**Detection and phylogenetic relationships of *Puccinia emaculata* and *Uromyces graminicola*
(Pucciniales) on switchgrass in New York State using rDNA sequence information**

Shawn C. Kenaley,^{*} George W. Hudler, and Gary C. Bergstrom

School of Integrative Plant Science, Plant Pathology and Plant-Microbe Biology Section, Cornell
University, Ithaca, NY 14853-5904, U.S.A

^{*}Corresponding author:
S.C. Kenaley
Tel: +1 (607) 339- 7366
Fax: +1 (607) 255-4471
E-mail: sck26@cornell.edu

Abstract

The species of rust fungi (Pucciniales) inciting disease on switchgrass (*Panicum virgatum*) grown in bioenergy feedstock systems across the north-central and eastern United States remain unclear. In the present study, the species number and phylogenetic relationships of rust species affecting switchgrass were examined in 2011–2013 at two sites in New York State as well as selected sites in Alabama, Iowa, Nebraska, Pennsylvania, South Dakota, and West Virginia using ribosomal RNA gene data (partial internal transcribed spacer [ITS] 1, complete 5.8 subunit [S] and ITS2, and partial 28S). Uredinial group and teliospore morphology were also utilized to delimit taxa in collection years 2012 and 2013. Maximum likelihood, maximum parsimony, and Bayesian analyses demonstrated two monophyletic clades. Clade I consisted of *Puccinia emaculata* and included the majority of single-sorus samples across sites, whereas, Clade II included multiple samples from Iowa, Nebraska, and South Dakota. Single-telial samples for Clade I possessed only two-celled teliospores while Clade II samples possessed only one-celled teliospores, and hence, were readily diagnosed morphologically to *P. emaculata* and *Uromyces graminicola*, respectively. No *U. graminicola* sequences exist in GenBank to compare with our Clade II samples; however, based on teliospore morphology, the identity of Clade II taxa is *U. graminicola*.

Keywords: ITS; *Panicum virgatum*; Rust fungi; Species diversity; Teliospores.

1. Introduction

Switchgrass (*Panicum virgatum* L) is a native C₄ perennial grass found throughout central and eastern North America (USDA-NRCS 2015). Over the last 15 years, switchgrass has received considerable interest in New York State and elsewhere in United States as a bioenergy feedstock for the production of lignocellulosic ethanol and grass pellets for combustion (Casler and Boe 2003; Casler 2005; Fike et al. 2006; Sanderson et al. 2006; Schmer et al. 2008; Wojner and Rutzke 2010). Continued research in switchgrass breeding and cultivation to optimize biomass and net energy yield while perfecting best management practices and improving economic returns likely will augment the value of switchgrass in an emerging bioeconomy (Das et al. 2004; Davis et al. 2009; Bhandari et al. 2011; Casler et al 2012; Cortese and Bonos 2013; Price and Casler 2014; Gamble et al. 2015). However, until recently, phytopathogenic fungi affecting switchgrass and their associated yield-reducing potential have received scant attention (Gravert et al. 2000; Gravert and Munkvold 2002; Gustafson et al. 2003; Krupinsky et al. 2004; Thomsen et al. 2008; Zale et al. 2008; Crouch et al. 2009; Hirsch et al. 2010; Frazier et al. 2013; Gilley et al. 2013; Uppalapati et al. 2013).

Because of their explosive epidemic potential, rust fungi (Pucciniales) historically are among the most serious and damaging pathogens of agricultural crops worldwide (Strange and Scott 2005). These obligate biotrophic fungi are particularly problematic in the production of clonal crops at high density and over large acreages as rust fungi can multiply rapidly, produce infectious propagules capable of long- and short-distance dispersal, and have extensive gene flow, thereby threatening crop health and the circumvention of control strategies across a large geographic area. Moreover, rusts are unique fungi as a single species can produce up to five, functionally and morphologically different spore stages (macrocytic O-IV; O = spermatium, I =

77   aceciospore, II = urediniospore, III = teliospore, and IV = basidiospore) and require at least two
78   taxonomically unrelated host plants to complete their life cycle (heteroecious; Cummins and
79   Hiratsuka 2003). Historically, the taxonomic classification of rust fungi in large part has been
80   based on morphologic discontinuities among telia and teliospores, and to a lesser extent,
81   urediniospores (Arthur 1934, Ramachar and Cummins 1963, 1965; Cummins 1971; Cummins
82   and Hiratsuka 2003). The alternate host(s) and morphology of the aecial state have also been
83   utilized to delimit heteroecious rusts. However, in temperate regions, aecia often occur in spring
84   to early summer and are short-lived, making their collection and correlation to the telial host
85   difficult (Jin et al. 2010).

86   Four species of rust fungi have been reported to incite disease on switchgrass: *Puccinia*
87   *emaculata* Schwein., *P. graminis* Pers., *P. huberi* Henn., and *Uromyces graminicola* Burrill
88   (Burrill 1884; Arthur 1934; Ramachar and Cummins 1963, 1965; Anonymous 1970; Cummins
89   1971). Presently, *P. emaculata* has received most of the attention as it is reportedly distributed
90   across the eastern two-thirds of N. America (Ramachar and Cummins 1965; Lenne 1990; Farr
91   and Rossman 2015) and has caused recurrent outbreaks on cultivated switchgrass in NY State
92   (S.C. Kenaley and G. C. Bergstrom, person. obsv.) and elsewhere in the central and eastern U.S.
93   (Gravert and Munkvold 2002; Gustafson et al. 2003; Zale et al. 2008; Hirsch et al. 2010; Gilley
94   and Tomaso 2011; Frazier et al. 2013; Uppalapati et al. 2013). However, the geographic
95   distribution and presence of *P. graminis*, *P. huberi*, and *U. graminicola* – either alone or in
96   combination with *P. emaculata* – on bioenergy switchgrass remains unclear. *Puccinia graminis*
97   recently was reported inciting disease on cultivated switchgrass in Iowa (Gravert and Munkvold
98   2002); a first report of this host-rust combination outside the state of Kansas. No study to date

has confirmed the occurrence of either *P. huberi* or *U. graminicola* on bioenergy switchgrass in the U.S.

Presently, no study has assessed the species number, geographies, and phylogenetic relationships of rust fungi affecting switchgrass across a large geographic area in the U.S. Therefore, the objective of study was to assess the morphological discontinuities and phylogenetic relationships among rust fungi affecting switchgrass in NY State as well as at selected sites in the north-central and eastern U.S. in order to delimit switchgrass-infecting rust fungi to species. Morphologic characters included uredinial group (Ramachar and Cummins 1965) as well as teliospore dimensions, cell number, and cell wall thickness (apical and side). Phylogenetic analyses were conducted for nuclear rRNA gene data (partial internal transcribed spacer [ITS] 1, complete 5.8 subunit [S] and ITS2, and partial 28S). The ITS region was chosen for phylogenetic analyses because it is the universal DNA barcode marker for species identification in fungi (Schoch et al. 2012) and has been demonstrated previously to provide sufficient variability (i.e., resolving-power) for the phylogenetic analysis of close-related rust fungi (Zambino and Szabo 1993; Weber et al. 2003; Szabo 2008; Liu and Hambleton 2010; Barilli et al. 2011; Feau et al. 2011; Kenaley et al. 2014).

