. emaculata* has not been well documented and presumably occurs on *Euphorbia* species; however, this has not been confirmed (1; 11). Additional studies, have reported variation in virulence among *P. emaculata* isolates from ornamental switchgrass cultivars, which appeared to be more susceptible than agronomic switchgrass cultivars (19). Potential race specificities and virulence determinants of *P. emaculata* are required for accurate analysis of host-pathogen interactions and detailed information on intraspecific pathogen diversity in different states is required for appropriately deploying resistant varieties (41). Understanding the genetic diversity of *P. emaculata* will be useful to breeders for the development of durable resistance in switchgrass cultivars.

Information on the genetic variation within *P. emaculata* is limited. Molecular markers have been widely used to map important resistance genes in crops and to study the population genetics of pathogens. One type of molecular marker used in population biology studies are simple sequence repeats (SSRs) or microsatellites. Microsatellites are regions of DNA consisting of short, tandemly repeated units (1-6 bp in length) found within the coding and noncoding regions of all eukaryotic organisms (28). Multiple SSRs can be used to characterize individuals in a population, creating a genetic profile or "fingerprint". Two approaches can be used to generate SSRs. In the first approach, SSR markers are identified and isolated from a repeat-enriched DNA library, while in the second approach SSRs are identified among expressed sequence tags (ESTs) generated in gene expression studies. To generate ESTs, mRNA from the organism of interest is isolated and used to synthesize cDNA by reverse transcription. This cDNA is then cloned into plasmid libraries and sequenced individually (24; 42). Or, the cDNA is directly sequenced using next generation sequencers in a massively parallel process referred to as RNA-Seq (13). Both approaches result in ESTs, though the latter approach produces many orders of magnitude more.

Microsatellites were selected for this *P. emaculata* genetic study, because of many beneficial characteristics. First, microsatellites reveal genetic variation and identify genes responsible for a special characteristic (e.g. pathogenicity). Second, microsatellites are conserved within species and between closely related species, allowing the use of SSR primer sets across species. Third, microsatellites analyses are relatively simple and easier to score than conventional genetic markers (e.g. RFLP [restriction fragment length polymorphism], AFLP [amplified fragment length polymorphism]). SSR band patterns are easy to detect, reproducible, and easy to compare accurately across gels (27). Also, *Puccinia emaculata* urediniospores and hyphae produced on switchgrass are dikaryotic, and codominant markers like SSRs are the most informative for the study of genetic diversity in such organisms (i.e. dikaryotic or diploid) (41). Many microsatellite markers have been described for rust fungi (2; 5-8; 10; 35; 36), but transferability to related rust fungi is low.

Wadl et al., 2011 reported ten microsatellite loci for *P. emaculata* (41). However, only three of the 10 microsatellite loci showed amplification and correct band sizes in the *P. emaculata* collections used in this study. The remaining primers did not produce amplicon or resulted in multiple bands. Thus, more molecular markers are required to study *P. emaculata* populations. The objectives of this study were to (*i*) develop and characterize new microsatellites for *P. emaculata*, (*ii*) evaluate the microsatellites in *P. emaculata* populations, and *(iii)* assess the transferability of microsatellite primers to other *Puccinia* species.

MATERIALS AND METHODS

Fungi material

Puccinia emaculata urediniospores were vacuum collected from symptomatic switchgrass leaves collected from fields in Iowa (IA), Mississippi (MS), South Dakota (SD) and Virginia (VA) in 2011 (Table 4.1). Collected urediniospores were dried over silica gel for 2 days at room

temperature and then flash frozen in liquid nitrogen for long term storage at -80°C. Oklahoma (OK) urediniospores were collected from field grown switchgrass leaves in 2009 and inoculated onto switchgrass plants (cvs. Kanlow, Blackwell, and Cave in Rock) by shaking sporulating leaves onto non-infected leaves, and inoculated plants were incubated in dew chamber at 25°C for 16 to 24 hours. A continuous uredinial culture of this Oklahoma rust population (OSU09-1) was maintained on switchgrass plants (same 3 cultivars mentioned above) in a growth chamber at 27°C, with a 12-h photoperiod. Occasionally, plants were re-inoculated with urediniospores shaken from sporulating leaves, incubated in a dew chamber and returned to the growth chamber to increase urediniospore loads. OSU09-1 urediniospores (OK) were regularly harvested since 2009, and stored long-term at -80°C. In this study Oklahoma urediniospores harvested in 2011 were used.

Genetic material

Genomic DNA was extracted according to Weising et al. (43), with some modifications. About 10 mg of urediniospores from each population were transferred into 2 mL screwcap tubes containing three 3 mm glass beads and ~0.2 ml 0.5 mm zirconium beads (BioSpec Products, Inc., Bartlesville, OK) and 1 mL of extraction buffer [140 mM Sorbitol, 220 mMTris-HCl, pH8.0, 22 mM EDTA, 800 mM NaCl, 0.8% cetyltrimethylammonium bromide (CTAB), and 1% Sodium Dodecyl Sulfate (SDS)] was added. Samples were homogenized in a bead beater (FastPrep®-24 Instrument, MP Biomedicals, Santa Ana, CA) for 20 sec at 4 m/s, three times. Then, 0.5 mL of chloroform: isoamyl alcohol (24:1, v/v) was added, and incubated at 58°C for 30 min. Phases were separated by centrifugation at 4°C for 20 min at 12,000 x *g*. The upper aqueous phase was transferred to a new tube, 700 µL isopropanol added, tubes gently mixed and placed at −20°C for 30 min. Precipitated DNA was pelleted by centrifugation, supernatant discarded, and the pellet washed with 70% ethanol. The pellet was air dried for 20 min and then suspended in 100 µL of TE (10mM Tris-HCl, pH 7.4; 1mM EDTA) (43). The quality of DNA was checked on TAE-1%

agarose gels and DNA concentrations were estimated using a NanoDrop ® 1000 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer.

Table 4.1. *Puccinia emaculata* accessions used in this study, switchgrass cultivar, origin and collection year.

***Puccinia emaculata* collections**	**Code**	**Switchgrass cv. or host**	**Origin**	**Collection year**
Pe-Iowa	ISU1	Cave in Rock	Woodruff Farm, Iowa State University, Story Co., IA	2011
Pe-Mississippi	MISS1	Alamo	H.H. Leveck Animal Research Center, Mississippi State University, Starkville, MS	2011
Pe-South Dakota	SDSU	Dacotah	SDSU Experiment Station Farm (Felt Farm), Brookings, SD	2011
Pe-Oklahoma	OSU09-1	Lowland genotype	Oklahoma State University Agronomy Farm, Stillwater, OK	2009[a]
Pe-Virginia	VT1-1	Alamo × Dacotah	Kentland Farm, Virginia Tech, Blacksburg, VA	2011

[a] maintained in growth chamber as urediniospores on cvs. Kanlow, Blackwell and Cave in Rock until 2011 when it was collected for this study.

Total RNA was extracted from four source materials, nongerminated and germinated OK and VA urediniospores, using a modified protocol of the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Approximately 30 mg urediniospores were flash frozen with liquid nitrogen and a subsample of urediniospores were germinated after exposure to 0.01 ppm nonanol solution. Separately samples were placed in 2 mL microcentrifuge tubes containing beads (as described above) and used immediately, or stored at -80°C, until used for RNA isolation.,Immediately after

liquid nitrogen was allowed to evaporate, 450 µL RLC buffer (guanidine hydrochloride) was added to urediniospores. β-mercaptoethanol was not added in RLC buffer. Then, samples were incubated for 5 min at 65°C, followed by bead beating three times at 4 m/s for 20 sec, and then incubated on ice for 2 min. The lysate was transferred to a QIAshredder spin column and a 0.5 volume of 96% (v/v) ethanol was added to the flow-through. Next, 650 µL of the ethanol-flow-through solution was transferred to a RNeasy spin column and centrifuged at 8,000 x *g* for 15 sec at 4°C. The flow-through was discarded and 700 µL Buffer RW1 was added to the column. The flow-through was discarded and the column was washed twice with 500 µL Buffer RPE, and centrifuged at 8,000 x *g* for 15 sec at 4°C. RNA was eluted with 50 µL of RNase-free water and stored at -80°C (15).

