

Phylogenetic relationships of sugarcane rust fungi

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Abstract The phylogenetic positions of *Puccinia* spp. infecting sugarcane (a complex hybrid of *Saccharum* spp.) were determined using 38 newly generated rust sequences and 26 sequences from GenBank. Rust specimens on sugarcane were collected from 164 locations in 23 countries and identified based on light microscopy. The morphology for all samples matched that of *Puccinia kuehnii* or *P. melanocephala*, the orange and brown rust pathogens of sugarcane, respectively. Nuclear ribosomal DNA sequences (rDNA) including portions of the 5.8S rDNA, the complete internal transcribed spacer 2 (ITS2) and 5' region of the large subunit (nLSU) rDNA were obtained for each species along with 36 additional rust taxa. Despite a shared host, the two *Puccinia* spp. on sugarcane are not closely related

within the *Pucciniales*. Phylogenetic analyses place *P. melanocephala* most closely to *P. miscanthi*, *P. nakanishikii*, and *P. rufipes* infecting *Miscanthus sinensis*, *Cymbopogon citratus*, and *Imperata cylindrica*, respectively. *Puccinia kuehnii* is basal to a clade of Poaceae-infecting rusts including *P. agrophila*, *P. polysora*, *P. substriata*, and *Uromyces setariae-italicae* infecting *Schizachyrium* spp., *Zea mays*, *Digitaria* spp., and *Urochloa mosambicensis*, respectively. Light and scanning electron microscopy images highlight morphological differences distinguishing the two sugarcane-infecting species. This study confirms the separation of rust species infecting *Poaceae* from *Cyperaceae*- and *Juncaceae*-infecting rusts and also provides support for the presence of an additional group that includes *P. kuehnii* and other grass-infecting relatives.

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Introduction

Two species of fungi are known to cause rust diseases on sugarcane (*Saccharum officinarum* L.). *Puccinia kuehnii* (W. Krüger) E.J. Butler, which causes orange rust, is more prevalent in high humidity, whereas *P. melanocephala* Syd. & P. Syd., which causes brown rust, occurs in cool, dry conditions (Braithwaite et al. 2009). Brown rust is found almost everywhere sugarcane is grown. After its first appearance in the Western Hemisphere in 1978, brown rust spread throughout the Americas within a year, and yield losses have been reported as high as 50% in Mexico (Purdy et al. 1983). In contrast, orange rust, once restricted to scattered outbreaks in Australia and Asia

(Egan 1980), was only recently reported for the first time in the western hemisphere, in Florida in 2007 (Comstock et al. 2008). Since then, orange rust has spread to several countries in Central America and appears to be moving throughout the Americas (Chavarria et al. 2009; Flores et al. 2009; Ovalle et al. 2008). Economic losses from orange rust during a single season have been estimated at US\$40 million in Florida and US\$177 million in Australia (Raid and Comstock 2006; Braithwaite et al. 2009). As a result of the recent movement of orange rust, there is great concern that the disease will spread to the sugarcane-growing areas of Hawaii, Louisiana and Texas in the United States and to South America. Important commercial sugarcane clones are susceptible to both orange and brown rust, and even resistant clones demonstrate increased susceptibility over time due to genetic variability within pathogen populations (Braithwaite et al. 2009; Magarey et al. 2001).

Puccinia kuehnii and *P. melanocephala* are the only species known to cause rust disease on commercial sugarcane and are commonly misidentified (Chona and Munjal 1950; Egan 1980; Roy et al. 1998; Virtudazo et al. 2001a). Virtudazo et al. (2001b) reviewed the taxonomy of these two species using freshly collected samples from Japan and the Philippines and dried specimens from seven herbaria worldwide. All specimens were classified as either *P. melanocephala* or *P. kuehnii*, the former distinguished by having paraphyses, dark brown urediniospores with uniformly thick cell walls, and 2-celled teliospores with upper cells darker than the lower cells (Virtudazo et al. 2001b). In contrast, *P. kuehnii* lacks paraphyses and has orange to cinnamon urediniospores with apically thickened walls, and 2- to 3-celled hyaline to whitish teliospores. Reported hosts for *P. melanocephala* include the following: *Aquilegia* spp. L., *Bambusa* spp. Schreb., *Eulalia fastigiata* (Nees ex Steud.) Haines, *Miscanthus floridulus* (Labill.) Warb. Ex K. Schum. & Lauterb., *M. sacchariflorus* (Maxim.) Benth., *M. sinensis* Andersson, *Phyllostachys aurea* Rivière & C. Rivière, *P. bambusoides* Siebold & Zucc., *P. glauca* McClure, *P. nigra* (Lodd. Ex Lindl.) Munro, *Saccharum barberi* Jeswiet, *S. narenga* (Nees ex Steud.) Wall. Ex Hack., *S. officinarum*, *S. robustum* E. W. Brandes & Jeswiet ex Grassl, *S. ravennae* (L.) L., *S. rufipilum* Steud., and *S. spontaneum* L.; and for *P. kuehnii* include the following grasses: *Saccharum arundinaceum* Retz., *S. barberi*, *S. edule* Hassk., *S. narenga*, *S. officinarum*, *S. robustum*, *S. sinense* Roxb., *S. spontaneum*, and *Sclerostachya fusca* (Roxb.) A. Camus (Farr and Rossman 2009; Virtudazo et al. 2001b). In some cases, the above-listed host names were changed according to USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network (GRIN) Online Database (National Germplasm Resources Laboratory, Beltsville, MD. URL: <http://www.ars-grin.gov/cgi-bin/npgs/html/index.pl> (27 August 2009)).

