Invasions of gladiolus rust in North America are caused by a widely-distributed clone of *Uromyces transversalis*

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ABSTRACT

Uromyces transversalis, the causal agent of Gladiolus rust, is an invasive plant pathogen in the United States and is regulated as a quarantine pathogen in Europe. The aim of this research was to: (i) determine the origin of introductions of *U. transversalis* to the United States, (ii) track the movement of genotypes, and (iii) understand the worldwide genetic diversity of the species. To develop molecular markers for genotyping, whole genome sequencing was performed on three isolates collected in the United States. Genomes were assembled *de novo* and searched for microsatellite regions. Primers were developed and tested on ten isolates from the United States resulting in the identification of 24 polymorphic markers. Among 92 isolates collected from Costa Rica, Mexico, New Zealand, Australia, and the United States there were polymorphisms within isolates with no genotypic diversity detected among isolates; however, missing data among the New Zealand and Australia isolates due to either poor amplification of degraded DNA or null alleles as a result of genetic differences made it difficult to generate conclusions about these populations. The microsatellite loci and flanking regions showed high diversity and two divergent genomes within dikaryotic individuals, yet no diversity among individuals, suggesting that the invasive *U. transversalis* populations from North America are strictly clonal.

Subjects Agricultural Science, Mycology, Population Biology
Keywords Clonal population, Invasion, Ornamental rust, Whole genome sequencing, Genetic diversity, Microsatellites

INTRODUCTION

Gladiolus rust, caused by the fungus *Uromyces transversalis,* was first identified in South Africa by von Thümen in 1876; however, little is known about the genetic diversity, center of origin, or historical dispersal patterns of *U. transversalis*. It was not until about a century after it was initially described that the fungus invaded northern Africa and then
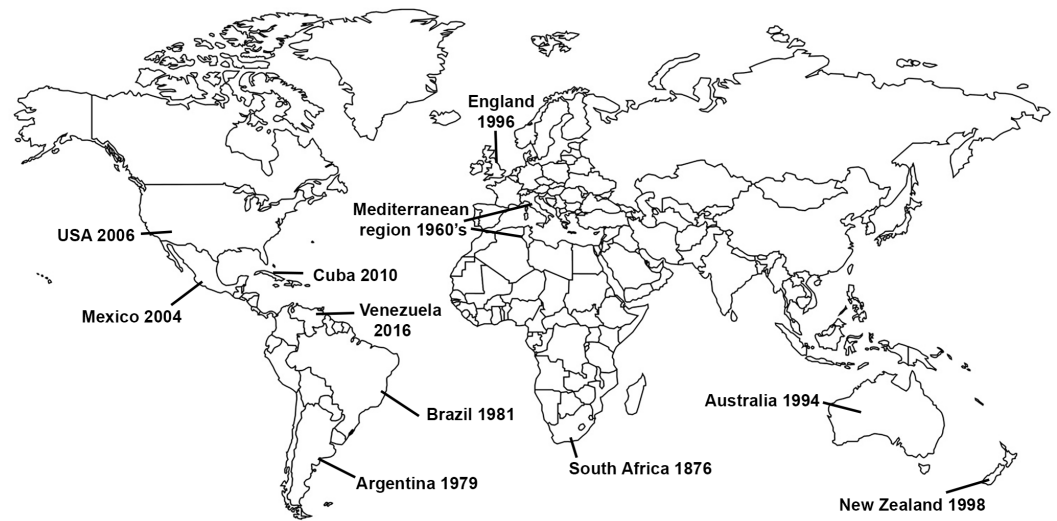
Figure 1 Invasion history of the Gladiolus rust fungus, *Uromyces transversalis*.

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southern Europe in 1966 (Fig. 1), reaching England by 1996 (*Beilharz, Parbery & Pascoe, 2001*). Subsequently, Gladiolus rust was detected in Argentina in 1979 (*Lindquist, Alippi & Medera, 1979*), Brazil in 1981 (*Pita et al., 1981*), Australia in 1994 (*Beilharz, Parbery & Pascoe, 2001*), New Zealand in 1998 (*McKenzie, 2000*), Mexico in 2004 (*Rodríguez-Alvarado et al., 2006*), the United States (USA) in 2006 (*Blomquist et al., 2007*), Cuba in 2010 (*Martínez-de la Parte et al., 2011*) and Venezuela in 2016 (*Mohali & Aime, 2018*). *Uromyces transversalis*, the causal agent of Gladiolus rust, can be devastating to species of *Gladiolus* and is difficult to eradicate once established.