Construction of genomic DNA libraries and isolation of microsatellites

Genomic DNA libraries highly enriched for SSR loci were constructed based on the biotin-labeled Dynabead enrichment strategy described by Glenn and Schable (13). Briefly, 20 µL OK (OSU09-1) urediniospore genomic DNA (100 ng/ µL) was digested with 10 U of RsaI (New England Biolabs, Ipswich, MA), and separated on a 2% agarose-TAE gel. DNA fragments ranging from 300 to 1000 bp were gel purified using GeneJET gel extraction kit (Thermo Scientific,Waltham, MA, USA) and ligated to linkers SuperSNX24 F+4PR. To perform Dynabead enrichment for the microsatellite-containing DNA fragments, DNA was incubated with each of the following 3'-biotinylated microsatellite probes (10 µL): $(AG)_{12,}$ $(TG)_{12}$, $(AAC)_6$ $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, and $(ATC)_{12}$, (mix of oligos at 1 µM each). Dynabeads (Invitrogen, Carlsbad, CA) were washed twice with TE (10 mM Tris pH 8.2, 2 mM EDTA) and twice with 1× Hyb solution (6× SSC, 0.1% SDS). Digested genomic DNA was incubated with the washed Dynabeads in Hyb solution at room temperature for 1 h. Beads were captured using a Dynal magnetic particle concentrator (MPC®-S, Invitrogen, Carlsbad, CA) and rinsed four time with washing solution (2x SSC, 0.1% SDS), followed by two more washes (1x SSC, 0.1% SDS), with

each of these solutions cooled to 5 to 10°C below the T_m for the oligo mix. The enriched fragments were denatured from beads by incubating beads in TLE (10 mM Tris, 0.1 mM EDTA, pH 8.0) at 95°C for 10 min. Enriched fragments were PCR amplified using the primers to the SuperSNX24 linkers and products ligated into cloning vector pCR2.1- TOPO, as described by the manufacturer (Invitrogen, Carlsbad, CA). Clones were transformed into *E. coli* cells (competent TOP10 cells, Invitrogen) and transformants selected on LB agar supplemented with ampicillin (50 µg/mL), X-gal (50 µg/mL) and incubated overnight at 37°C. Positive colonies (white) were each hand-picked into 50 µL water and boiled for 10 min at 90°C. Plasmid inserts were screened by PCR with M13 primers (M13-F: GTAAAACGACGGCCAG, M13-R: CAGGAAACAGCTATGAC) before sequencing. PCR products were purified using ExoSAP-IT® reagent (USB Corporations, Cleveland, OH, USA) and submitted for automated sequencing using the M13 primers at the Recombinant DNA/Protein Resource Facility (Oklahoma State University, Stillwater, OK). Sequences were assembled using Geneious 6.0.1.R (Biomatters, Aukcland, NZ) and contigs used later for primer design.

Construction and sequencing of cDNA library for EST-SSRs

Purification of mRNA and cDNA synthesis were performed using TruSeq RNA Sample Preparation Kit v2 (Illumina Inc., San Diego, CA). Four hundred ng total RNA was diluted with nuclease-free water to a final volume of 50 µL. Fifty microliters of RNA Purification (oligoT) beads were added to the total RNA and the entire volume was pipetted up and down gently 6 times. RNA was denatured at 65°C for 5 min, to facilitate binding of the poly-A RNA to the beads, and the suspension placed on the magnetic stand at room temperature for 5 min to allow RNA to bind to the beads. Without disturbing the beads, the supernatant was removed and discarded and the beads were washed with 200 µL Bead Washing Buffer by gently pipetting the entire volume up and down 6 times. Then the solution was placed again on the magnetic stand at room temperature for 5 min and the supernatant was removed and discarded, without disturbing

the beads. Then 50 µL elution buffer was added to the beads, which were suspended by gently pipetting up-and-down 6 times. This suspension was incubated at 80°C for 2 min to elute mRNA from the beads. Fifty microliters of Bead Binding Buffer was added to the solution and the solution was gently mixed, incubated at 8°C for 5 min and then placed on the magnetic stand at room temperature for 5 min. The supernatant was removed and discarded and 200 µL Bead Washing Buffer were added to the beads, mixed gently, incubated at 4°C and the suspension once again placed on the magnetic stand at room temperature for 5 min. The supernatant was removed and discarded and 19.5 µL of Elute, Prime, and Fragment Mix (1st cDNA synthesis reaction buffer) was added to clean beads, gently mixed, and incubated at 94°C for 8 minutes. First strand cDNA was prepared at Oklahoma State University using a TruSeq RNA Sample Preparation Kit v2 (Illumina Inc., San Diego, CA) following manufacturer's instructions. The resulting single stranded cDNAs were shipped to the Center for Genome Research and Biocomputing (Oregon State University, Corvalis, OR) on dry ice where second strand synthesis of the cDNA was performed and the cDNA libraries were sequenced using a Illumina HiSeq™ 2000. Sequences were filtered by source, assembled and potential SSRs identified.

EST-SSR sequences of less than 100 bp were discarded and sequences above that size were aligned using Geneious 6.0.1.R, to determine which EST-SSRs occurred in both OK and VA urediniospores. Obtained contigs also were screened for similarities against GenBank's database. BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer design

SSR primers (from SSR-enriched DNA library $n = 49$ and EST-SSRs $n = 33$) were designed from regions flanking at least six di, tri, tetra and pentanucleotide repeats using the Websat software (http://wsmartins.net/websat) (22) and Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) (31). Primer design considerations included: an amplicon size from 100

to 350 bp, a Tm between 50°C to 65°C, GC content between 40 and 60%, and primer sizes between 18 and 21 bp (30; 34). Microsatellite primers designed from sequences of clones from the repeat-enriched genomic library were named with a three-letter code "OPE" followed by a number (Table 4.2). OPE is an abbreviation for OSU *Puccinia emaculata*. Primers developed from EST-SSRs were abbreviated "OEPE" for OSU EST *P. emaculata*, followed by a number (Table 4.3).

Table 4.2. Loci, primer sequences, repeat motifs, (Tm), and fragment sizes of *Puccinia emaculata* SSR loci (n= 49) developed from repeat enriched DNA libraries.

Locus	Repeat Motif		Primer sequences (5' to 3')	Tm	Size (bp)
OPE1	$(CATC)_5$	F:	ATCCATCCATCCATCCACTC	60	183
		R:	CCAGGGCAGTATTTGCTT		
OPE2	$(GA)_{10}$	F:	TGATGGGGAAACAGTGAAAG	59	126
		R:	AAGGCAAAGACGAAAGCAAA		
OPE3	$(CA)_{22}$	F:	CAAGCAATAAAATGGGGACA	58	157
		R:	TGTGTGTGTGTGAGGGAGAG		
OPE4	$(AT)_4$	F:	CCTTCTTTCCTTTCTTTGGA	56	273
		R:	AGGCTAAGTTGAGTATGAGG		
OPE5	$(TAG)_{10}$	F:	AAAGGTTGAGTGGTAGTGGT	54	174
		R:	GCTAATGATGACGAAGTTGT		
OPE6	$(TAC)_9$	F:	TGAAGAGGAAGGTGTTGCTG	59	138
		R:	GAGGAGAAGAACGATGAGGA		
OPE8	$(TG)_{12}$	F:	ATCACCTCTGTTTCCGTCCA	61	348
		R:	CACACACACACACACACACA		
OPE10	$(TAG)_5$	F:	CCTCAATCCAACTTTTCCA	56	296
		R:	ATGGTGGGTGATGATGTT		
OPE11	$(GT)_8$	F:	GGCGTGGATGAAATGTGT	58	154
		R:	GAGAAGAGACCCTCAGAAGA		
OPE12	$(GAT)_7$	F:	GCTTTCTTTGAGGGGGCTA	59	178
		R:	GCTACAGGAGGGGTTAGTGG		
OPE13	$(TC)_8$	F:	CGAACGCCCTACATCTTATG	58	233
		R:	GGGAAAATCTGGACACCACT		
OPE14	$(TTC)_{15}$	F:	GTGGTGGGTCTTGTATTC	51	129
		R:	CAGTATCCATCCCTTTCCTG		
OPE15	$(AC)_7$	F:	GATTCTCTTGCTCGTCAT	51	107
		R:	GAGTGTGTGTGTGTGTTA		
OPE17	$(TTC)_5$	F:	GCGGCAAGAACAGTAGATGT	50	205