Though differences in morphology of the sugarcane rusts have been clarified previously, their phylogenetic relationships to each other and other rust fungi remain unknown. In response to the threat of rusts on sugarcane production, a morphological assessment of rust fungi causing diseases on sugarcane was conducted using globally distributed samples to determine the current distribution of the two sugarcane-infecting species. In addition, DNA sequence data were obtained to clarify the phylogenetic relationships of sugarcane infecting rust fungi to each other and to other rust fungi.

Materials and methods

Origins of fungal strains and microscopy

Rust specimens on sugarcane were sent from 164 different field locations in 23 countries to the Systematic Mycology and Microbiology Laboratory in Beltsville, MD, between 2007 and 2008, and specimens were deposited in the United States National Fungus Collection (Table 1). Microscopic features of pustules, urediniospores, and paraphyses were examined for all specimens. Representative isolates of *P. kuehnii* and *P. melanocephala* were observed under the scanning electron microscope (BPI 878930 and 878929, respectively). To increase taxon sampling, rust specimens from grasses and other hosts were obtained from specimens intercepted by USDA, APHIS, Plant Health, Plant Protection and Quarantine mycologists or collected in the field, pressed and dried until further processing in the laboratory. Additional DNA sequences from rusts were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>) (Table 2).

DNA isolation, amplification, and sequencing

Portions of the ITS2 and nLSU locus were sequenced from a representative specimen of *P. kuehnii* (BPI 878930) and *P. melanocephala* (BPI 878929). For each sampled individual, 5–6 sori were excised from dried symptomatic leaf material and placed in 2-ml bead solution tubes of the UltraClean Plant DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA). Tubes were processed twice for 30 s at 5.0 m/s in a FastPrep™ (Bio101) instrument with 10-min room-temperature incubations between runs (BIO101, Vista, CA, USA). Tubes were incubated at 55°C overnight (15–17 h) with solution P1 followed by the remaining DNA extraction steps according to the manufacturer's instructions. PCR amplifications were performed with forward primer Rust2inv and reverse primer LR6 according to the procedures outlined in Aime (2006). PCR products were cleaned by adding 2 µl ExoSAP-IT (Affymetrics/USB, Santa Clara,

Table 1 Sugarcane rust samples identified in this study

Country	<i>Puccinia</i> sp.	Variety and specimen voucher number
Australia	<i>melanocephala</i>	Q117 (BPI879669), Q124 (BPI879693), Q190 (BPI879692)
	<i>kuehni</i>	Q124 (BPI879696)
Barbados	<i>melanocephala</i>	DB9627 (BPI879727), HQ3097 (BPI879726)
Brazil	<i>melanocephala</i>	B8008 (BPI879657), RB835486 (BPI879651), BPI879654, BPI879656), RB918639 (BPI879653), RB92579 (BPI879655)
China	<i>kuehni</i>	RB72-454 (BPI879703), BPI879704, BPI879705)
Colombia	<i>melanocephala</i>	CC84-75 (BPI879618, BPI879622, BPI879623), CC85-92 (BPI879613, BPI879614, BPI879615), CC92-2804 (BPI879620), CC93-3895 (BPI879617, BPI879628), MZCV44-275 (BPI879658, BPI879616, BPI879621), CC85-92 (BPI879619, BPI879624, BPI879735, BPI879627, BPI879626), CC93-3895 (BPI879625, BPI879609, BPI879610, BPI879611), CC94-5446 (BPI879608), CC94-5827 (BPI879607)
Costa Rica	<i>kuehni</i>	CPCL02-2130 (BPI879677, BPI879752), Q132 (BPI879682), Q138 (BPI879680), RB73-9735 (BPI879676), SP71-5574 (BPI879672, BPI879678, BPI879681), SP79-2233 (BPI879679), SP79-2233 (BPI879707), CPL02-0487 (BPI879751), CP86-1633 (BPI879753), CP04-1705 (BPI879754), Q96 (BPI879755)
	<i>melanocephala</i>	CP71-5574 (BPI879749)
Dom. Republic	<i>melanocephala</i>	CR8230 (BPI879645), CR74250 (BPI879648), CR83323 (BPI879646), CR9303 (BPI879647)
DR Congo	<i>melanocephala</i>	SP70-1284 (BPI879765)
Ecuador	<i>melanocephala</i>	BJ68-11 (BPI879756), B76-592 (BPI879757), BJ65-152 (BPI879758), B43-62 (BPI879762), B54-142 (BPI879763), BJ65-152 (BPI879762), POJ22-22 (BPI879761), POJ (BPI879760)
El Salvador	<i>kuehni</i>	CP72-2086 (BPI879135, BPI879136, BPI879137) CP80-1557 (BPI879667), CP84-1198 (BPI879740), Mex79-431 (BPI879738), PR75-2002 (BPI879739)
Guadeloupe	<i>melanocephala</i>	B86517 (BPI879723), DB60377 (BPI879722), FG05261 (BPI879725), FG05265 (BPI879724)
Guatemala	<i>melanocephala</i>	B43-63 (BPI879659), CG96-135 (BPI879660), CG97-97 (BPI879661, BPI879666), Mex67-2969 (BPI879662), PR68-3120 (BPI879663), CG03-123 (BPI879748)
	<i>kuehni</i>	BT65-152 (BPI879697)
Jamaica	<i>kuehni</i>	BT9087 (BPI879721), BT9186 (BPI879720)
Japan	<i>melanocephala</i>	F177 (BPI879691), N19 (BPI879701), RK96-6049 (BPI879702)
	<i>kuehni</i>	Co301 (BPI879708, BPI879709, BPI879710), Ni17 (BPI879700, BPI879737), Ni6 (BPI879711), Ni9 (BPI879698), NiTn19 (BPI879699)
Mauritius	<i>melanocephala</i>	M1030/71 (BPI879674), M1176/77 (BPI879675), M1202/01 (BPI879750), M442/51 (BPI879652), M515/01 (BPI879673)
Mexico	<i>kuehni</i>	Mex57-1285 (BPI879741, BPI879742), Mex61-230 (BPI879139, BPI879743, BPI879744), Co301 (BPI879140, BPI879745, BPI879746, BPI879747)
Nicaragua	<i>kuehni</i>	CP72-2086 (BPI879650, BPI879668, BPI879690), CPISA 00-1075 (BPI879649)
Pakistan	<i>melanocephala</i>	PR-1000 (BPI879670), SPF-234 (BPI879664, BPI879665, BPI879671)
Panama	<i>melanocephala</i>	BT34152 (BPI879736)
Philippines	<i>kuehni</i>	56-226 (BPI879631, BPI879694), 80-13 (BPI879632, BPI879637, BPI879638, BPI879641, BPI879695), 86-550 (BPI879633, BPI879636, BPI879643, BPI879644), 95-09 (BPI879629, BPI879634, BPI879642), 95-152 (BPI879630, BPI879640), 97-45 (BPI879635, BPI879639)
Réunion	<i>melanocephala</i>	B65/220 (BPI879713), CP75-361 (BPI879766), H49-5 (BPI879715), LF54/6032 (BPI879714), LF56/26 (BPI879719), M99/48 (BPI879717), N 55/805 (BPI879712), Q76 (BPI879718), R86/591 (BPI879716), R92/6261 (BPI879767)
South Africa	<i>melanocephala</i>	N29 (BPI879683, BPI879685, BPI879686, BPI879687), N39 (BPI879684)
USA, Hawaii	<i>melanocephala</i>	H49-5 (BPI879688), H65-7052 (BPI879689)
USA, Louisiana	<i>melanocephala</i>	HoCP 96-540 (BPI879706) Ho95-988 (BPI879734, BPI879728, BPI879729), HoCP 96-540 (BPI879731, BPI879732), L97-128 (BPI879733), L99-226 (BPI879730)