2. Materials and methods

2.1 Fungi and plant material

Rust samples (N= 70 total) were collected with switchgrass leaves in September-October 2011 and 2012 across two yield trails in NY State (Table 1) conducted at Cornell University in Ithaca (Tompkins County, NY) and the USDA-NRCS Plant Material Center in Big Flats (Chemung Co., NY; Fig. 1). Additional samples also were procured in 2012 and 2013 throughout the

growing season from rust-infected switchgrass collected in Alabama (AL; N= 6), Iowa (IA; N= 11), Nebraska (NE; N= 16), Pennsylvania (PA; N= 13), South Dakota (SD; N= 3), and West Virginia (WV; N= 2). Voucher specimens for each of the aforementioned samples were deposited at the Cornell University Plant Pathology Herbarium (CUP). Fungus material collected in 2011 was subjected only to DNA analysis, whereas, samples procured in 2012-2013 were used for DNA and morphological analyses.

2.2 Molecular identification and phylogeny

2.2.1. DNA isolation

For 2011 collections, a single-sorus (uredinium or telium) for each sample was carefully excised using a sterile surgical scalpel and transferred to a pre-labeled, sterile 1.5mL, safe-lock centrifuge tube for DNA extraction. However, for 2012-2013 samples, a single-sorus was excised from host tissue and halved using sterile techniques; one-half was used for DNA extraction, whereas the other half was utilized for morphologic examination (see below, section *2.3 Morphological identification*). Genomic DNA extractions were performed using a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's protocol with modifications as described in Kenaley et al. (2014). Before following the manufacturer's protocol, individual samples were flash-frozen in liquid nitrogen and ground separately to a fine powder in their respective safe-lock tube using a mini-pestle, and thereafter, incubated at 55°C with periodic inversions (x5; every 0.5 h) for 2.5 h in 500 mL of the kit-provided lysis buffer (AP1) amended with 10 mL of Proteinase K (10 mg mL⁻¹; QIAGEN). The tubes of lysed tissue were then placed in boiling water for 5 min, amended with 150 mL P3 buffer, and centrifuged for 1 min at 13000 rpm. The lysate was then incubated for an additional 10 min at -20°C and

centrifuged again for 5 min (13000 rpm) to pelletize cell debris and ease transfer of the supernatant (ca. 660 mL) to a QIAshredder Mini spin column. The remaining procedures were identical to that provided in the DNeasy Plant Handbook (steps 9–19 of the Mini protocol).

2.2.2. PCR amplification, DNA sequencing, and cloning

Full-length (partial 18S rRNA gene; complete ITS1, 5.8S rRNA gene, and ITS2; and partial 28S rRNA gene) or partial ITS sequences (partial ITS1; complete 5.8S and ITS2; and partial 28S) sequences were PCR-amplified using the primer pair ITS1rustF10d (Barnes and Szabo 2007) and RUST1 (Kropp et al. 1995) or PRITS1f (5′-AGTGCACTTTATTGTGGCTCGA-3′; L. Castlebury, pers. comm.) and ITS4B (Gardes and Bruns 1993), respectively. PCRs were conducted in a 25 mL reaction volume consisting of 2.5 mL AccuPrime PCR buffer II (Invitrogen, Carlsbad, CA; 600 mM Tris-SO₄ [pH 8.9], 180 mM [NH₄]₂SO₄, 20 mM MgSO₄, 2 mM dNTPs, thermostable AccuPrime protein, 10 % glycerol), 1.5 mL 50 mM MgSO₄, 0.5 mL (10 mM) of each primer, 0.1 mL AccuPrime Taq DNA polymerase High Fidelity (Invitrogen) with 3′-to-5′ exonuclease activity, 20.4 mL PCR-grade water (Ambion, Austin, TX), and 1.0 mL undiluted, DNA template. PCRs were performed using a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA) with the following cycling parameters: initial hold for 3 min at 95° C, 35 cycles at 95° C for 30s, 50° C for 30 s, and 72° C for 2 min with final extension step of 72° C for 10 min. The risk of cross-contamination across PCRs was reduced by adhering strictly to sterile techniques as well as performing DNA extractions and PCR reactions in separate laboratories free of rust-infected plant material. Controls (i.e., minus genomic DNA) were also included in each PCR run. Amplicon size for each PCR product was checked via electrophoresis in 1.2% (w/v) agarose gels followed by ethidium bromide staining (1.2 mg/L 0.5X Tris-acetate

EDTA [TAE]) and visualization under ultraviolet fluorescence. PCR-ITS products were purified using a DNA Clean and Concentrator Kit -5 (Zymo Research, Irvine, CA) or enzymatically using the reagent illustra ExoStar (GE Healthcare, Piscataway, NJ) according to the manufacturers' protocol. Purified amplicons were normalized (130 ng per reaction) and sequenced bidirectionally – forward and reverse – using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, California), BigDye terminator DNA sequencing kit (Applied Biosystems), and the referred primers.

To quality check the single-sorus DNA extract method utilized herein, a representative sample of PCR products (N= 18 single-sorus samples) across 2011–2013 collections and geographic locations was cloned and sequenced. PCR products selected for cloning were chosen according to the collection's geographic origin. Secondary PCR and electrophoresis were performed as described previously; however, PCR-ITS products were purified using the Wizard SV PCR Clean-up System (Promega, Madison, Wisconsin) and ligated to a cloning vector with the TOPO TA cloning kit (Invitrogen) following the manufacturers' instructions. After transformation, plasmid DNA from four-to-five positive, randomly chosen sequenced-derived clones per single-sorus sample were extracted, using the Wizard SV DNA Purification System (Promega) according to system-provided protocol, and sequenced as described above using the universal primer pair M13.

2.2.3 Phylogenetic analysis

Raw ITS sequences (N=121; 747-759 bp) were assembled and edited in CodonCode Aligner v5.1.5 (CodonCode Corporation, Dedham, MA). A primary dataset consisting of all new ITS sequence data – including the 18 PCR-ITS sequence-derived clones – was aligned in Clustal X

v2.1 (Larkin et al. 2007) and adjusted by eye in Geneious v.6.0.6 (Biomatters Ltd., Auckland, New Zealand) to correct ambiguously aligned sites and erroneous gaps, and filtered by collection year, geographic location, and host. Redundant sequences were pruned – retaining only unique rDNA data by year-location-host combination – and, whenever possible, sequences derived from cloning were selected as representative taxa. For the latter sequences, cloned PCR-ITS products (N= 4 or 5 products per single-sorus) from each sample were identical and differed by at least one or more nucleotides across samples. The resultant (pruned) dataset included 35 sequences representing the breadth of DNA, geographic, and host information amassed in the present study. Separate BLASTN searches (<https://blast.ncbi.nlm.nih.gov>) were then performed for each of the 35 sequences to procure a total of eight nearest matches in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>): *Puccinia* spp. (GenBank EU014051); *P. andropogonis* Schwein (DQ344516); *P. asparagi* D.C. (AY217137); *P. graminis* Pers. (AF468044); *P. horiana* Henn. (EU816912); and, *P. striiformis* Westend. (GQ457306).

The final ITS dataset, consisting of 43 ingroup sequences and the outgroup taxon *Puccinia coronata* f. sp. *avenae* (GenBank AY114290), was aligned in Clustal X v2.1 and edited manually as needed (TreeBase, <http://purl.org/phylo/treebase/phyloids/study/TB2:S18667>). The phylogenetic analysis integrated three approaches: maximum-likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) of phylogeny. ML and MP analyses were performed using PAUP* v4.0b10 (Swofford 2003), whereas BI was executed via the Markov Chain Monte Carlo (MCMC) method in MrBayes v3.2.0 (Ronquist et al. 2012). For the ML analysis, the best model of sequence evolution for the final alignment was the transition model 2 plus gamma distribution (TIM2+G; Posada 2003) and was selected by the Akaike Information Criterion (AIC; Akaike 1974) implemented in jModelTest 2.1.4 (Guindon and Gascuel 2003;

Darriba et al. 2012). The heuristic search was performed with 1000 random-addition-sequence (RAS) replicates, tree bisection-reconnection (TBR) branch swapping, and MULTREES in effect. All nucleotides were included in the phylogenetic analysis and gaps were treated as missing characters. Branch support was evaluated using 1000 bootstrap replicates and 100 RAS per pseudo-replicate. MP analysis was conducted using PAUPrat (Sikes and Lewis 2001) in conjunction with PAUP* by executing 20 replicate searches of 200 iterations and perturbing 15% of the informative characters per search. Non-parametric bootstrap values were estimated with 1000 psuedoreplicates, TBR swapping with each consisting of 100 RAS, and holding ten trees at each step while saving no more than ten trees. Nodes receiving ML and MP bootstrap values of $\geq 70\%$ were considered significantly supported.