Locus	Repeat Motif		Primer sequences (5' to 3')	Tm	Size (bp)
		R:	TGATGACTCCGATGAGGATG		
OPE18	$(TGA)_4$	F:	CTTGTGGGTCTCGTTGTGTC	55	101
		R:	TCCTCATTCGTGCCTTTC		
OPE19	$(TAC)_8$	F:	GATGGCAGATTACAAGACAGAG	57	228
		R:	CAACTTCGTCAAAAACAGTCC		
OPE21	$(TAC)_{10}$	F:	TGGCTGATTACAAGACAGAGTT	57	390
		R:	GGTTGAGTGGTAGTGGTAGAGA		
OPE28	$(TG)_{10}$	F:	CCCGAAATGACACATCAAAA	59	328
		R:	ACACACACACACACACAGCAC		
OPE29	$(TG)_{12}$	F:	CGTAGAAAAATCATAAAAATGC	54	208
		R:	CACACACACACACACACACA		
OPE30	$(TG)_{12}$	F:	TGTTTCTAGGGGCTTTGCTG	60	192
		R:	ACTGGCCATAAGCCCATATT		
OPE32	$(GAT)_{12}$	F:	ACAAGCCATCCAAGGGAGT	60	171
		R:	CAAGTTCATCGGCATCGTT		
OPE34	$(TCT)_{13}$	F:	TTTGTGGTGGGTCTTGTATTC	57	153
		R:	TGTCATTCCTGTTTTTCGTG		
OPE44	$(ACT)_4$	F:	GACTGTTGTTGACGAAGTTGTTG	60	250
		R:	ATGTAAAACGAGGCCAGTGAAT		
OPE45	$(TGA)_6$	F:	GAAAAGAAAAGAAGAGTCGCTG	58	375
		R:	AGTTTGTGAGTGCTTGCGT		
OPE46	$(TAG)_6$	F:	GCAGTAAATGACCAGCAAAA	56	396
		R:	AGACCCAACACAAACTCCTAAT		
OPE47	$(TATCA)_2$	F:	ACTCATCTACTCATCACGCATC	57	349
		R:	CATACCACAGGATTCTATCACG		
OPE48	$(AGA)_4$	F:	TATGGTAGCCCAGAGAACGA	58	339
		R:	GCCTTTCGGGATGAGGAT		
OPE49	$(TAC)_7$	F:	GATGGCAGATTACAAGACAGAG	57	245
		R:	CAACTTCGTCCAAAACAGTCC		
OPE50	$(TA)_8$	F:	GAGAGAGTAGACACGGACTTCA	56	399
		R:	TACCTTGAGACTTCCATACCAG		
OPE51	$(TG)_{54}$	F:	GCTCCACAAGATAAATGTGTGC	60	292
		R:	CCTTGAGACTTCCATACCAGGA		
OPE52	$(AG)_{10}$	F:	TACATCACAAATCCTAAGACCC	56	361
		R:	CACTATCCTCATGCTTCAGTTT		
OPE53	$(AC)_5$	F:	CCAATTTAGGGAATCTGAACTC	57	312
		R:	TTCATCACTATCCTCATGCTTC		
OPE53b	$(TCA)_5$	F:	CACACCACACCTCTCATTACTC	57	285
		R:	GTGATGCCTCTGTCTTCTGTAA		
OPE54	$(TCT)_7$	F:	GCAGGTTGTTATGAAGCTAATC	56	164
		R:	GTGTGGTGTCCTCTCAATCTAT		

Locus	Repeat Motif		Primer sequences (5' to 3')	Tm	Size (bp)
OPE55	$(AC)_5$	F:	ACACACACAGACACACACACAT	57	232
		R:	AGCATGTTCCAGTCCTTAATCT		
OPE56	$(TGA)_7$	F:	CTAGCAGAATCACATCAACAAC	55	383
		R:	ATGGCAGATTACAAGACAGAGT		
OPE57	$(TAC)_7$	F:	GCCCTTAAATCACTTGTAACCT	57	124
		R:	CCTTGTTTGAGTCGTAGTTAGC		
OPE58	$(ATT)_7$	F:	AGATGGTATGCCTTGAGGTAG	56	266
		R:	TTAGTTTTCCTCTCACGAGTGT		
OPE59	$(TAC)_7$	F:	ATGGCAGATTACAAGACAGAGT	56	378
		R:	CTAGCAGAATCACATCAACAAC		
OPE60	$(AC)_{44}$	F:	TTGCTTAGAGGAGGCTGAAA	58	346
		R:	TTTTTGGTTAGCGGAGAAGG		
OPE61	$(TG)_{25}$	F:	GTTTTGAGGAGGGAGGGAGT	60	246
		R:	GGCTGTGGTGTAGTGAGAGAA		
OPE62	$(TCT)_3$	F:	TCAGCGAGATTGCCGTTAC	60	236
		R:	TGAAGATGGCTCAGAAAAGG		
OPE62'	$(ATTT)_3$	F:	TCGTCTTCTTCTGTGCATGTCT	60	345
		R:	TGATGACTCGTTGGCTCATTAC		
OPE65	$(ATAAT)_2$	F:	GGTGATGATGAGGAGTAGGAGT	57	355
		R:	CGCATTAAACTGACTAACTTGC		
OPE66	$(TATTA)_2$	F:	GCTCTGAATTAACCCCTCACTA	58	342
		R:	CGAATGAAGAAAAGACAACTCC		
OPE67	$(TCA)_9$	F:	GTCCAAACCCTTGCTTGTTC	60	399
		R:	GGCTCCATCACTCTTCTCTACA		
OPE68	$(AG)_{35}$	F:	ACGAGGATAAACTACTGCCAT	56	397
		R:	ACTGGCCGACTATGTTACG		
OPE69	$(TAC)_7$	F:	ATAAGTAGCCCCTATCAGACCT	55	439
		R:	GATTACGCCAACGCTCAGA		
OPE70	$(TCA)_8$	F:	GCAACTCCCTTGGATGGAT	60	236
		R:	CTGCTTGGTTCATTTTACCC		

Table 4.3. Loci, primer sequences, repeat motifs, melting temp (Tm), and fragment sizes of *Puccinia emaculata* SSR loci (n= 33) developed from RNA-Seq expressed sequence tags (ESTs).