Table 2 Rust taxa used for phylogenetic analysis

Taxa	Genbank and specimen voucher number	Host	Location ^a	Author
<i>Aecidium kalanchoe</i> J.R. Hern.	AY463163, BPI843633	<i>Kalanchoe blossfeldiana</i> Poelln. (Crassulaceae)	ID, USA	Hernandez et al. (2004)
<i>Coleosporium asterum</i> (Diétel) Syd. & P. Syd.	GU058009, BPI879270	<i>Solidago</i> sp. L. (Asteraceae)	MD, USA	This study
<i>Cumminsiaella mirabilissima</i> (Peck) Nannf.	DQ354531, BPI871101	<i>Berberis aquifolium</i> Pursh (Berberidaceae)	Germany	Aime (2006)
<i>Dietelia portoricensis</i> (Whetzel & L.S. Olive) Burticá & J.F. Hennen	DQ354516, BPI844288	<i>Mikania micrantha</i> Kunth (Asteraceae)	Costa Rica	Aime (2006)
<i>Gymnoconia peckiana</i> (Howe) Trotter	GU058010, BPI879271	<i>Rubus</i> sp. L. (Rosaceae)	MD, USA	This study
<i>Gymnosporangium confusum</i> Diétel	GU058011, BPI879272	<i>Juniperus</i> sp. (Cupressaceae)	Turkey	This study
<i>Gymnosporangium yamadai</i> Miyabe ex G. Yamada	GU058012, BPI879273	<i>Juniperus chinensis</i> L. (Cupressaceae)	DE, USA	This study
<i>Kiehnella uredinis</i> (Link) Arthur	GU058013, BPI879274	<i>Rubus</i> sp. (Rosaceae)	MD, USA	This study
<i>Melampsora</i> sp. (Melampsoraceae)	GU058014, BPI879275	<i>Salix geveryana</i> Andersson (Salicaceae)	UT, USA	This study
<i>Miyagia pseudosphaeria</i> (Mont.) Jorst.	DQ354517, BPI842230	<i>Sonchus oleraceus</i> L. (Asteraceae)	CA, USA	Aime (2006)
<i>Phakopsora pachyrhizi</i> Syd. & P. Syd.	DQ354537, BPI871755	<i>Glycine max</i> (L.) Merr. (Fabaceae)	Zimbabwe	Aime (2006)
<i>Phragmidium violaceum</i> (Schultz) G. Winter	GU058015, BPI879276	<i>Rubus parviflorus</i> Nutt (Rosaceae)	WA, USA	This study
<i>Puccinia agrophila</i> Syd.	GU058016, PUR120	<i>Schizachyrium</i> sp. (Poaceae)	Nigeria	This study
<i>Puccinia andropogonis</i> Schwein.	GU057993, BPI871763	<i>Andropogon</i> sp. (Poaceae)	MN, USA	This study
<i>Puccinia arundinariae</i> Schwein.	GU057997, BPI843964	<i>Arundinaria</i> sp. (Poaceae)	VA, USA	This study
<i>Puccinia caricis</i> Rebert.	DQ354514, BPI871515	<i>Ribes</i> sp. (Grossulariaceae)	ND, USA	Aime (2006)
<i>Puccinia carthami</i> Corda	AY787782	<i>Carthamus tinctorius</i> L. (Asteraceae)	Oman	Deadman et al. (2005)
<i>Puccinia encici-oleracei</i> Pers.	GU058017, BPI879277	<i>Aster</i> sp. (Asteraceae)	Mexico	This study
<i>Puccinia convolvuli</i> (Pers.) Castagne	GU058018, BPI879278	<i>Calystegia sepium</i> (L.) R. Br. (Convolvulaceae)	MD, USA	This study
<i>Puccinia coronata</i> Corda	DQ354526, BPI844300	<i>Rhamnus cathartica</i> L. (Rhamnaceae)	ND, USA	Aime (2006)
<i>Puccinia dioicae</i> Magnus	GU058019, BPI879279	<i>Oenothera fruticosa</i> L. subsp. <i>glauca</i> (Michx.) Straley (Onagraceae)	MD, USA	This study
<i>Puccinia duthiei</i> Ellis & Tracy	GU057998, BPI863454	<i>Dichanthium</i> sp. (Poaceae)	Guam	This study
<i>Puccinia graminis</i> Pers.	AF522177	Unknown	Unknown	Szaro and Bruns
<i>Puccinia hemerocallidis</i> Thüm.	GU058020, BPI879280	<i>Dianella tasmanica</i> Hook. f. (Hemerocallidaceae)	FL, USA	This study
<i>Puccinia heucherae</i> (Schwein.) Diétel	DQ359702, RHS5296/05	<i>Heuchera</i> sp. L. (Saxifragaceae)	United Kingdom	Henricot et al. (2007)
<i>Puccinia hordei</i> G.H. Oth	DQ354527, BPI871109	Unknown Poaceae	CA, USA	Aime (2006)
<i>Puccinia kuehnii</i> (W. Krüger) E.J. Butler	GU058021, BPI878930	<i>Saccharum</i> sp. L. (Poaceae)	Mexico	This study
<i>Puccinia magnusiana</i> Körn.	GU058000, BPI879281	<i>Phragmites</i> sp. Adams. (Poaceae)	China	This study
<i>Puccinia malvacearum</i> Bertero ex Mont.	EF561641, PBM2572	<i>Alcea</i> sp. L. (Malvaceae)	MA, USA	Matheny and Hibbett (unpub.)
<i>Puccinia mariae-wilsoniae</i> Clinton	GU058022, BPI879282	<i>Claytonia virginica</i> L. (Portulacaceae)	MD, USA	This study
<i>Puccinia melanocephala</i> Syd. & P. Syd.	GU058001, BPI878929	<i>Saccharum</i> sp. (Poaceae)	South Africa	This study
<i>Puccinia menthae</i> Pers.	DQ354513, BPI871110	<i>Cunila origanoides</i> (L.) Britton (Lamiaceae)	MD, USA	Aime (2006)
<i>Puccinia miscanthi</i> Miura	AJ296546, THS-R11503	<i>Miscanthus sinensis</i> Andersson (Poaceae)	Japan	Virtudazo et al. (2001a)