The TIM2+G model of sequence evolution cannot be executed in MrBayes. Therefore, the next best-fit model, general time reversal plus gamma distribution (GTR+G; Rodriquez et al. 1990) was selected using jModelTest 2.1.4 as previously described. One cold and three heated Markov chain(s) were run, and samples were taken every 100 generations over 5.0×10^6 generations for a total of 50,000 sampled generations. The potential scale reduction factor (PSRF) for each of the model parameters was >1.0 when MrBayes terminated. Chain convergence was accessed via examining the average standard deviations of split frequencies and likelihood values. A 10% burn-in value was determined using Tracer v1.5 (Rambaut and Drummond 2009); removing 5000 sampled generations. The remaining trees were utilized to calculate a 50% majority rule consensus tree and calculate probabilities.

To further compare inter- and intraspecific genetic variability among rust taxa, mean pairwise genetic distances were calculated among taxa and six of the nearest GenBank taxa using the final ITS alignment, custom distance matrix option in PAUP*, the GTR+G model of

sequence evolution, and the statistical software JMP Pro 11 (SAS Institute Inc., Cary, NC).
GenBank taxa *P. graminis* (AF468044) and *Puccinia* spp. (EU014051) were removed from
statistical comparisons as each sequence consisted of only 711 characters in the final 816-
character alignment. Mean nucleotide difference (N) and nucleotide similarity (%) within and
between taxa were assessed also in Geneious.

2.3 Morphological identification

For 2012 and 2013 collections, all single-sorus samples were identified to either uredinia
grouping as described by Ramachar and Cummins (1965) or the genus *Puccinia* or *Uromyces*
based on cell number per teliospore (Arthur 1934; Cummins and Hirtatsuka 2003). To do this,
under a stereomicroscope, fungus material was hand sectioned, mounted separately in
lactophenol alone and with cotton blue (3–5 sections per mounting solution), and examined via
light microscopy. Between samples, instruments and materials utilized in preparing mounts (i.e.,
dissecting needle, scalpel, sterile ultra-fine razor blade, and glass microscopy slide overlaid with
time tape) were either sterilized or changed out. The surrounding bench top was also surface-
sanitized using Vesphene IIse (STERIS Corporation, Mentor, OH) between processing each
sample to further prevent cross-contamination among morphologic and/or DNA samples.
Uredinial group (I-VIII) was determined according the presence/absence of paraphyses in
combination with urediniospore ornamentation (echinulate or verrucose) and the position of
germ pores (basal, equatorial, or scattered).

Teliospore morphologies – including spore length and width, cell number, and cell wall
thickness (apical and side) – were measured for 20 and 10 single-telial samples (N= 20
teliospores/sample) collected in 2012-2013 and assigned phylogenetically and based on

teliospores cell number to separate clades. One single-telium sample with two-celled teliospores and was unresolved to a clade following phylogenetic analyses was also included in the morphologic analysis. Digital micrographs were taken with a Spot Insight FireWire 4 Megasample Color Mosaic camera (Diagnostics Incorporated, Sterling Heights, MI) mounted to a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) and controlled by Spot software v4.6 (Diagnostics Incorporated). Micrographs were analyzed in Adobe Photoshop Creative Cloud 2014 (Adobe Systems Inc., San Jose, CA) using the image analysis function and record measurement tool. Thereafter, teliospore morphology per telium was compared to species descriptions (Arthur 1934; Ramachar and Cummins 1963, 1965; Cummins 1971). One-way analysis of variance (1-way ANOVA) was also used to examine the variance in the quantitative characters among single-telial samples and significant differences between means were determined via contrast comparisons ($\alpha = 0.05$) or a Tukey's Honestly Significant Difference (HSD) test ($\alpha = 0.05$). The frequency of one- versus two-celled teliospores by clade was also assessed statistically using contingency table analysis and a one-tailed Fisher's Exact Test ($\alpha = 0.05$). Statistical analyses were executed in JMP Pro 11.

3. Results

3.1. Phylogenetic analyses

The final alignment including ingroup taxa and the outgroup taxon *P. coronata* f. sp *avenae* consisted of 816 total characters (227 ITS1, 156 5.8S, 270 ITS2, and 163 28S) of which 148 were parsimony informative, 138 variable and parsimony uninformative, and 530 invariable. Maximum likelihood (ML), maximum parsimony (MP), and Bayesian (BA) analyses effectively delimited all but one taxon (i.e., PUC-24) to two, separate monophyletic clades (Clade I and II);

each strongly supported by ML, MP, and BA credibility values (Fig. 2). The taxon PUC-24 collected in 2013 from switchgrass cultivar Summer grown in Lincoln, Nebraska was not resolved to either Clade I or Clade II; however, according to genetic distance and nucleotide similarity, it was more closely-related genetically to taxa in Clade II (N= 11 pairwise comparisons, 816 nucleotides; mean genetic distance = 0.0440 ± 0.0030 ; mean percent nucleotide similarity = 90.9 ± 0.3 ; mean nucleotide difference = 69.7 ± 2.9) than to Clade I taxa (N= 24 pairwise comparisons, 816 nucleotides; mean genetic distance = 0.0457 ± 0.0014 ; mean percent nucleotide similarity = 89.3 ± 0.3 ; mean nucleotide difference = 82.4 ± 2.2). Likewise, based on phylogenetic analyses, PUC-24 was most closely-related – albeit distantly – to a *Puccinia* spp. (EU014051) isolated from an unspecified poaceous weed by Alaei et al. (2009).

Clade I consisted of *P. emaculata* EU915294 (Zale et al. 2008) and comprised 99% (69/70) of single-sorus samples analyzed from NY State. Similarly, all rust samples examined on infected switchgrass from Alabama (6/6 samples), Pennsylvania (13/13), and West Virginia (2/2) also fell into Clade I – *P. emaculata*. Taxa circumscribed to Clade I were genetically identical or similar to *P. emaculata* according to genetic distance (GTR+G), percent nucleotide similarity (%), and nucleotide difference (N; Table 2). The mean intraspecific genetic distance, mean percent nucleotide similarity, and mean nucleotide difference among Clade I taxa (N= 276 pairwise comparisons, 816 nucleotides), including *P. emaculata* EU915294, was 0.0057 substitutions/site, 98.5%, and 12.0 nucleotides, respectively. Moreover, comparing directly the sequence data for Clade I taxa to that of *P. emaculata* EU915294 (i.e., the reference sequence for Clade I), they shared a mean percent nucleotide identity of 99.1%, differed on average by only eight nucleotides, and were separated by a mean genetic distance of 0.0030 substitutions/site (N= 23 pairwise comparisons, 816 nucleotides). In contrast, the mean interspecific genetic distance

between Clade I and Clade II taxa was 0.0398 substitutions/site. Clade I and Clade II sequences also shared only 91.1% nucleotide identity and differed by an average of 69.1 nucleotides. Likewise, sequences for Clade II taxa were remarkably different when compared to *P. emaculata* EU915294 (N= 11 pairwise comparisons, 816 nucleotides): mean genetic distance = 0.0378 substitutions/site; mean percent nucleotide similarity = 91.3%; and, mean nucleotide difference = 67.5. Taxa in both clades, however, were more closely related to each other than to their nearest matches in GenBank (Table 2).