Locus	Repeat Motif		Primer sequences 5' to 3'	Tm	Size (bp)
OEPE2	$(ATTCA)_8$	F:	CGTGAGTGAAAGATAACGAGTTG	60	195
		R:	TTTCTTACTTCGCCTATTTCGG		
OEPE6a	$(AAACT)_{12}$	F:	GTAATAAGAGCCAACACGGAGG	60	381
		R:	TAGGATTAGGCATGGCGTACTT		
OEPE6b	$(TG)_7$	F:	AACTTTTCCTGCACCCTTTT	59	244
		R:	CTGTGATTAGCCCTTGAAACAC		
OEPE7a	$(GAG)_6$	F:	GGACGATGGAATACCGCTC	60	383
		R:	GAGAGAGAGTCAAGAGCCTTCG		
OEPE8b	$(GTT)_7$	F:	GGTGGTTTGGGCATTATCAG	60	319
		R:	ACTCACTACAGCACTCACTCGG		
OEPE26b	$(TTA)_6$	F:	CTATAAATCGTCCAAAGCAGGG	60	162
		R:	GCAGCGAAACTATGATGTGTGT		
OEPE28	$(GGT)_6$	F:	AACGTCTCCAAAAGCTGATCTC	60	278
		R:	GATTGCAGTAAGACAAGGGGAC		
OEPE37	$(CAG)_7$	F:	CAAGCTCATTCTTCCAGTTTCA	59	299
		R:	CTTTGCTGTGGTACTTGCTGAT		
OEPE47	$(AG)_7$	F:	AAAAGTACAGGAAATCGCAAGC	60	167
		R:	CCTAGTATGAGCACCAGCTTCA		
OEPE48	$(TA)_8$	F:	ATCACAACCCAACAGTAAATCG	59	226
		R:	AGACCTTCCATTTTCCCTCC		
OEPE55	$(GGT)_6$	F:	GTGGTGGTGTTGATGATGATG	59	139
		R:	CGACAAGGAGATATGGAGGTTT		
OEPE58	$(CTG)_7$	F:	GAACCAAGTTAGAGGGGAGGTT	60	337
		R:	TACGTCTTCTTTGATCCCATGA		
OEPE66	$(ATACC)_{10}$	F:	TTAGACCTTTACACACCTACCCC	60	396
		R:	GAGATGTTCTGCGAGGTACAGA		
OEPE67	$(AT)_7$	F:	GCGTATGGTTTTGTAGCTTGTG	60	245
		R:	TTTCGCTTCTCTCTTCGACTCT		
OEPE68	$(GTT)_8$	F:	TGGAGAGATAGACCCAATAGCC	60	313
		R:	CAACTCATCAACACACAACCCT		
OEPE71	$(AG)_8$	F:	AGCGTGACAAGTGAACAAGAGA	60	345
		R:	TACAACCCGAAACTCCTCAACT		
OEPE78	$(GGA)_6$	F:	TTCCTATTGAAGCTAGTGGGGA	60	176
		R:	TACTACTACGCACAGACACCGC		
OEPE79	$(ATG)_6$	F:	TGGACAAAGGGTGTGTAGAATG	60	125
		R:	TTTACCATCCCAACCGATTAAG		
OEPE92	$(GA)_{18}$	F:	TTGTTAAAGGATACGAGCCGAT	60	167
		R:	ACCAAACCAGATTGAGCAGATT		
OEPE103	$(CAA)_7$	F:	AGGCAACCCAACAGACAGTAGT	59	173

Locus	Repeat Motif		Primer sequences 5' to 3'	Tm	Size (bp)
		R:	TTGATGTACGAAGCACCATGTA		
OEPE104	$(CCT)_6$	F:	GCAGAGACTGAACATCCTGTGA	60	270
		R:	AAACAACTCTTCCAGCCAAAAC		
OEPE106	$(AC)_7$	F:	TCCTACACAAACAGGAAGACGA	61	308
		R:	GATGATGAGCGTCGATGAAAC		
OEPE109	$(GAA)_8$	F:	TAGCATTACGGAAACTGAAGCA	60	283
		R:	GTGAAGTGGTTGGATTTGATGA		
OEPE111	$(GCT)_6$	F:	TATGATTGAGGAGATGCTGGTG	59	346
		R:	GAGACGGACTTCCAGGTGAT		
OEPE115	$(ATC)_8$	F:	GCCAGAATACTACCATCAACCC	60	201
		R:	AGGTTAAGCCGAATGTGGTG		
OEPE118	$(GAA)_6$	F:	GATCCTCCACAACAAACCAGAC	60	283
		R:	GTGCAGAACTTTCAGGGGATAA		
OEPE128	$(GAG)_7$	F:	TTTGCGACGACCGAGGGG	67	128
		R:	TCACCAACCGCTGCCCTATCTT		
OEPE141	$(TGA)_6$	F:	ATCGGCTTCACATCTGGTATCT	61	275
		R:	ATCAGGTTCTGCGTCTTCTGTC		
OEPE156	$(GA)_7$	F:	GAAAGAGCAACCAAGTGAAACC	60	271
		R:	CCTCATCAACATCCACAACAAC		
OEPE159	$(CTT)_7$	F:	GTTGCGGCTGCTTCTTCTT	60	106
		R:	CAAGATCCAACGAACTCAAGG		
OEPE160	$(TG)_7$	F:	TACCGAGAGCTTTTGAGAGACC	60	126
		R:	TTTCCACCAAGAACCACTACAA		
OEPE161	$(GA)_{10}$	F:	ATCAATGTAAGCACCAAAGCAG	60	123
		R:	CAGGAACCATACAAGCCTAACC		
OEPE162	$(CT)_8$	F:	CTCCTCTCGCTTCCCAGTC	60	263
		R:	TTACCTACATCTTCATCCGCCT		

Microsatellite validation

Microsatellite primers were pre-screened using genomic DNA isolated from bulked *P. emaculata* urediniospores collected in five different states: Iowa, Mississippi, Oklahoma, South Dakota, and Virginia (Table 4.1). PCR was performed in a total volume of 20 µL with 2 µL of DNA (30 ng), 10 µL of GoTaqGreen Master Mix (Promega, Madison, WI), and 1 µL of each forward and reverse primer (5 µM) and 6 µL of sterile nuclease-free water. The following PCR program was used: 5 min initial denaturation at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s annealing at the optimal Tm for each primer (Tables 4.2 and 4.3), and 30 s at 72°C. PCR was

completed with a 10 min final extension at 72°C. Allele amplicons were resolved and visualized by electrophoresis on a 2.5% agarose-TAE gel.

Microsatellites that amplified DNA fragments and were informative (variable fragment sizes) for urediniospore populations from the five states were tested on whole genome amplifications (WGAs) from 25 single urediniospores of *P. emaculata* isolated from the same five state populations, IA, MS, SD, VA and OK (5 singles spores/state). WGAs were prepared from single urediniospores as described previously (see Chapter 3). Briefly, single germinated urediniospores were isolated using a modified cylinder loop-needle method adapted from Hildebrand (1938) (16). WGAs were carried out using an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) following the manufacturer's instructions with minor modifications (see Chapter 3). PCR conditions for amplifying microsatellites were the same as described above. Allele sizing was determined using a 2100 Bioanalyzer with a DNA 1000 kit (Agilent Technologies, Inc., Santa Clara, CA) located at Recombinant DNA/Protein Resource Facility (OSU).

Cross-species amplification of SSR markers

Cross-transferability of microsatellites (16 from SSR-enriched DNA library and 33 from EST-SSRs) was examined using genomic DNA from four multi-urediniospore samples of *Puccinia* spp. (Table 4.4): *P. andropogonis* (PA-01), *P. sorghi* (PS-01), *P. striiformis* f.sp. *tritici* (PST-01), and *P. triticina* (PT-01). The identifications of these *Puccinia* species were previously confirmed using sequences of the ITS region (see Chapter 3). Microsatellite PCR conditions were identical to those described for *P. emaculata*. Alleles were visualized after electrophoresis on 2.5% agarose-TAE gels.

Table 4.4. Isolates of four *Puccinia* species used to test cross transferability of microsatellites.