<i>Puccinia nakanishikii</i> Dietel	GU058002, BPI879283	<i>Cymbopogon citratus</i> (DC.) Stapf (Poaceae)	HI, USA	This study
<i>Puccinia physalidis</i> Peck	DQ354522, BPI844306	<i>Physalis lanceolata</i> Michx. (Solanaceae)	ND, USA	Aime (2006)
<i>Puccinia pimpinellae</i> (F. Strauss) Link	GU058023, BPI879284	<i>Osmorhiza berteroi</i> DC. (Apiaceae)	WA, USA	This study
<i>Puccinia pittieriana</i> Henn.	EU851138	<i>Solanum tuberosum</i> L. (Solanaceae)	Colombia	Zuluaga et al. (unpub.)
<i>Puccinia polysora</i> Underw.	GU058024, BPI863756	<i>Zea mays</i> L. (Poaceae)	Puerto Rico	This study
<i>Puccinia purpurea</i> Cooke	GU057999, PUR129	<i>Sorghum</i> sp. (Poaceae)	Nigeria	This study
<i>Puccinia rufipes</i> Dietel	AJ296545, THS-R11602	<i>Imperata cylindrica</i> (L.) P. Beauv. (Poaceae)	Japan	Virtudazo et al. (2001a)
<i>Puccinia silvatica</i> J. Schröt.	AY222048, TUB011528	<i>Taraxacum</i> sp. (Asteraceae)	Germany	Lutz et al. (2004)
<i>Puccinia sorghi</i> Schwein.	GU057994, BPI878071	<i>Zea</i> sp. (Poaceae)	South Africa	This study
<i>Puccinia</i> sp. (Pucciniaceae)	GU058026, MCA3259	Poaceae unknown	Uganda	This study
<i>Puccinia</i> sp. (Pucciniaceae)	GU058025, MCA2969	Poaceae unknown	Hawaii, USA	This study
<i>Puccinia sparganioides</i> Ellis & Tracy	GU058027, BPI879285A	<i>Spartina patens</i> (Aiton) Muhl. (Poaceae)	DE, USA	This study
<i>Puccinia spgazzinii</i> De Toni	EU851150	<i>Mikania micrantha</i> Kunth (Asteraceae)	Colombia	Zuluaga et al. (unpub.)
<i>Puccinia sporoboli</i> var. <i>robusta</i> Cummins & H.C. Greene	GU058003, BPI871549	<i>Calamovilfa</i> sp. (Poaceae)	ND, USA	This study
<i>Puccinia striiformis</i> Westend.	GU058005, BPI871785	<i>Triticum</i> sp. (Poaceae)	ND, USA	This study
<i>Puccinia substriata</i> Ellis & Barthol.	GU058028, BPI863747	<i>Digitaria</i> sp. (Poaceae)	Honduras	This study
<i>Puccinia symphoricarpi</i> Harkn.	GU058006, BPI879286	<i>Symphoricarpos albus</i> (L.) S. F. Blake (Caprifoliaceae)	WA, USA	This study
<i>Puccinia triticina</i> Erikss.	GU058007, BPI878079	<i>Triticum</i> sp. (Poaceae)	ND, USA	This study
<i>Puccinia vexans</i> Farl.	GU058008, BPI879287	<i>Fouquieria</i> sp. (Fouquieriaceae)	Mexico	This study
<i>Puccinia violae</i> (Schumach.) DC.	GU058029, BPI879288	<i>Viola pedata</i> L. (Violaceae)	MD, USA	This study
<i>Puccinia windsoriae</i> Schwein.	GU057995, BPI871790	<i>Tridens</i> sp. (Poaceae)	VA, USA	This study
<i>Puccinosira pallidula</i> (Speg.) Lagerh.	DQ354534, BPI863541	<i>Triumfetta semitriloba</i> Jacq. (Malvaceae)	Venezuela	Aime (2006)
<i>Uromyces acuminatus</i> Arthur	GU058004, BPI879285B	<i>Spartina patens</i> (Aiton) Muhl. (Poaceae)	RI, USA	This study
<i>Uromyces appendiculatus</i> (Pers.) Link	AF522182	Unknown	Unknown	Szaro and Bruns (unpub.)
<i>Uromyces ari-triphylli</i> (Schwein.) Seeler	DQ354529, BPI871111	<i>Arisaema triphyllum</i> (L.) Schott (Araceae)	MD, USA	Aime (2006)
<i>Uromyces ciceris-arietini</i> (Grognot) Jacz. & Boyd	GU058030, BPI879192	<i>Cicer</i> sp. (Ciceraceae)	CA, USA	This study
<i>Uromyces galegae</i> (Opiz) Sacc.	DQ250133, BPI863535	<i>Galega officinalis</i> L. (Fabaceae)	Turkey	Aime (2006)
<i>Uromyces linearis</i> Berk. & Broome	GU057996, BPI871072	<i>Panicum</i> sp. (Poaceae)	Hawaii, USA	This study
<i>Uromyces neotropicalis</i> J.R. Hern. & Aime	DQ021885, MCA2533	Unknown Cucurbitaceae	Ecuador	Hernandez et al. (2004)
<i>Uromyces novissimus</i> Speg.	EU851147	<i>Cayaponia</i> sp. (Cucurbitaceae)	Colombia	Zuluaga et al.
<i>Uromyces viciae-fabae</i> (Pers.) J. Schröt.	AY745695, PBM2573	<i>Pisum</i> sp. (Fabaceae)	MA, USA	Matheny and Hibbett (unpub.)