Clade II included 11 unique taxa limited to Iowa, Nebraska, NY State, and/or South Dakota. Mean intraspecific genetic distance among Clade II taxa was 0.0081 substitutions/site yielding a mean percent nucleotide similarity of 98.0% and mean difference of 16.4 nucleotides. No ITS sequence exists in GenBank with $\geq 98\%$ nucleotide similarity to Clade II taxa.

3.2. Uredinia group and teliospore morphology

Across all collection years (N= 121 total samples), 31.4% (N= 38) and 68.6% (N= 83) of the single-sorus samples examined immediately prior to DNA extraction were in the uredinial and telial life stage, respectively. Approximately 32% of the latter single-uredinial samples (31.6%; 12/38) were collected in years 2012–2013 and classified to uredinial Group V (Ramachar and Cummins 1965). Uredinial group, however, was not informative phylogenetically as all uredinial samples examined in 2012–2013 as well as those classified to either Clade I or Clade II possessed uredinia and urediniospores corresponding to Group V (Table 1; Ramachar and Cummins 1965). Uredinia were amphigenous, cinnamon brown in color, lacked paraphyses, and possessed similarly colored echinulate urediniospores with three-to-four germ pores along the equator. In contrast, teliospore cell number was a discrete, morphological, and phylogenetically,

informative discontinuity separating PUC-24 and Clade I taxa from Clade II taxa (Table 1; Fig. 3).

Approximately 65.1% of single-telial samples (54/83) were collected between 2012 and 2013; 78.2% (43/55) of said samples possessed only two-celled teliospores and were assigned phylogenetically to Clade I. Likewise, 20% (11/55) of telial samples possessed only one-celled teliospores and, based on molecular analysis of the partial ITS region, were ascribed to Clade II. PUC-24 – the taxon unresolved to Clade I or Clade II – produced only two-celled teliospores. Thus, unlike uredinial group, teliospore cell number readily separated Clade I taxa and PUC-24 from taxa in Clade II. Accordingly, Clade I taxa and PUC-24 were classified to the genus *Puccinia* whereas Clade II taxa were classified to the genus *Uromyces*. These results also were consistent with the more thorough morphologic examination of teliospores from PUC-24 as well as taxa in Clade I and II.

Collectively, the occurrence of one- versus two-celled teliospores differed significantly among PUC-24 and taxa from Clade I and II (Likelihood ratio $\chi^2 = 733.0$, $df = 2$, $P < 0.001$) as well as for the direct comparison of teliospores from Clade I and Clade II taxa (Likelihood ratio $\chi^2 = 718.5$, $df = 1$, $P < 0.0001$; Fisher's exact test $P < 0.0001$). PUC-24 (20/20 spores) and Clade I taxa (400/400 spores) produced only two-celled teliospores, whereas, taxa examined from Clade II possessed only one-celled teliospores (180/180 spores). Observations of other qualitative characters of telia and teliospores overlapped considerably among PUC-24 and taxa from Clade I and Clade II, including teliospore color (brown to chestnut-brown) and shape (oblong to ellipsoid) as well as telium position in host tissue (mostly epiphyllous). However, significant differences were evident between Clade I and Clade II taxa in teliospore length (1-way ANOVA, $F_{1, 578} = 458.0$, $P < 0.0001$) and width (1-way ANOVA, $F_{1, 578} = 9.6$, $P = 0.0021$) as

well as the thickness of the cell wall apically (1-way ANOVA, $F_{1,578} = 115.6$, $P < 0.0001$) and at the sides (1-way ANOVA, $F_{1,578} = 440.7$, $P < 0.0001$). The mean length and width for Clade I taxa were $35.6 \mu\text{m}$ ($\pm 0.6 \mu\text{m}$) and $18.0 \mu\text{m}$ ($\pm 0.2 \mu\text{m}$), respectively, and were significantly greater (contrast comparisons, $P \leq 0.0021$) than those measured for teliospores of Clade II taxa (mean length = $26.0 \pm 0.4 \mu\text{m}$; mean width = $17.5 \pm 0.3 \mu\text{m}$). In contrast, the cell walls of teliospores for taxa in Clade II were significantly thicker (contrast comparisons, $P < 0.0001$), both apically (mean = $7.4 \pm 0.2 \mu\text{m}$) and at the sides (mean = $2.6 \pm 0.1 \mu\text{m}$), when compared to the apical (mean = $6.0 \pm 0.1 \mu\text{m}$) and side (mean = $1.9 \pm 0.0 \mu\text{m}$), cell wall thickness of teliospores for Clade I taxa. PUC-24 teliospores were significantly wider (mean = $19.1 \pm \mu\text{m}$;) than those of Clade I (Tukey's HSD, $P = 0.0229$) and Clade II taxa (Tukey's HSD, $P = 0.0006$); however, the length (mean = $38.0 \pm 1.7 \mu\text{m}$) and cell wall thicknesses of PUC-24 teliospores (mean apical thickness = $5.6 \pm 0.4 \mu\text{m}$; mean side thickness = $1.8 \pm 0.1 \mu\text{m}$) were similar to those measured for teliospores of Clade I taxa.

According to species descriptions (Arthur 1934; Ramachar and Cummins 1963, 1965; Cummins 1971), single-telial samples examined morphologically and placed in Clade I were identified to the species *Puccinia emaculata*: teliospores brown to chestnut-brown, two-celled, oblong to ovoid, and $19.6\text{-}53.6 \times 11.0\text{-}26.1 \mu\text{m}$; cell wall $1.3\text{-}4.0 \mu\text{m}$ thick at sides, $2.9\text{-}12.9 \mu\text{m}$ apically (Fig. 3A). As noted previously, no identical or near identical ITS sequence(s) exist in GenBank to compare with our Clade II taxa. However, based on morphological results, the ten taxa examined from Clade II were identified to *U. graminicola* (Arthur 1934; Ramachar and Cummins 1963; Cummins 1971): teliospores brown to chestnut-brown, one-celled, oblong to ovoid, and $21.0\text{-}34.5 \times 12.8\text{-}23.3 \mu\text{m}$; cell wall $1.7\text{-}4.8 \mu\text{m}$ thick at sides, $4.5\text{-}10.4 \mu\text{m}$ apically (Fig. 3B). Although the teliospores of PUC-24 were significantly wider than those of Clade I

taxa, the morphology of this taxon was consistent with that described for *P. emaculata* (Arthur 1934; Ramachar and Cummins 1965; Cummins 1971). Hereafter, the taxa assigned morphologically and phylogenetically to Clade I and Clade II are referred to as *P. emaculata* and *U. graminicola*, respectively.

3.3. Host-rust species combinations

Taxa placed into the *P. emaculata* and *U. graminicola* were found infecting seven and four, different cultivars of switchgrass, respectively (Table 1; Fig.2). Collectively across study sites, taxa identified as *P. emaculata* (105/121, 86.8% total single-sorus samples) were found infecting the cultivars Dallas Blues (N= 6 samples), Cave-in-Rock (N= 64), Forestburg (N= 1), Kanlow (N= 19), Summer (N= 1), and Sunburst (N= 12) as well as a landscape ornamental cultivar (N= 2). Cave-in-Rock (N= 10), Kanlow (N= 1), Summer (N= 2), and Sunburst (N= 2) were also susceptible to *U. graminicola* (15/121, 12.4% total single-sorus samples). The unknown *P. emaculata*-like taxon (PUC-24) was collected once from the cultivar Summer grown in Lancaster Co., Nebraska.