Isolate	Code	Host	Origin	Collection year
Puccinia striiformis f.sp. *tritici*	PST-01	*Triticum aestivum*	Stillwater, OK	2013
Puccinia triticina	PT-01	*T. aestivum*	Stillwater, OK	2013
Puccinia andropogonis	PA-01	*Andropogon gerardii*	Wilburton, Oklahoma	2013
Puccinia sorghi	PS-01	*Zea mays*	Eastern Oklahoma	2013

Data Analysis

Estimation of polymorphism informativeness, measured as polymorphism information content (PIC), and gene diversity of individual SSR loci were determined using PowerMarker version 3.25 (20). Additionally, individual population distribution and genetic analyses were performed using GenAlex 6.5 (26). Genetic differentiation (difference in allele frequencies among populations) within *P. emaculata* single spores was analyzed using F_{ST} statistic estimates (F_{ST} value of 0 to 0.05 = low, 0.05 to 0.15 = moderate, 0.15 to 0.25 = high, >0.25 = very high genetic differentiation) (44). Gene flow between populations was assessed by estimation of the number of migrants per generation (Nm < 0.5, no gene flow, populations isolated; 0.5 > Nm > 1, gene flow is weak; Nm > 1, frequent gene flow among populations) (44). Genotype clustering based on genetic distances, illustrated by principal coordinate analysis (PCoA), allelic patterns across *P. emaculata* populations, and private alleles were estimated using GenAlex 6.5.

RESULTS

Isolation of SSR loci from enriched libraries

Transformation of *E. coli* was successful and of the 630 positive colonies,150 colonies were selected for PCR amplification, of which 60 were sequenced. From the 60 sequences obtained, 56

transformants contained unique sequences. A total of 54 sequences contained microsatellites, of which, 49 contained a number of repeat motifs ranging from 5 to 12.

Assembly and characterization of *P. emaculata* ESTs

A total of 704 SSR containing sequences were obtained from RNA-seq of cDNA libraries from OK and VA urediniospores (germinated and nongerminated). These sequences assembled into 163 contigs. Similarity searches (BLASTx) returned hits with significant identities. Most of the *P. emaculata* contigs showed hits to hypothetical proteins in the genomes of *Puccinia graminis* (>78% identity) and *P. horiana* (>80% identity). From these 163 contigs, 33 SSRs were found to occur in both OK and VA and were used to design microsatellite primers.

Microsatellite validation

SSR primers were designed only for the clones and ESTs carrying more than 6 dinucleotide or trinucleotide repeats. A total of 82 SSR primer pairs (49 from genomic library and 33 from EST- SSR) were initially developed and screened with *P. emaculata* genomic DNA from urediniospores collected in IA, MS, OK, SD, and VA (Table 4.1). Developed primers are listed in Tables 4.2 and 4.3. In all five *P. emaculata* urediniospore populations, 16 out of 49 primers pairs from the SSR-enriched library (32.7%) and 32 out of 33 primer pairs generated from EST-SSR sequences (97.0%) produced amplicons of the expected sizes. The rest of the primers did not produce amplicons or produced multiple bands. Of these 48 SSR primer pairs, 20 primer pairs identified polymorphic microsatellite loci in multispore genomic DNAs from the five state populations (8 from the SSR-enriched library and 12 from the EST-SSRs), and 18 primer pairs identified polymorphic SSRs in single spore WGAs (two SSR primers, OPE19 and OPE21, were monomorphic in WGAs). The sizes of SSR alleles ranged from 120 to 380, with most of the alleles being 100 to 350 bp long. The average observed heterozygosity of *P. emaculata* SSR

markers was 0.1390, and the polymorphic information content (PIC) value ranged from 0.65 to 0.91, (Table 4.5).

Table 4.5. Allele diversity and genetic characteristics of 18 polymorphic SSR loci for single *Puccinia emaculata* urediniospores.

	Locus name[a]	Sample No.	Allele No.	Availability	*P*[b]	Heterozygosity[b]	PIC[b]
repeat-enriched library	OPE2	25	9	1.0000	0.7622	0.1250	0.7289
	OPE5	25	17	1.0000	0.9123	0.1667	0.9062
	OPE19	25	3	0.9583	0.5709	0.0000	0.4977
	OPE21	25	4	0.9583	0.3062	0.0000	0.2907
	OPE28	25	4	0.9583	0.7108	0.0000	0.6550
	OPE32	25	7	1.0000	0.7622	0.0833	0.7223
	OPE59	25	4	1.0000	0.6424	0.0000	0.5827
	OPE60	25	6	1.0000	0.7292	0.0833	0.6816
from ESTs	OEPE6a	25	6	1.0000	0.7257	0.0833	0.6784
	OEPE6b	25	6	1.0000	0.7786	0.1250	0.7438
	OEPE28	25	7	0.9583	0.6853	0.0435	0.6383
	OEPE68	25	10	1.0000	0.8646	0.0833	0.8501
	OEPE71	25	6	1.0000	0.7986	0.2500	0.7690
	OEPE92	25	14	1.0000	0.8429	0.9583	0.8272
	OEPE128	25	11	0.9167	0.8399	0.3182	0.8252
	OEPE141	25	9	1.0000	0.7865	0.1250	0.7620
	OEPE156	25	9	0.9583	0.7836	0.0435	0.7580
	OEPE160	25	12	1.0000	0.8628	0.0833	0.8490
	OEPE161	25	10	1.0000	0.8325	0.0833	0.8139
	OEPE162	25	12	1.0000	0.7977	0.1250	0.7818
	Mean	25	8	0.9854	0.7497	0.1390	0.7181

a. SSR loci for *P. emaculata*, OPE (from repeat enriched library OEPE (from EST sequences)
b. *P*: major allele frequency, *H*: heterozygosity, and PIC: polymorphic information content

The genetic analyses of the 18 SSR loci among 25 single urediniospores of *P. emaculata* showed moderate genetic variation among states ((F_{st}=0.123)) and frequent gene flow between populations (Nm>1.858) (Table 4.6). SSR genotypes among the five single urediniospores from each state tightly clustered together, with IA and SD appearing most similar, and VA most

distinct (Figure 4.1). The highest numbers of private alleles were found in VA, SD, and IA (Figure 4.2).

Table 4. 6. Genetic diversity within *Puccinia emaculata* populations Iowa (IA), Mississippi (MS), Oklahoma (OK), South Dakota (SD) and Virginia (VA).

Pop1	Pop2	*Fst*[a]	*Nm*[a]
OK	VA	0.155	1.362
OK	MS	0.119	1.851
VA	MS	0.117	1.879
OK	IA	0.134	1.619
VA	IA	0.147	1.456
MS	IA	0.094	2.422
OK	SD	0.146	1.461
VA	SD	0.13	1.68
MS	SD	0.085	2.685
IA	SD	0.104	2.16

[a] *Fst:* genetic variation (F_{ST} value of 0 to 0.05 = low, 0.05 to 0.15 = moderate, 0.15 to 0.25 = high, >0.25 = very high genetic differentiation;; *Nm:* gene flow (*Nm* < 0.5 isolation of the groups, 0.5 > *Nm* > 1 gene flow is weak, *Nm* > 1 indicates constant gene flow)