^a CA California, DE Delaware, FL Florida, HI Hawaii, ID Idaho, MD Maryland, MN Minnesota, ND North Dakota, RI Rhode Island, UT Utah, VA Virginia, WA Washington

CA, USA) and incubating tubes at 37°C for 60 min and 80°C for 15 min. Cleaned PCR products were sequenced in 10- μ l reaction volumes with 0.25 μ l Big Dye Terminator sequencing enzyme v.3.1 (Applied Biosystems, Foster City, CA, USA), 1 μ l of 1 μ M primer, 2 μ l of 5 \times sequencing buffer, 5.75 μ l water, and 2 μ l cleaned PCR product. Sequencing primers were the same as those used for PCR, and LR3 (Vilgalys and Hester 1990) and LROR (Moncalvo et al. 1995) were used as internal sequencing primers. Cycle sequencing conditions were: 96°C for 1 min, 35 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Sequencing reactions were cleaned with the Big Dye XTerminator purification kit (Applied Biosystems) and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems). DNA sequences are deposited in GenBank (Accessions GU057993–GU058030).

Sequence alignment and phylogenetic analysis

Sequences were assembled and edited in Sequencher v.4.8 (Gene Codes, Ann Arbor, MI, USA). Edited sequences were aligned with *Puccinia* sequences retrieved from Genbank (Table 2) using ClustalX 2.0.11 and the alignment was adjusted manually in MacClade 4.08 (Maddison and Maddison 2001). Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) analyses were implemented in PAUP* vers. 4.0d106 (Swofford 2002). The best fitting model was determined using AIC as implemented by Modeltest 3.7 (Posada and Crandall 1998). Characters were equally weighted and unordered with gaps treated as missing data. Heuristic searches were performed with 1,000 random addition sequence replicates and tree-bisection-reconnection branch swapping, collapse and Mul-Trees (saving all optimal trees) options in effect. Outgroups were defined as *Coleosporium asterum* (Dietel) Syd. & P. Syd., *Gymnoconia peckiana* (Howe) Trotter, *Gymnosporangium confusum* Dietel, *G. yamadae* Miyabe ex G. Yamada, *Kuehneola uredinis* (Link) Arthur, *Melampsora* sp., *Phakopsora pachyrhizi* Syd. & P. Syd., and *Phragmidium violaceum* (Schultz) G. Winter, as prior studies showed these to belong to families outside the *Pucciniaceae* Chevall. (Aime 2006). Maximum parsimony bootstrap support was calculated using the same settings as above with 1,000 replicates, each with 100 random taxon addition replicates (Felsenstein 1985). Bayesian (BI) analyses were conducted with MrBayes 3.1 (Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2001) using the appropriate model with four incrementally heated Markov chains and two concurrent runs of 10,000,000 generations sampled every 10,000 generations for a total of 1,000 trees saved. The outgroup taxon was defined as *Phakopsora pachyrhizi*. The initial 25% of trees sampled was discarded as burn-in. A majority rule consensus tree was calculated from the remaining pool of trees.