4. Discussion

Four rust fungi – *Puccinia emaculata*, *P. huberi*, *P. graminis*, and *U. graminicola* – previously have been characterized on and reported to infect switchgrass in N. America (Arthur 1934; Ramachar and Cummins 1963, 1965; Cummins 1971; Tiffany et al. 1990; Gravert and Munkvold 2002; Gustafson et al. 2003; Farr and Rossman 2015). Phylogenetic analyses of the partial ITS region of rust taxa infecting switchgrass in NY State as well as elsewhere in the north-central and eastern U.S. clearly demonstrated the occurrence of two distinct species – *P. emaculata* and *U.*

graminicola (Fig. 2). These analyses were also supported statistically by pairwise, inter- and intra-specific comparisons among taxa and nearest matches in GenBank by genetic distance (GTR+G), nucleotide identity (%), and nucleotide difference (N; Table 2). Moreover, single-teliospore samples diagnosed morphologically as *P. emaculata* or *U. graminicola* were circumscribed to separate monophyletic clades and teliospore morphologies were significantly different between species, particularly spore length, cell number, and cell wall thicknesses (Fig. 3). *Uromyces graminicola* is the only one-celled teliospore-producing rust fungus known to infect switchgrass (Arthur 1934; Ramachar and Cummins 1963). *Puccinia graminis* and *P. huberi* were not recovered in the phylogenetic analyses or via morphological identification. The recovery of the latter species, *P. huberi*, was particularly doubtful from the onset of the present study as reports of this species affecting switchgrass have been limited to outside the U.S., including Brazil, Cuba, Mexico, and Puerto Rico (Lenne 1990). However, *Puccinia graminis* – the stem rust fungus – historically is known to occur on switchgrass in Kansas (Anonymous, 1970) and, more recently, was reported in Iowa by Gravert and Munkvold (2002). This species was an ingroup taxon utilized in the present phylogenetic analyses (Fig. 2); yet, none of the taxa from Iowa or elsewhere grouped with *P. graminis*. Interestingly, Ramachar and Cummins (1965) reclassified *P. graminis* Pers. var. *brevicarpa* Peck described by Peck (1873) in synonymy with *P. emaculata* and, stated *P. graminis* “...is recorded from Iowa on *Echinochloa crus-galli* (L.) Beauv [barnyardgrass], but is not otherwise known to occur on the Paniceae.” Peck (1873) likely would have agreed with Ramachar and Cummins’ treatment as he noted this species “...especially occurs on *Panicum capillare* L. [witchgrass] and may be *P. emaculata* Schw.” Similarly, the known host distribution of *P. graminis* in the U.S. includes only three host taxa in the tribe Paniceae (Farr and Rossman 2015) – *E. crus-galli*, *Panicum virgatum*, and

Paspalum bushii Michx. (thin paspalum, syn. *Paspalum stramineum* Nash). Therefore, additional morphologic and molecular analyses of rust-infected switchgrass collected from across the central and north-central U.S. will be required to either definitively confirm or refute the occurrence *P. graminis* on switchgrass or allied taxa of Paniceae.

The detection of at least two species of rust fungi affecting switchgrass in NY State as well as Iowa, Nebraska, and South Dakota is concerning in so much as the future development of rust resistant cultivars will require breeding programs that select for and integrate not only yield-defining agronomic/horticultural traits but also multiple sources (genes) of rust resistance. Because the majority of rust resistance genes reported to date in agricultural crops appear to be species- and/or race-specific (Ellis et al. 2014; Hulbert and Pumphrey 2014; Mundt 2014), knowledge regarding the genetic basis of rust resistance in switchgrass is critical in attaining durable resistance (Cornelius and Johnson 1941; Uppalapati et al. 2013; Serba et al. 2015). In addition, epidemiological data on the spread and intensification of rust disease in switchgrass bioenergy cropping systems, incited by *P. emaculata* and/or *U. graminicola*, is also of great importance toward monitoring culture conditions that may favor or attenuate disease development (McDonald and Linde 2002). However, such epidemiological information will require elucidating further the geographic distribution of *P. emaculata* and *U. graminicola* as well as conducting new studies into the contribution of their telial and aecial hosts in the long-distance spread and manifestation of disease.

Albeit an exploratory study, the work presented here demonstrated the occurrence of *P. emaculata* on switchgrass in seven states: Alabama, Iowa, Nebraska, NY State, Pennsylvania, South Dakota, and, West Virginia. This rust fungus-host combination has been reported previously in six of these states with the exception being West Virginia (Farr and Rossman

2015), and hence, this finding represents the first report of *P. emaculata* affecting switchgrass in West Virginia. *Puccinia emaculata*, including synonyms (e.g., *P. graminis* var. *brevicarpa*, *P. pammelii* (Trel.) Arthur, and *P. panici* Dietel), however, has a considerably large geographic distribution in N. America spanning 28 states in the U.S. from New Hampshire through the eastern provinces of Quebec and Ontario (Canada) to North Dakota, South into northern Mexico, and East to the state of Florida (Ramachar and Cummins 1965; Lenne 1990; Farr and Rossman 2015). The geographic distribution of *P. emaculata* corresponds neatly with that of its telial hosts including switchgrass and at least 10 other perennial grasses. The isotype of *P. emaculata* (Arthur Herbarium, Purdue University [PUR], PUR000614) was collected by Von Schweinitz (date unknown) on witchgrass (*Panicum capillare*) – a native *Panicum* spp. with an extensive geographic range covering all of southern Canada and the lower 48 U.S. states. Barnyard grass, bitter panicgrass (*P. amarum* Elliot), coastal panicgrass (*Panicum amarulum* Elliott), decorative millet (*E. holciformis* [Kunth] Nash), Gattinger’s panicgrass (*P. gattingeri* Nash), Hall’s panicgrass (*P. hallii* Vasey), Philadelphia panicgrass (*P. philadelphicum* Bernh. ex. Trin.), proso millet (*P. miliaceum* L.), and variable panicgrass (*Dichanthelium commutatum* [Schult.] Gould) also serve as telial hosts for *P. emaculata* and are found widely throughout the central, eastern, and/or southeastern U.S. (USDA-NRCS 2015). However, the contribution and relationships among telial hosts in native landscapes to disease progress on cultivated switchgrass in agronomic settings remains unclear. The importance of native telial hosts in supporting effective inoculum production and, hence, serving as inoculum reservoirs for the dispersal of airborne urediniospores, is also unknown. To our knowledge, no studies to date have examined the inoculum-producing potential, or susceptibility, of known telial hosts of *P. emaculata* other than switchgrass (Johnston et al. 1952; Gustafson et al. 2003; Uppalapati et al. 2013). Moreover, given

its range of telial hosts and large geographic distribution, studies into the winter survival of *P. emaculata* are also warranted. Ecological zones for winter survival may exist – similar to that described for rust fungi of cereals (Roelfs 1989) – where *P. emaculata* overwinters in the southern portion of its geographic distribution (e.g. Mexico and southern Gulf States of the U.S.) and in the spring and summer, spreads northward in a stepwise-fashion on prevailing winds to susceptible hosts as they become available. *Puccinia emaculata* typically first appears late in the growing season (late August – early October) at sites in NY State and Pennsylvania (S.C. Kenaley and G.C. Bergstrom, unpub. data) likely indicating that overwinter survival of *P. emaculata* in switchgrass plantations in the Northeast is limited, if it occurs at all, and the long-distance dispersal of windborne urediniospores along a “*Puccinia* pathway” may be contributing annually to the spread of this rust fungus to northern temperate regions of the U.S.