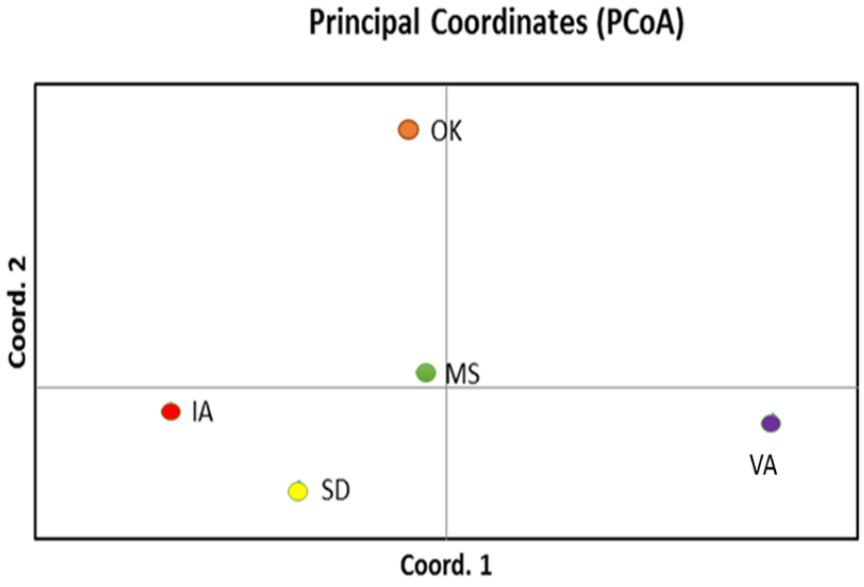

Figure 4.1. Principal coordinate analysis of 25 *Puccinnia emaculata* single spores from Iowa (IA), Mississippi (MS), Oklahoma (OK), South Dakota (SD) and Virginia (VA) using 18 polymorphic microsatellite loci.

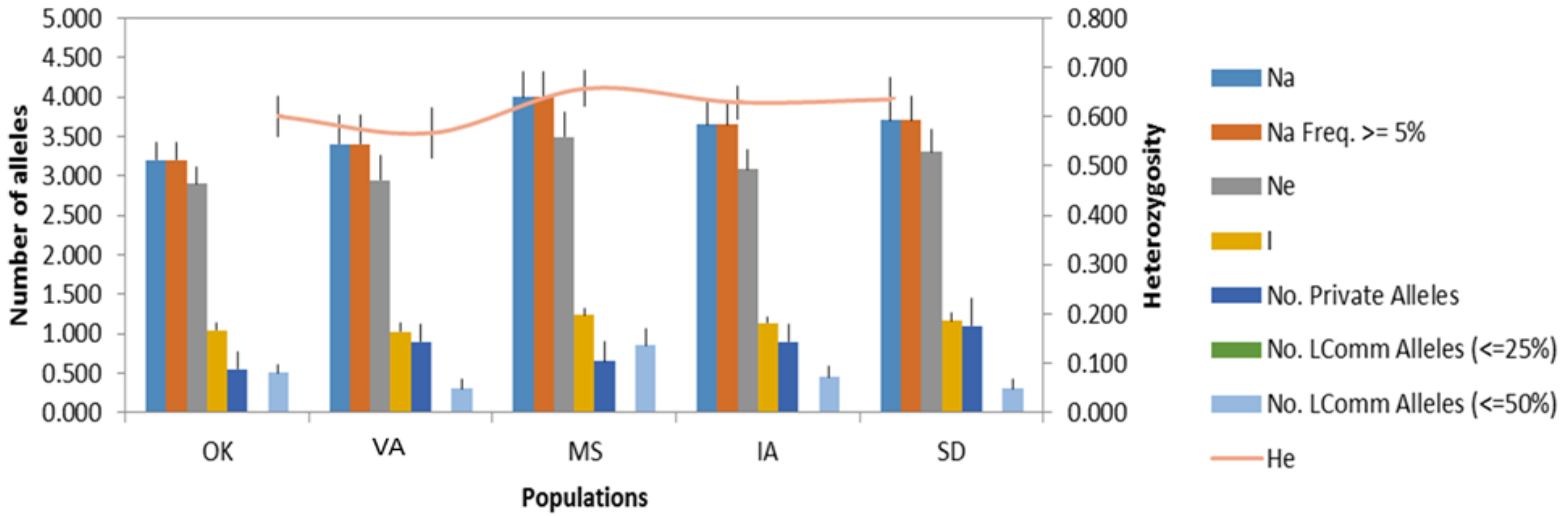

Figure 4.2. Allelic patterns across *P. emaculata* populations, Oklahoma (OK), Virginia (VA), Mississippi (MS), Iowa (IA) and South Dakota (SD). Na = No. of Different Alleles, Na (Freq >= 5%) = No. of Different Alleles with a Frequency >= 5%, I = Shannon's Information Index = -1* Sum (pi * Ln (pi), No. Private Alleles = No. of Alleles Unique to a Single Population, No. LComm Alleles (<=25%) = No. of Locally Common Alleles (Freq. >= 5%) Found in 25% or Fewer Populations, No. LComm Alleles (<=50%) = No. of Locally Common Alleles (Freq. >= 5%) Found in 50% or Fewer Populations, He = Expected Heterozygosity = 1 - Sum pi^2.

Transferability of SSR markers across related *Puccinia* species

Of the 16 enriched library SSR primer pairs tested, 11 were transferable to other *Puccinia* species (68.8%) and 2 primer pairs, OPE5 and OPE21, produced PCR products in all the *Puccinia* species evaluated. Higher marker transferability was found among EST-SSRs. Of the 33 EST-SSR primer pairs tested, 27 were transferable to other *Puccini*a species (81.71%), and 8 (OEPE2, 6b, 26b, 58, 66, 106, 160, and 161) were transferable to all *Puccinia* species tested (Table 4.7).

Table 4.7. Transferability of SSR loci from *Puccinia emaculata* to four *Puccinia* species. SSR primers amplifying a band or bands in those species are indicated with an "X".

SSR Locus[a]	***P. graminis* f.sp. *tritici***	***P. triticina***	***P. sorghi***	***P. andropogonis***
OPE2		X	X	X
OPE5	X	X	X	X
OPE19			X	X
OPE21	X	X	X	X
OPE28		X	X	
OPE32				
OPE46				X
OPE49	X			
OPE50		X		
OPE52		X		X
OPE54	X			
OPE58		X	X	
OPE59				
OPE60				
OPE67				
OPE69				
OEPE2	X	X	X	X
OEPE6a	X	X		X
OEPE6b	X	X	X	X
OEPE7a				X
OEPE8b				X
OEPE26b	X	X	X	X
OEPE28		X	X	X
OEPE37	X		X	X
OEPE47		X	X	X
OEPE48				
OEPE55				
OEPE58	X	X	X	X

SSR Locus[a]	*P. graminis* f.sp. *tritici*	*P. triticina*	*P. sorghi*	*P. andropogonis*
OEPE66	X	X	X	X
OEPE67				
OEPE68		X	X	X
OEPE71				X
OEPE78				
OEPE79				
OEPE92				X
OEPE103		X		X
OEPE104	X			X
OEPE106	X	X	X	X
OEPE109		X		X
OEPE111				X
OEPE115				X
OEPE118	X			
OEPE128	X			X
OEPE141				
OEPE156				X
OEPE159	X	X	X	
OEPE160	X	X	X	X
OEPE161	X	X	X	X
OEPE162		X		X
Total	18	23	20	31

[a] OPE (from repeat enriched libraries) and OEPE (from EST sequences)

DISCUSSION

SSR markers have been widely used for genetic studies in plants and animals, because of the advantages they present. SSRs are informative markers revealing genetic variation in diploid or dikaryotic organisms, such as *Puccinia* species (35; 36; 41; 45). The characterization of SSR markers requires isolating genome fragments with repetitive sequence motifs, designing primers to amplify randomly selected SSR loci, and screening these with a representative collection of individuals to assess SSR polymorphisms (28).

More recently, ESTs have begun to be used not only for gene discovery, but also as a resources to identify useful molecular markers, such as SSRs. EST libraries are already among

the most diverse and abundant type of sequence data available (3; 9; 18). The number of EST-SSRs reported in *Puccinia* species are 47,500 and 168,200 ESTs from Pucciniales (NCBI database) (2; 7; 35; 36; 42; 45). Once SSR markers are developed either from repeat-enriched DNA libraries or from ESTs, they provide an effective means to study the population genetics of the target organism.