Results

Morphology

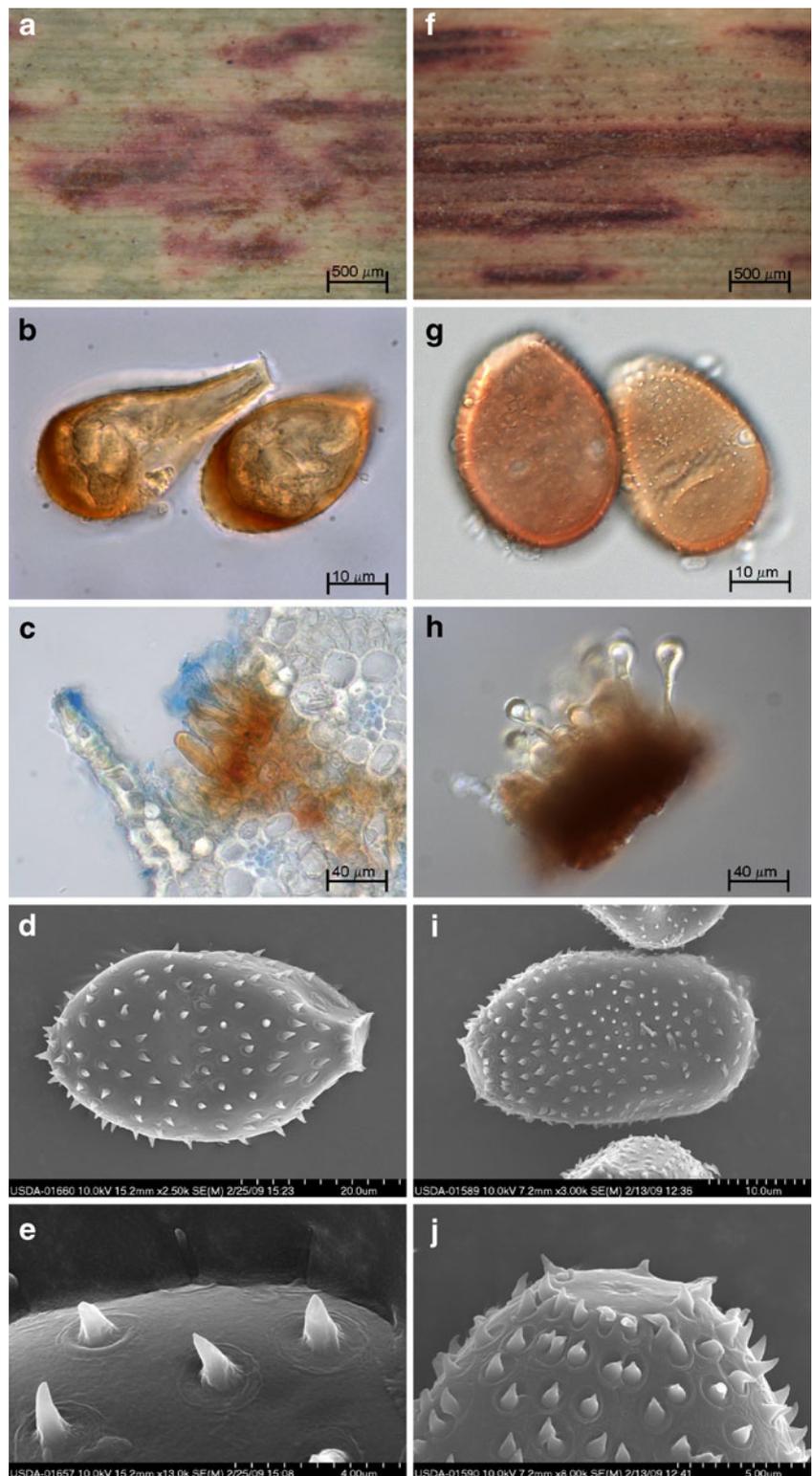
Though it can be difficult to distinguish between infections caused by *P. kuehnii* and *P. melanocephala* on sugarcane tissue in the field, under the dissecting microscope, orange rust pustules are bright orange and surrounding necrotic tissue is reddish/pink (Fig. 1a), compared to brown rust which has darker pustules and orange/brown necrotic host tissue (Fig. 1f). Urediniospores are the most commonly observed spores for both *P. kuehnii* (Fig. 1b) and *P. melanocephala* (Fig. 1g). Urediniospores of *P. kuehnii* are distinguished by their bright orange color, compared to the brown-colored spores of *P. melanocephala*. Urediniospores of *P. melanocephala* have evenly spaced cell walls, while those of *P. kuehnii* are thickened up to 13 μ m at the apex. Urediniospores of *P. kuehnii* are larger in size, ranging from 35–68 μ m in length (Fig. 1d), compared to those of *P. melanocephala*, which are 24–43 μ m (Fig. 1i). In addition, paraphyses are lacking in uredinia of *P. kuehnii* (Fig. 1c), whereas they are abundant in those of *P. melanocephala* (Fig. 1h). Under the SEM, the more densely spaced surface ornamentation of *P. melanocephala* is the most prominent distinguishing character (Fig. 1e, j). Of the 59 samples of *P. kuehnii* collected (Table 1), no teliospores were observed, perhaps due to their rareness or the fact that the majority of samples were collected in November, before the formation of telia.

Phylogenetic analysis

Thirty-eight rust taxa were newly sequenced and aligned with 26 rust sequences from GenBank to determine the phylogenetic position of *P. kuehnii* and *P. melanocephala* within the *Pucciniales* Clem. & Shear. The aligned ITS2 and nLSU dataset contained 1,639 characters. The entire rDNA ITS2 region and some regions in the nLSU were excluded due to difficulty in alignment, leaving 1,254 total characters for analysis of which 223 were parsimony informative. A variation of the general time reversible (GTR) model of nucleotide substitution (GTR+I+ Γ) was selected by Modeltest. Tree topologies resulting from NJ, MP, ML and BI resulted in essentially the same well-supported nodes. Maximum parsimony analysis resulted in 54 equally parsimonious trees with lengths of 1,089 steps, consistency index (CI) of 0.614 and retention index (RI) of 0.916. The maximum likelihood analyses generated a single topology with a $-\ln=7,196.119$ (Fig. 2).

All analyses grouped both sugarcane rusts within the family *Pucciniaceae*, the crown group of extant rusts containing the majority of described species. Within the *Pucciniaceae*, three larger groups were identified (Fig. 2).