The importance of telial hosts in the spread or local and regional persistence of *U. graminicola* in the continental U.S. has also received no attention. In addition to switchgrass, the primary hosts of *U. graminicola* in the U.S. overlap partially with *P. emaculata* as bitter and coastal panicgrasses are reported telial hosts for both rust fungi. *Uromyces graminicola*, however, also has been found in the U.S. to occur on starved panicgrass (*Dichanthelium depauperatum* [Muhl.] Gould, winged panicgrass (*Panicum altum* Hitchc. & Chase), beaked panicgrass (*P. anceps* Michx.), maidencane (*P. hemitomom* Schult.), and guineagrass (*Urochloa maxima* [Jacq.] R. Webster) as well as the jointed goatgrass (*Aegilops cylindrica* Host; Preston 1945; Ramachar and Cummins 1963; Anonymous 1970; McCain et al. 1990; Farr and Rossman 2015). As with *P. emaculata*, the geographic distribution of *U. graminicola* closely follows that of its telial hosts and, hence, is expansive. *Uromyces graminicola* has been reported to occur in at least 18 states, including the four states – Iowa, Nebraska, NY State, and South Dakota – from

which it was identified on switchgrass in the present study (Table 1; Fig. 2; Ramachar and Cummins 1963; Farr and Rossman 2015). Collectively, the geographic distribution of *U. graminicola* in the continental U.S. appears to be nearly identical to that of *P. emaculata* extending from southern New England and states of the northern mid-west to New Mexico and Florida. *Uromyces graminicola* affecting switchgrass, however, has not yet been confirmed to exist in Alabama and several southeastern states including Arkansas, where, *P. emaculata* is known to infect switchgrass (Hirsch et al. 2010; Farr and Rossman 2015). The geographic distribution and number of telial hosts for *U. graminicola* and *P. emaculata* across natural landscapes in the central and eastern U.S. suggest that both rust fungi have a large effective population size and, hence, high-levels of evolutionary potential (McDonald and Linde 2002). Therefore, future research projects into the metapopulation genetics of *P. emaculata* and *U. graminicola* are needed to examine the potential exchange of asexual inoculum (genotype flow) among telial hosts in cultivation and natural landscapes. Moreover, in addition to the dispersal and admixture of asexual inoculum among telial host populations, a key concern for switchgrass breeders and growers is that local and regional populations of these fungi may also be reproducing sexually thereby providing sources of novel recombinant, and possibly more virulent, genotypes (gene flow). Sexual reproduction and the resultant generation of recombinant genotypes and virulence patterns have been reported for several rust fungi including *Puccinia graminis* f.sp. *tritici* Erikss. & Henning (Roelfs and Groth 1980; Burdon and Roelfs 1985) and *P. striiformis* f.sp. *tritici* Erikss. (Duan et al. 2010; Ali et al. 2014) as well as the poplar rust fungus *Melampsora larici-populina* Klebahn (Gérard et al. 2006; Xhaard et al. 2011). Plant pathogens with a large effective populations size, efficient long-distance dispersal mechanisms, and capable of asexual as well as sexual reproduction pose the greatest risk to

thwart disease resistance and cultural control strategies (McDonald and Linde 2002; Barrett et al. 2009; Mundt 2014). *Puccinia emaculata* and *Uromyces graminicola* reportedly host-alternate (i.e., mate; Arthur 1905, 1906, 1908, 1934; Ramachar and Cummins 1963); however, for both species, where this occurs geographically has not been fully explored and the identity of the aecial host(s) is questionable (Arthur 1915, 1917; Jackson 1917).

Puccinia emaculata and *U. graminicola* are characterized as heteroecious, macrocytic rusts; thus, their life cycles include five spore stages (O-IV) and host-alternation between switchgrass (III-IV) – and other grasses (see above) – and taxa in the spurge family, Euphorbiaceae Juss. (Stuart 1901; Arthur 1905, 1906, 1914; Ramachar and Cummins 1963). The host-alternation for *P. emaculata* sensu lato was first confirmed by Stuart (1901), who successfully inoculated potted switchgrass using the aecial state *Aecidium pammellii* Trel. (syn. *P. pammellii*, *P. panici*) on flowering spurge (*Tithymalopsis corollata* (L.) Klotzsch & Garcke, syn. *Euphorbia corollata* L.). Thereafter, Arthur (1905, 1908) demonstrated primary-to-alternate host switching for *P. pammellii* and *P. panici* – which, at that time, he considered separate species – from switchgrass to flowering spurge and snow on the mountain (*Euphorbia marginata* Pursh), respectively. Host-alternation for *P. emaculata* sensu stricto, however, has not been determined. Throughout the early 20th century, J.C. Arthur conducted numerous inoculation tests to identify the likely alternate host for *P. emaculata*, including the aecial hosts of *P. pammellii* and *P. panici* as well as at least 24 additional plant species (Arthur 1902, 1903, 1905, 1907, 1908, 1915, 1917). All tests utilized inoculum identified to *P. emaculata* on witchgrass (i.e., *Panicum capillare*), yet, failed to yield infection. Thus, the life cycle of *P. emaculata* sensu lato is in large part attributable to circumscribed taxa – *P. pammellii* and *P. panici*. The latter taxon was synonymized with *P. emaculata* by Ramachar and Cummins (1965) whereas *P. pammellii* was

circumscribed to *P. panici* by Arthur (1934). It is of interest to note that the ITS sequence PUC-24 was isolated once from switchgrass grown in Lancaster Co., Nebraska in the present study failed to group phylogenetically with either the *P. emaculata* or *U. graminicola* (Table 1; Fig. 2). The single-telial sample from which PUC-24 was produced was also identified morphologically as a *P. emaculata*; however, according to genetic distance and nucleotide similarity, it was more closely-related genetically to *U. graminicola* than the *P. emaculata*. This is of interest because Jackson (1917) and Arthur (1917) indicated that *U. graminicola* is morphologically “inseparable” and “correlated to” *P. panici*. Given evidence of a possible second *P. emaculata*-like species in combination with the morphological similarities among *P. pammelii*, *P. panici*, and *P. emaculata* sensu stricto (Arthur 1905, 1934), morphologic and multigene molecular analyses comparing authenticated and type material from the latter species with that of *A. pammelii* – the purported aecial state of *P. emaculata* sensu lato – are justified and will further provide needed insight into the biology of *Puccinia* affecting switchgrass and related *Panicum* spp.

The purported euphorbious hosts of *U. graminicola* are prairie tea (*Croton monanthogynus* Michx.), linear rushfoli (*Crotonopsis linearis* Michx.), gulf sebastian bush (*Ditrysinia fruticosa* [Bartram] Govaerts & Frodin; syn. *Sebastiana ligustrina* [Michx.] Müll.Arg.), and queen’s delight (*Stillingia sylvatica* L.; syn. *Stillingia angustifolia* [Torr.] Wats.) (Arthur 1934). However, to the best of our knowledge, no inoculation test(s) has demonstrated whether the aeciospores of the reported aecial state, *Aecidium crotonopsidis* Burrill (syn. *A. stillingiae* Tracy & Earle), are infectious on switchgrass or other telial hosts and, vise versa, whether basidiospores of *U. graminicola* upon inoculation of the reported aecial hosts can incite infection and yield spermatia and aecia (Burrill 1884; Olive 1895; Tracy and Earle 1899). From

1906–1916, J.C Arthur attempted several rounds of inoculation tests aimed at demonstrating host-alternation of *U. graminicola* (Arthur 1906, 1909, 1910, 1917); however, all tests produced negative results. Whether other scientists performed and/or reported additional inoculations tests with positive results between 1917 and the publication of Arthur (1934) is unclear as there is no indication in the scientific literature that such report(s) exist. The only prior description of the likely association between *U. graminicola* and its aecial hosts before Arthur (1934) appeared in Jackson (1917). The latter author clearly states that as of the publication of his work, “...no cultures of *U. graminicola* have been successfully carried out.” Jackson (1917) also noted, “...it is probable that the aecial stage will be found on some member of the Euphorbiaceae... *A. stillingiae* Tracy & Earle, which occurs on various species of *Stillingia* and *Sebastiana*... is a very probable aecial connection.” Did J.C Arthur have unpublished data or personal communications that demonstrated *U. graminicola*’s aecial connection with euphorbs? We likely will never know the answer to this question; therefore, future studies should reexamine the life cycle of *U. graminicola* to determine whether it can indeed infect and sporulate (stages 0-I) on gulf sebastian bush, linear rushfoli, queen’s delight, and/or prairie tea. Molecular analyses similar to that proposed herein for establishing the aecial state of *P. emaculata* sensu stricto should also be carried out as a first step toward identifying the aecial host(s) of *U. graminicola*.