Prior to the present study, Wadl et al. 2011 (41) characterized 10 SSR markers in 20 single-pustule isolates of *P. emaculata* from AR, LA, MS, NC, and TN. However, *P. emaculata* urediniospores from IA, MS, OK, SD, and VA, collected during this study, resulted in amplification with only three (Pe2-005, Pe2-018 and Pe4-032) of the 10 SSR primer pairs. In order to assess the population genetics of switchgrass rust, more SSR markers were needed. Thus, twenty polymorphic SSR markers for *P. emaculata* from a repeat-enriched genomic library and RNA-Seq EST libraries were developed in this study.

Short repeats can produce monomorphic PCR products displaying very low polymorphism (44), for example microsatellite OPE55 (AC_5) and OPE44 (ACT_4), demonstrated this effect when evaluated with multispore DNA. Also, of the 60 sequenced microsatellite clones from the repeat-enriched library, primer design was not possible for 11 clones, which had poor quality sequences or contained SSRs either too near the start or end of the clone insert, leaving insufficient flanking DNA for primers. The 49 microsatellites captured from the repeat-enriched library possessed perfect di, tri, tetra and pentanucleotide repeats, with trinucleotide repeats being the most common. Di- and trinucleotide repeats have been demonstrated to be common in some *Puccinia* species, such as *P. triticina* and *P. graminis* f.sp. *tritici* (36; 45)

Molecular diversity among the different urediniospore populations from five states all show high levels of polymorphism with all SSRs. Among the 25 single spores of *P. emaculata*, two primer pairs, OPE19 and OPE21, identified only monomorphic alleles. SSR primer set OPE5

identified as many as 17 alleles per locus, while OEPE92 identified 14 alleles and primer sets OEPE160 and OPE162 each identified 12 alleles. Sizing alleles using the Bioanalyzer identified four samples showing an absence of bands. This may be due to low concentrations of PCR products. Or, the concentration and integrity of DNA in these four samples may have affected the amplification of alleles (12).

PIC value is an indicator predicting the usefulness of DNA markers for gene mapping, molecular breeding, and germplasm evaluation (33). Markers with higher PIC values possess greater potential to reveal allelic variation. The average PIC value of SSR markers tested by different researchers for crops varies based on the number of SSR markers used and the number of genotypes tested. The PIC values of SSRs in the present study were 0.7893 and 0.6646 from the repeat-enriched library and from ESTs, respectively. SSR markers are able to detect genetic polymorphism better than other molecular markers because the allelic diversity is caused by repeated sequences rather than single nucleotide substitutions or indels (37; 38). The polymorphisms seen in the present study are high and could be due to the following: 1) the large number of SSRs used, 2) adequate genome coverage, and 3) variability among populations used.

The 18 SSRs used to analyze the 25 single spores of *P. emaculata* collected from five different state populations show significant genetic differentiation among populations. VA single spores differed from OK single spores by 15% and from MS single spores by 12%. The least genetic differentiation (10%) occurred between single spores from IA and SD, which was expected because both states and sample sites within these states were near one another. MS also had less genetic variation with IA (9%) and SD (8%), which corroborated the "*Puccinia Pathway*" hypothesis established in previous wheat rust studies (23). Likewise, phylogenetic analyses using the three conserved loci, ITS, bTub, and TEF1a revealed that *P. emaculata* haplotype diversity declines moving north and east along "*Puccinia Pathway*" (see Chapter 3). This, along with higher numbers of haplotypes in southern states OK and MS, suggests a possible

southern inoculum source. Also, allelic patterns show fewer private alleles in OK and MS, suggesting that these two states have gene flow and high migrant exchange occurring each generation.

Also, many of the SSRs developed for *P. emaculata* were transferable to other *Puccinia* species. Transferability of microsatellites appears to be proportional to the phylogenetic relatedness to *P. emaculata*, with 36.7% of microsatellite markers amplifying in *P. striiformis* f. sp. *tritici* DNA, 46.9% in *P. triticina*, 40.8% in *P. sorghi*, and 63.3% in *P. andropogonis*, without any modification to the PCR conditions. The higher percentage of transferability to *P. andropogonis* could be due to its close phylogenetic relationship with *P. emaculata* (see Chapter 3). While these results represent progress in the population studies of rust fungi, further studies using a broader collection of rust fungi with other *Puccinia* species, especially those specializing on C3 or C4 grasses, will be necessary to further assess questions of transferability and polymorphism.

Two different sources for SSR marker isolation were used in this study, 1) repeat-enriched DNA libraries and 2) ESTs. The enriched library technique may seem more labor-intensive. However, to successfully sequence ESTs using RNA-seq with the Illumina HiSeq™ 2000, very high quality RNA is required, the isolation of which is also very labor intensive.

In this study, highly polymorphic SSR markers in *P. emaculata* were identified and evaluated for application in population genetic studies. These SSR markers are highly transferable to related taxa and provide useful tools for assessing genetic diversity within and among species. The highly informative SSR markers in *P. emaculata* should serve as valuable molecular tools to understand the etiology of switchgrass rust, for tracking races in switchgrass production fields, and to initiate the development of effective management strategies.

REFERENCES

1. Arthur, J. C., and Cummins, G.B., 1962. Manual of the rust in the United States and Canada. Hafner, New York. 438 p.

2. Bahri, B., Leconte, M., de Vallavieille-Pope, C., and Enjalbert, J. 2009. Isolation of ten microsatellite loci in an EST library of the phytopathogenic fungus *Puccinia striiformis* f. sp. *tritici*. Conservation Genetics 10:1425-1428.

3. Bouck, A., and Vision, T. 2007. The molecular ecologist's guide to expressed sequence tags. Molecular Ecology 16:907-924.

4. Bouton, J. 2008. Improvement of switchgrass as a bioenergy crop. In: Vermis W (ed), genetic improvement of bioenergy crops. Springer science and bussiness media, pp 295-308.

5. Cristancho, M., and Escobar, C. 2008. Transferability of SSR markers from related Uredinales species to the coffee rust *Hemileia vastatrix*. Genetic Molecular Resources 7:1186-1192.

6. Dambroski, H. R., and Carson, M. L. 2008. Development and characterization of novel, polymorphic microsatellite markers for oat crown rust, *Puccinia coronata*. Molecular Ecology Resources 8:1395-1398.

7. Dracatos, P. M., Dumsday, J. L., Olle, R. S., Cogan, N. O., Dobrowolski, M. P., Fujimori, M., Roderick, H., Stewart, A. V., Smith, K. F., and Forster, J. W. 2006. Development and characterization of EST-SSR markers for the crown rust pathogen of ryegrass (*Puccinia coronata* f. sp. *lolii*). Genome 49:572-583.

8. Duan, X., Enjalbert, J., Vautrin, D., Solignac, M., and Giraud, T. 2003. Isolation of 12 microsatellite loci, using an enrichment protocol, in the phytopathogenic fungus *Puccinia triticina*. Molecular Ecology Notes 3:65-67.

9. Ellis, J., and Burke, J. 2007. EST-SSRs as a resource for population genetic analyses. Heredity 99:125-132.

10. Enjalbert, J., Duan, X., Giraud, T., Vautrin, D., Vallavieille-Pope, D., and Solignac, M. 2002. Isolation of twelve microsatellite loci, using an enrichment protocol, in the phytopathogenic fungus *Puccinia striiformis* f. sp. *tritici*. Molecular Ecology Notes 2:563-565.

11. Freshour, L., Agarwal, S., Sorochan, J., Zale, J., Ownley, B., Gwinn, K., Castlebury, L. A., and Carris, L. M. 2008. First report of *Puccinia emaculata* on switchgrass (*Panicum virgatum* L.) in Tennessee. Plant Disease 92:1710.