Fig. 1 Distinguishing features of *Puccinia kuehnii* (a–e) and *P. melanocephala* (f–j). **a, f** Color of pustules and host necrosis **b, g** Urediniospore shape, color, and wall thickness. The absence (c) or presence (h) of paraphyses. **d, i** Urediniospore size, shape, and surface ornamentation



Group 1 consists of a large, well-supported clade containing all *Cyperaceae*- or *Juncaceae*-infecting rusts along with other angiosperm-infecting rusts. *Puccinia melanocephala* is placed in group 2, a clade of grass-infecting rusts, including *P. miscanthi* Miura, *P. nakanishikii* Dietel, and *P.*

rufipes Dietel infecting *Miscanthus sinensis*, *Cymbopogon citratus* (DC.) Stapf (lemongrass), and *Imperata cylindrica* (L.) P. Beauv., respectively. Only one point mutation and two deletions separate *P. miscanthi* from *P. melanocephala* in the region of the nLSU locus sequenced. *Puccinia*

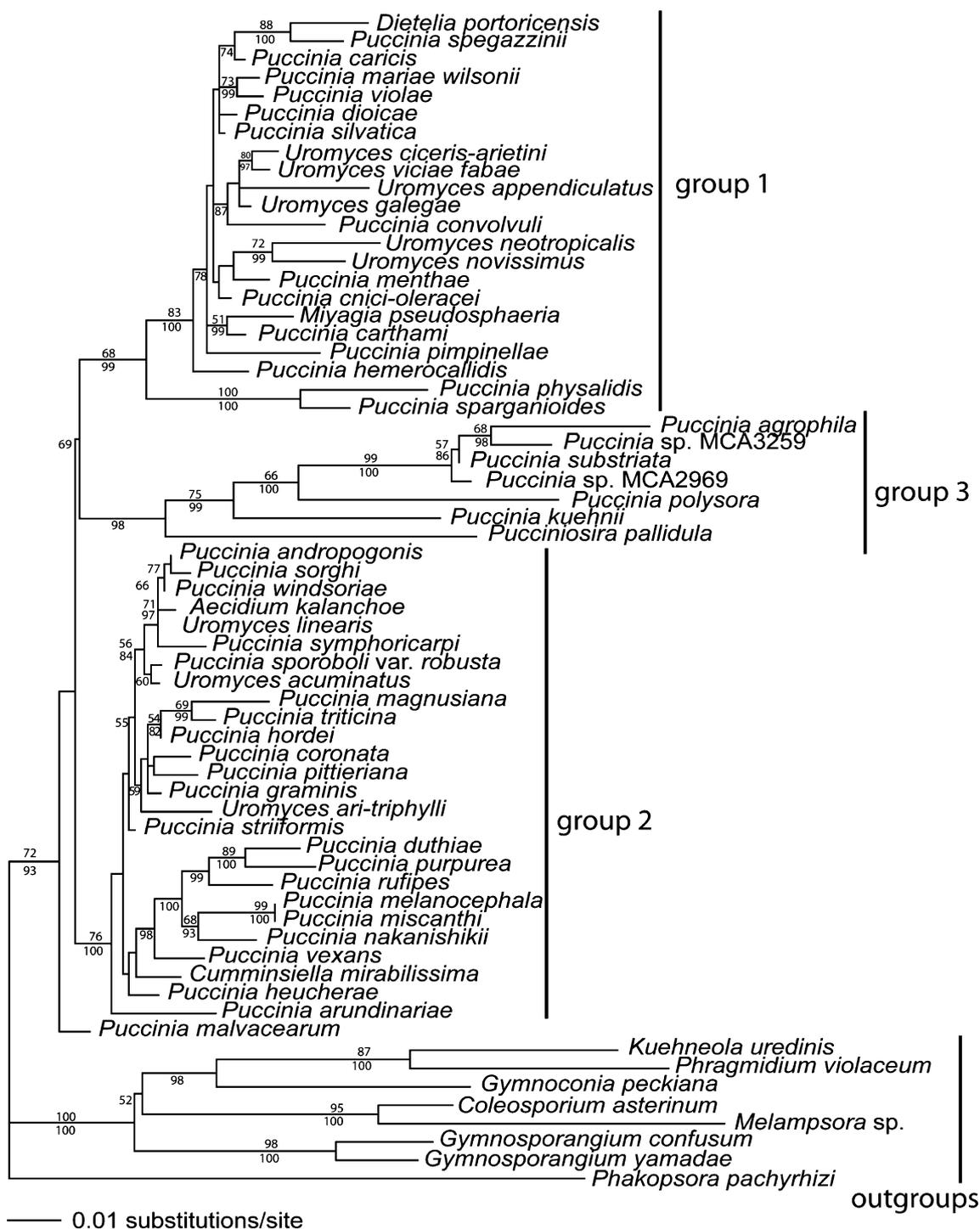


Fig. 2 Maximum-likelihood *Pucciniaceae* phylogeny based on the nuclear rLSU locus. Branch support >50% from maximum parsimony bootstrap (*above*) and posterior probabilities from Bayesian analysis (*below*) is indicated

kuehnii is placed outside both larger groups in another assemblage of grass-infecting species (group 3) including *P. polysora* Underw., *P. agrophila* Syd., and *P. substriata* Ellis & Barthol. infecting *Zea mays* L., *Schizachyrium* spp. Nees, and *Digitaria* spp. Haller, respectively. Two *Puccinia* spp. infect-

ing unidentified *Poaceae* from Uganda and Hawaii are also placed in this well-supported group. Support for *Puccinosira pallidula* (Speg.) Lagerth. (infecting *Triumfetta semitriloba* Jacq.) as basal to group 3 was found with Bayesian inference posterior probability, but not parsimony bootstrap.