The work presented supports the resolving power of rDNA data for species identification in rust fungi (Zambino and Szabo 1993; Weber et al. 2003; Szabo 2008; Liu and Hambleton 2010; Barilli et al. 2011; Feau et al. 2011; Kenaley et al. 2014). Moreover, this research was the first to explore the species diversity of rust fungi affecting switchgrass across a large geographic area as well as delve into the reported ecology and taxonomic history of *P. emaculata* and *U. graminicola* – highlighting unresolved questions pertinent to present day mycologists and plant

pathologists charged with studying the ecology and management of these fungi. Collectively, the results presented provide sequence data for plant breeders and plant pathologists alike to develop and apply molecular detection tools (e.g., species-specific primers) for *P. emaculata* sensu lato and *U. graminicola* in the future genetic improvement of switchgrass. Our review of the historical reports surrounding the relationships of *P. emaculata* and *U. graminicola* with their aecial hosts should also stimulate interested scientists to reevaluate, if not revise, the current state of knowledge regarding the biology of these fungi.

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Table 1 – List of single-sorus samples by ITS sequence identity (Taxon ID) including voucher number, GenBank accession number, life stage (II – uredinial, III – telial), uredinia group, teliospore cell number (Cell No.), species determination based on DNA analysis and teliospore morphology (Morphology), and collection date (Date). Host data including switchgrass cultivar and geographic location (county and state) are also provided. A single representative for each taxon was utilized for phylogenetic and statistical analyses – see Fig. 2 and Table 2, respectively. The geographic locations from which samples were collected is illustrated in Fig. 1.

Taxon ID	Voucher	GenBank No.	Life stage ^a	Uredinia group ^b	Cell No.	DNA analysis	Morphology ^c	Cultivar	Location; Date; Collector
PUC-1	CUP-068048	KR264595	II	V		<i>Puccinia emaculata</i>		Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
PUC-1	CUP-068049	KR264596	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
PUC-2	CUP-068050	KR264597	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
PUC-3	CUP-068051	KR264598	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Sunburst	Saunders, NE; 1-Oct-13; C. Jochum
PUC-3	CUP-068052	KR264599	II	V		<i>Puccinia emaculata</i>		Sunburst	Saunders, NE; 1-Oct-13; C. Jochum
PUC-3	CUP-068053	KR264600	III		2	<i>Puccinia emaculata</i>		Sunburst	Saunders, NE; 1-Oct-13; C. Jochum
PUC-3	CUP-068054	KR264601	III		2	<i>Puccinia emaculata</i>		Sunburst	Saunders, NE; 1-Oct-13; C. Jochum
PUC-4	CUP-068055	KR264602	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Sunburst	Brookings, SD ; 24-Sept-12; A. Boe
PUC-5	CUP-068056	KR264603	III			<i>Puccinia emaculata</i>		Sunburst	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-5	CUP-068057	KR264604	III			<i>Puccinia emaculata</i>		Sunburst	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-5	CUP-068058	KR264605	III			<i>Puccinia emaculata</i>		Sunburst	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-5	CUP-068059	KR264606	III			<i>Puccinia emaculata</i>		Sunburst	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-6	CUP-068060	KR264607	II			<i>Puccinia emaculata</i>		Kanlow	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-6	CUP-068061	KR264608	II	V		<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-6	CUP-068062	KR264609	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-6	CUP-068063	KR264610	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-6	CUP-068064	KR264611	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-7	CUP-068065	KR264612	II			<i>Puccinia emaculata</i>		Kanlow	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-7	CUP-068066	KR264613	III			<i>Puccinia emaculata</i>		Kanlow	Tompkins, NY; 20-Sep-11; S. Kenaley

PUC-7	CUP-068067	KR264614	II	V		<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-7	CUP-068068	KR264615	II	V		<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-7	CUP-068069	KR264616	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-7	CUP-068070	KR264617	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-7	CUP-068071	KR264618	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-7	CUP-068072	KR264619	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-8	CUP-068073	KR264620	III		2			Forestburg	Crawford, PA; 27-Sep-12; C. Layton
PUC-9	CUP-068074	KR264621	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Kanlow	Saunders, NE; 1-Oct-13; C. Jochum
PUC-9	CUP-068075	KR264622	II			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-9	CUP-068076	KR264623	II			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-9	CUP-068077	KR264624	II			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-9	CUP-068078	KR264625	II			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-9	CUP-068079	KR264626	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-9	CUP-068080	KR264627	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-9	CUP-068081	KR264628	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-9	CUP-068082	KR264629	II			<i>Puccinia emaculata</i>		Kanlow	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-9	CUP-068083	KR264630	II			<i>Puccinia emaculata</i>		Sunburst	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-9	CUP-068084	KR264631	III			<i>Puccinia emaculata</i>		Kanlow	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-10	CUP-068085	KR264632	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
PUC-10	CUP-068086	KR264633	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
PUC-10	CUP-068087	KR264634	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
PUC-10	CUP-068088	KR264635	II	V		<i>Puccinia emaculata</i>		Cave-in-Rock	Crawford, PA; 27-Sep-12; C. Layton
PUC-11	CUP-068089	KR264636	II	V		<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 27-Sep-12; C. Layton
PUC-12	CUP-068090	KR264637	II			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-12	CUP-068091	KR264638	II			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-12	CUP-068092	KR264639	II			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-12	CUP-068093	KR264640	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 13-Sep-11; S. Kenaley
PUC-12	CUP-068094	KR264641	II			<i>Puccinia emaculata</i>		Sunburst	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-12	CUP-068095	KR264642	II			<i>Puccinia emaculata</i>		Sunburst	Tompkins, NY; 20-Sep-11; S. Kenaley

PUC-12	CUP-068096	KR264643	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068097	KR264644	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068098	KR264645	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068099	KR264646	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068100	KR264647	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068101	KR264648	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068102	KR264649	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068103	KR264650	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068104	KR264651	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068105	KR264652	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068106	KR264653	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068107	KR264654	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068108	KR264655	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-12	CUP-068109	KR264656	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-13	CUP-068110	KR264657	III	2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Summer	Saunders, NE; 1-Oct-13; C. Jochum
PUC-14	CUP-068111	KR264658	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068112	KR264659	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068113	KR264660	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068114	KR264661	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068115	KR264662	II		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068116	KR264663	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068117	KR264664	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068118	KR264665	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068119	KR264666	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068120	KR264667	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068121	KR264668	III		<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-14	CUP-068122	KR264669	II	V	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-14	CUP-068123	KR264670	III	2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-14	CUP-068124	KR264671	III	2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley

PUC-14	CUP-068125	KR264672	II	V		<i>Puccinia emaculata</i>		Ornamental	Jefferson, WV; 5-Sep-12; S. Kenaley
PUC-15	CUP-068126	KR264673	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Ornamental	Jefferson, WV; 5-Sep-12; S. Kenaley
PUC-15	CUP-068127	KR264674	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-15	CUP-068128	KR264675	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 20-Sep-11; S. Kenaley
PUC-15	CUP-068129	KR264676	III		2	<i>Puccinia emaculata</i>		Cave-in-Rock	Tompkins, NY; 5-Oct-12; S. Kenaley
PUC-15	CUP-068130	KR264677	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-15	CUP-068131	KR264678	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-16	CUP-068132	KR264679	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Kanlow	Saunders, NE; 1-Oct-13; C. Jochum
PUC-16	CUP-068133	KR264680	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Dallas Blues	Lee, AL; 13-Oct-12; A. Hagan
PUC-16	CUP-068134	KR264681	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-16	CUP-068135	KR264682	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-16	CUP-068136	KR264683	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-16	CUP-068137	KR264684	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-16	CUP-068138	KR264685	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-16	CUP-068139	KR264686	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-16	CUP-068140	KR264687	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-17	CUP-068141	KR264688	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Dallas Blues	Lee, AL; 13-Oct-12; A. Hagan
PUC-17	CUP-068142	KR264689	III		2	<i>Puccinia emaculata</i>		Kanlow	Crawford, PA; 20-Sep-13; G. Bergstrom
PUC-18	CUP-068143	KR264690	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Dallas Blues	Lee, AL; 13-Oct-12; A. Hagan
PUC-19	CUP-068144	KR264691	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Dallas Blues	Lee, AL; 13-Oct-12; A. Hagan
PUC-20	CUP-068145	KR264692	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Dallas Blues	Lee, AL; 13-Oct-12; A. Hagan
PUC-21	CUP-068146	KR264693	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Dallas Blues	Lee, AL; 13-Oct-12; A. Hagan
PUC-22	CUP-068147	KR264694	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-22	CUP-068148	KR264695	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-22	CUP-068149	KR264696	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-22	CUP-068150	KR264697	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-22	CUP-068151	KR264698	III			<i>Puccinia emaculata</i>		Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
PUC-23	CUP-068152	KR264699	III		2	<i>Puccinia emaculata</i>	<i>P. emaculata</i>	Kanlow	Saunders, NE; 1-Oct-13; C. Jochum
PUC-24	CUP-068153	KR264700	III		2	<i>Puccinia</i> species	<i>P. emaculata</i>	Summer	Saunders, NE; 1-Oct-13; C. Jochum

URO-1	CUP-068154	KR264701	II	V		<i>Uromyces graminicola</i>		Cave-in-Rock	Saunders, NE; 1-Oct-13; C. Jochum
URO-1	CUP-068155	KR264702	III		1	<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Cave-in-Rock	Saunders, NE; 1-Oct-13; C. Jochum
URO-1	CUP-068156	KR264703	III		1	<i>Uromyces graminicola</i>		Cave-in-Rock	Saunders, NE; 1-Oct-13; C. Jochum
URO-2	CUP-068157	KR264704	III		1	<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Kanlow	Saunders, NE; 1-Oct-13; C. Jochum
URO-3	CUP-068158	KR264705	II			<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Cave-in-Rock	Chemung, NY; 5-Oct-11; S. Kenaley
URO-4	CUP-068159	KR264706	III		1	<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Sunburst	Brookings, SD ; 24-Sept-12; A. Boe
URO-5	CUP-068160	KR264707	III		1	<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
URO-6	CUP-068161	KR264708	II	V		<i>Uromyces graminicola</i>		Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
URO-6	CUP-068162	KR264709	III		1	<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
URO-7	CUP-068163	KR264710	III		1	<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Summer	Saunders, NE; 1-Oct-13; C. Jochum
URO-8	CUP-068164	KR264711	II	V		<i>Uromyces graminicola</i>		Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
URO-8	CUP-068165	KR264712	III		1	<i>Uromyces graminicola</i>	<i>U. graminicola</i>	Sunburst	Brookings, SD ; 24-Sept-12; A. Boe
URO-9	CUP-068166	KR264713	III		1		<i>U. graminicola</i>	Summer	Saunders, NE; 1-Oct-13; C. Jochum
URO-10	CUP-068167	KR264714	III		1		<i>U. graminicola</i>	Cave-in-Rock	Madison, IA; 10-Jul-13; E. Saalau-Rojas
URO-11	CUP-068168	KR264715	III		1			Cave-in-Rock	Saunders, NE; 1-Oct-13; C. Jochum

^a – Life stage: II - uredinium and III - telium

^b – For 2012 and 2013 collections, single-uredinial isolates were classified according to uredinial group (Ramachar and Cummins 1965) prior to DNA analysis.

^c – Fungus species was determined based on morphometric examination of teliospores (20 teliospores/isolate) including cell number, length, and width as well as cell wall thickness at the sides and top of teliospores.

Table 2 – Inter- and intraspecific pairwise comparisons of ITS sequences utilized in phylogenetic reconstruction: mean genetic distances (GTR+G model); mean nucleotide similarities (%), and mean nucleotide differences (N). Ninety-five percent (95 %) confidence intervals were calculated for comparison of mean differences.

Pairwise comparison	Mean and 95% confidence interval ($\pm$)			
	Comparisons (N)	Genetic distance (substitutions/site)	Nucleotide similarity (%)	Nucleotide difference (N)
<i>Intraspecific</i>				
Clade I	276	0.0057 ($\pm$ 0.0007)	98.5 ($\pm$ 0.1)	12.0 ($\pm$ 1.1)
Clade I versus <i>Puccinia emaculata</i> (EU915294)	23	0.0030 ($\pm$ 0.0018)	99.1 ($\pm$ 0.4)	8.0 ($\pm$ 3.1)
Clade II	55	0.0081 ($\pm$ 0.0014)	98.0 ($\pm$ 0.2)	16.4 ($\pm$ 1.9)
<i>Interspecific</i>				
Clade I versus Clade II	264	0.0398 ($\pm$ 0.0006)	91.1 ($\pm$ 0.09)	69.1 ($\pm$ 0.8)
Clade I versus GenBank taxa ^a	121	0.0783 ($\pm$ 0.0065)	85.5 ($\pm$ 1.0)	112.9 ($\pm$ 7.5)
Clade II versus GenBank taxa ^a	55	0.0848 ($\pm$ 0.0010)	85.6 ($\pm$ 1.4)	111.4 ($\pm$ 11.2)
Clade II versus <i>Puccinia emaculata</i> (EU915294)	11	0.0378 ($\pm$ 0.0025)	91.3 ($\pm$ 0.2)	67.5 ($\pm$ 2.0)
GenBank taxa ^a	10	0.1091 ($\pm$ 0.0228)	82.4 ($\pm$ 3.5)	135.8 ($\pm$ 27.4)

^a – Comparisons included *Puccinia andropogonis* (DQ344516), *P. asparagi* (AY217137), *P. horiana* (EU816912), *P. sorghi* (DQ344516), and, *P. striiformis* (GQ457306). Sequence data for GenBank accessions *P. graminis* (AF468044) and *Puccinia* spp. (EU014051) were not included as each the latter sequences consisted of only 711 characters in the final, 816 character alignment.

A map of the United States showing the distribution of the American black bear. Black triangles indicate the range of the species, which is primarily in the western and southern regions, including Montana, Wyoming, Colorado, New Mexico, Texas, Oklahoma, Kansas, Nebraska, South Dakota, North Dakota, Minnesota, Iowa, Missouri, Arkansas, Louisiana, Mississippi, Alabama, Georgia, Florida, and parts of the Northeast. A north arrow is located in the bottom right corner.

Fig. 2

Fig. 3