12. Gilbert, J., Lewis, R., Wilkinson, M., and Caligari, P. 1999. Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theoretical and Applied Genetics 98:1125-1131.

13. Glenn, T. C., and Schable, N. A. 2005. Isolating microsatellite DNA loci. Methods in Enzymology 395:202-222.

14. Gravert, C., and Munkvold, G. 2002. Fungi and diseases associated with cultivated switchgrass in Iowa. Journal Iowa Academy of Science 109:30-34.

15. Handbook, R. M. 2006. Concurrent mRNA and protein extraction from the same experimental sample using a commercially available column-based RNA preparation kit. BioTechniques 40:54-58.

16. Hildebrand, E. M. 1938. Techniques for the isolation of single microorganisms. The Botanical Review 4:627-664.

17. Hirsch, R. L., TeBeest, D. O., Bluhm, B. H., and West, C. P. 2010. First report of rust caused by *Puccinia emaculata* on switchgrass in Arkansas. Plant Disease 94:381-381.

18. Kalia, R. K., Rai, M. K., Kalia, S., Singh, R., and Dhawan, A. 2011. Microsatellite markers: an overview of the recent progress in plants. Euphytica 177:309-334.

19. Li, Y., Windham, M., Trigiano, R., Windham, A., Ownley, B., Gwinn, K., Zale, J., and Spiers, J. 2009. Cultivar-specific interactions between switchgrass and *Puccinia emaculata*. Phytopathology 99: S72.

20. Liu, K., and Muse, S. V. 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 21:2128-2129.

21. Madden, L. 1983. Measuring and modeling crop losses at the field level. Phytopathology 73.

22. Martins, W., de Sousa, D., Proite, K., Guimarães, P., Moretzsohn, M., and Bertioli, D. 2006. New softwares for automated microsatellite marker development. Nucleic Acids Research 34:e31-e31.

23. Nagarajan, S., and Singh, D. 1990. Long-distance dispersion of rust pathogens. Annual Review of Phytopathology 28:139-153.

24. Parkinson, J., and Blaxter, M. 2009. Expressed sequence tags: an overview. Methods Mol Biol 533:1-12.

25. Parrish, D. J., and Fike, J. H. 2005. The biology and agronomy of switchgrass for biofuels. Critical Reviews in Plant Science 24:423-459.

26. Peakall, R., and Smouse, P. E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537-2539.

27. Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., and Rafalski, A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding 2:225-238.

28. Queller, D. C., Strassmann, J. E., and Hughes, C. R. 1993. Microsatellites and kinship. Trends in ecology & evolution 8:285-288.

29. Ramachar, P., and Cummins, G. B. 1965. The species of *Puccinia* on the Paniceae. Mycopathologia et Mycologia Applicata. 25:7-60.

30. Roux, K. H. 1995. Optimization and troubleshooting in PCR. Genome Research 4:S185-S194.

31. Rozen, S., and Skaletsky, H. 1999. Primer3 on the WWW for general users and for biologist programmers. Bioinformatics methods and protocols, Springer, 365-386.

32. Sharma, N., Piscioneri, I., and Pignatelli, V. 2003. An evaluation of biomass yield stability of switchgrass *Panicum virgatum* L.) cultivars. Energy conversion and management 44:2953-2958.

33. Shete, S., Tiwari, H., and Elston, R. C. 2000. On estimating the heterozygosity and polymorphism information content value. Theoretical Population Biology 57:265-271.

34. Singh, V. K., and Kumar, A. 2001. PCR primer design. Molecular Biology Today 2:27-32.

35. Szabo, L. J. 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus, *Puccinia graminis*. Molecular Ecology Notes 7:92-94.

36. Szabo, L. J., and Kolmer, J. A. 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus *Puccinia triticina*. Molecular Ecology Notes 7:708-710.

37. Tautz, D., and Renz, M. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Research 12:4127-4138.

38. Tautz, D., Trick, M., and Dover, G. A. 1986. Cryptic simplicity in DNA is a major source of genetic variation. Nature 322: 652- 656.

39. Tiffany, L., Shearer, J., and Knaphus, G. 1990. Plant parasitic fungi of four tallgrass prairies of northern Iowa: distribution and prevalence. JIAS, Journal of the Iowa Academy of Science 97:157-166.

40. Uppalapati, S. R., Serba, D. D., Ishiga, Y., Szabo, L. J., Mittal, S., Bhandari, H. S., Bouton, J. H., Mysore, K. S., and Saha, M. C. 2012. Characterization of the rust fungus, *Puccinia emaculata*, and evaluation of genetic variability for rust resistance in switchgrass populations. BioEnergy Research. 6:458-68.

41. Wadl, P. A., Dean, D., Li, Y., Vito, L. M., Scheffler, B. E., Hadziabdic, D., Windham, M. T., and Trigiano, R. N. 2011. Development and characterization of microsatellites for switchgrass rust fungus (*Puccinia emaculata*). Conservation Genetics Resources 3:185-188.

42. Wang, X., Mulock, B., Guus, B., and McCallum, B. 2010. Development of EST-derived simple sequence repeat markers for wheat leaf rust fungus, *Puccinia triticina* Eriks. Canadian Journal of Plant Pathology 32:98-107.

43. Weising K, Nybom H, Wolff K, Meyer W (1995). DNA Fingerprinting in plants and fungi. CRC Press, Boca Raton, FL.

44. Wright, S. 1978. Evolution and the genetics of populations: a treatise in four volumes: Vol. 4: variability within and among natural populations. University of Chicago Press, 1978.

45. Zhong, S., Leng, Y., Friesen, T., Faris, J., and Szabo, L. 2009. Development and characterization of expressed sequence tag-derived microsatellite markers for the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici*. Phytopathology 99:282-289.

VITA

Gabriela Karina Orquera Delgado

Candidate for the Degree of

Master of Science/Arts

Thesis: POPULATION BIOLOGY OF SWITCHGRASS RUST (*Puccinia emaculata* Schw.)

Major Field: Entomology and Plant Pathology

Biographical:
Gabriela K. Orquera was born in Quito, Ecuador in August 1986. Gabriela went to the Escuela Politécnica del Ejército (ESPE) (currently Universidad de las Fuerzas Armadas ESPE) for her undergraduate degree in Biotechnology Engineering. The title of her undergraduate research project was "Analyses of bacterial communities associated with suppression of *Phytophthora infestans* (Mont.)". Gabriela graduated in October 2010, and in April 2011 she interned at Oklahoma State University as a research assistant under Dr. Stephen Marek and Dr. Carla Garzon in the Department of Entomology and Plant Pathology. Due to the outstanding results obtained during her intern period, a graduate research assistant (GRA) position was offered by Oklahoma State University under the supervision of Dr. Stephen Marek. Working as a GRA, Gabriela participated in international, national, and regional conferences related to biofuels and phytopathology. In April 2014, Gabriela presented the Phoenix Award for outstanding master's student at OSU. After obtaining her Master's degree in Entomology and Plant Pathology, and Gabriela will continue her PhD program at Oklahoma State University in Plant Pathology.

Education:
Completed the requirements for the Master of Science in Entomology and Plant Pathology at the Oklahoma State University, Stillwater, Oklahoma- USA, July 2014.
Completed the requirements for the Bachelor of Science in Biotechnology Engineering at the Escuela Politécnica del Ejército (ESPE), Quito- Ecuador, October 2010.

Experience:
Graduate Research Assistant. Entomology and Plant Pathology, Noble Research Center, Oklahoma State University, Stillwater- Oklahoma, USA, January 2012 to July 2014.
Biotechnology Assistant, Biocontrol and Bioscience S.A, Quito- Ecuador. October 2011 to January 2012.

Professional Memberships:
American Phytopathological Society (APS)
National Honor Society Phi Kappa Phi