Discussion

This study is the first to use morphological data obtained from globally sampled sugarcane rust infections to confirm that *P. kuehnii* and *P. melanocephala* are the most widely occurring sugarcane rust pathogens. Out of the 164 samples collected, 67 were identified as *P. kuehnii* and 97 were identified as *P. melanocephala*, indicating that *P. melanocephala* is still the most common and widespread rust on sugarcane (Table 1). Still, 36% of the samples collected in the Western Hemisphere were identified as *P. kuehnii* (compared to 49% in the Eastern Hemisphere) demonstrating that orange rust is already an important disease in the Western Hemisphere and its incidence is likely to continue increasing. The majority of major breeding lines show susceptibility to at least one of the diseases, and six cultivars were found to harbor both *P. kuehnii* and *P. melanocephala* in this study (Table 1).

The presence of uredinia was sufficient for identification to species by confirming the presence or absence of paraphyses and apically thickened urediniospores. The fact that no teliospores were found in all *P. kuehnii* samples is not surprising considering teliospores of *P. kuehnii* were not reported until Hennen (1986) on sugarcane nor on any host since their initial description by Butler (1914). The expansion in the reported range of urediniospore size is likely due to increased sampling.

Rust fungi consist of more than 7,000 species, the majority of which do not have associated sequence data. Rather, rust taxonomy has been based almost entirely on morphology, despite limited characters, phenotypic variability, convergent evolution, and complicated life cycles. However, using two nuclear rDNA genes (18S and 28S), the order *Pucciniales* (rusts) was determined to be a monophyletic group with three lineages, or sub-orders: (1) *Uredinineae* Aime (rusts in which the aecial stage, when present, is formed on angiosperms), (2) *Melamporineae* Aime (rusts on which the aecial stage, when present, is formed on members of the Pinaceae and which contains the fern rusts and many conifer pathogens), and (3) *Mikronegeriineae* Aime (Type 12 spermogonia characterized by deep-seated hymenia, indeterminate growth, and well-developed beaks). Eight families in the order *Pucciniales* are supported, five of which are within the sub-order *Uredinineae* (Aime 2006).

The family *Pucciniaceae* is estimated to contain ~5,000 of the ~7,800 described species (Kirk et al. 2008) and includes the vast majority of *Puccinia* Pers. ex Pers. and *Uromyces* (Link) Unger species, as well as the genera *Aecidium* Pers., *Cumminsia* Arthur, *Dietelia* Henn., *Endophyllum* Lév., *Miyagia* Miyabe ex Syd. & P. Syd., *Puccinosira* Lagerh., *Sphenospora* Dietel, and *Uredo* Pers. (Aime 2006). The speciose genera *Uromyces* (~800 spp.) and *Puccinia* (~4,000 spp.), distinguished by 1- or 2-celled

teliospores, respectively, are not monophyletic (Maier et al. 2003). Rather, the phylogeny within the family *Pucciniaceae* seems influenced to some extent by host associations. Species infecting *Cyperaceae* or *Juncaceae* in their uredinial or telial state are found in a highly supported monophyletic group (group 1) among other angiosperm rusts. In contrast, species infecting *Poaceae* in their uredinial and telial stages are found in various unsupported clades outside group 1 and are referred to as group 2 (Aime 2006; Maier et al. 2007). This study found that, although the sugarcane-infecting *Puccinia* species are not closely related to each other, they both fall outside the highly supported group 1 rust clade, further maintaining the separation of rust species infecting *Poaceae* from *Cyperaceae*- and *Juncaceae*-infecting rusts and their evolutionary derivatives. This study presents statistical support for both group 1 and 2 as a monophyletic clade and also provides support for the presence of an additional group 3 that includes *P. kuehnii* and other grass-infecting relatives.

This study highlights that *P. melanocephala* and *P. miscanthi* are closely related; *P. miscanthi*, sequenced by Virtudazo et al. (2001a), differs from the *P. melanocephala* isolate sequenced in this study by only two deletions and one point mutation across 600 base pairs of the nLSU locus. The lack of distinguishing morphological characters between the species and their overlapping host ranges may cause confusion in their identification. Both species are reported to infect *Miscanthus* spp., *S. ravennae*, and *S. spontaneum* in their uredinial and telial states, potentially leading to misdiagnosis. The rDNA ITS sequence of *P. miscanthi* obtained by Virtudazo et al. (2001a) differs from the *P. melanocephala* isolate used in this study by 14 point mutations and 6 indels. Additional isolates will help clarify whether sequence differences are a result of interspecific or intraspecific variation. In the literature, host differences are largely what separate the two species. The uredinial and telial states of *P. miscanthi* are found on *Imperata cylindrica*, *Miscanthus* spp. Andersson, *Phacelurus* sp. Griseb., *Saccharum narenga* (Nees ex Steud.) Wall. ex Hack., *S. ravennae*, *S. spontaneum*, *Sorghum bicolor* (L.) Moench subsp. *bicolor*, and *Thysanolaena latifolia* (Roxb. ex Hornem.) Honda (Farr and Rossman 2009; Virtudazo et al. 2001b). *Puccinia miscanthi* is reported to produce spermogonia and aecia on *Lysimachia clethroides* Duby and *Plantago* spp. L. The spermogonial and aecial hosts of *P. melanocephala* are unknown. The slight variations in reported telial size and thickness of pedicel walls between the two species are similar to morphological differences based on host or environment and these variations have not yet shown to correlate with molecular differences (Hiratsuka et al. 1992). Phylogenetic analyses with highly variable loci will help clarify whether or not *P. miscanthi* is distinct from *P. melanocephala*.

This study will allow more accurate identification of the rust species causing sugarcane diseases and the resulting phylogeny will assist in making predictions regarding potential rust pathogen host jumps to sugarcane and identifying sources of resistance to these diseases. Our results indicate the need for more detailed phylogenetic analyses within the grass-infecting rust clades, which will require the inclusion of additional taxa and loci.

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