The fungus *U. transversalis* is an obligate biotrophic pathogen that grows and reproduces on members of the family Iridaceae in arid, Mediterranean, and tropical climates (*Garibaldi & Aloj, 1980*; *Hernández, 2004*; *Peterson & Berner, 2009*; *Rizvi et al., 2007*). In regions where Gladiolus rust is established the disease can cause crop losses of 10–100%, unless fungicide applications are used (*Beilharz, Parbery & Pascoe, 2001*; *Ferreira & Nevill, 1989*; *Hernández, 2004*; *Littlejohn & Blomerus, 1997*; *Valencia-Botin et al., 2013*). As a consequence, the pathogen is considered of quarantine significance in Europe and was regulated in the USA from 2007 to 2015 (*Peterson & Berner, 2009*; *Rizvi et al., 2007*).

Gladiolus flowers are imported into the USA from multiple countries including Mexico, where *U. transversalis* is prevalent in *Gladiolus* production areas (*Valencia-Botin et al., 2013*). Shipments of *Gladiolus* flowers infected with *U. transversalis* arriving to the United States from Mexico have been repeatedly intercepted at border stations in California and Texas (*Brown, 2005*; *Hernández, 2004*; *Rizvi et al., 2007*; *Valencia-Botin et al., 2013*), and at a Florida border station with imports arriving from Mexico and Brazil (*Schubert et al., 2007*). A quarantine and national management plan strategy was followed by both federal and state quarantine officials in an attempt to contain and manage *U. transversalis* in the USA (*Rizvi et al., 2007*; *Valencia-Botin et al., 2013*). Despite quarantine measures, severe outbreaks of Gladiolus rust occurred in 2014 in the United States, leading the U.S.

Department of Agriculture, Animal and Plant Health Inspection Services (USDA-APHIS) to revise its response requirements in 2015 (*US Department of Agriculture APHIS, 2015*).

Uromyces transversalis primarily infects the leaves and stem of its host; however, under heavy inoculum pressure it can also infect the flowers (*Ferreira & Nevill, 1989*). Visibly infected plants lose economic value as an ornamental cut flower (*Valencia-Botin et al., 2013*). Infection by the rust fungus reduces the plant's vigor, resulting in reduced flower production (*Wise, Mueller & Buck, 2004*). The initial symptoms of *U. transversalis* on *Gladiolus* leaves are small chlorotic spots, which eventually break the leaf surface to reveal small yellow-orange uredinia. The uredinia coalesce to form large lesions (3–7 mm) laterally across the leaf surface (*Beilharz, Parbery & Pascoe, 2001*; *Martínez-de la Parte et al., 2011*; *Rizvi et al., 2007*; *Rodríguez-Alvarado et al., 2006*; *Valencia-Botin et al., 2013*). *U. transversalis* produces urediniospores and teliospores, but has no known alternate host (*Hernández, 2004*) or other spore types (*Hernández, 2004*; *Rizvi et al., 2007*). This suggests that sexual reproduction does not occur in *U. transversalis* due to an incomplete life cycle. As with many rusts, the urediniospores are the dispersal and infection spores. *U. transversalis* spores may be disseminated locally by wind or water splash (*Hernández, 2004*). Long-distance dispersal of urediniospores may occur naturally by wind, but it is primarily attributed to human-mediated movement of infected plants, including potted flowers, cut flowers and corms (*Beilharz, Parbery & Pascoe, 2001*; *Wise, Mueller & Buck, 2004*).

Molecular markers for genotyping isolates are necessary to understand the genetic diversity and historical dispersal patterns of *U. transversalis*. Due to the high variability, multiplexing capacity, ease of reproducibility, and relatively low cost associated with processing a large number of isolates (*Frenkel et al., 2012*; *Leclercq, Rivals & Jarne, 2007*), microsatellite markers are the ideal marker choice for determining the genetic diversity and population structure of *U. transversalis*. The objectives of this research were to: i) develop microsatellite markers to genotype isolates of *U. transversalis*, ii) determine the geographic origin and track the movement of introduced genotypes of *U. transversalis* in the USA, and iii) understand the genetic diversity of *U. transversalis* collected from a wide geographical area in order to understand historical dispersal patterns of this invasive fungus. We hypothesize that *U. transversalis* was introduced into the USA from Mexico, and that the invasive populations have low genetic diversity.

MATERIALS & METHODS

Isolate collection and DNA extraction

DNA was extracted from a total of 92 samples of *Uromyces transversalis* (Table 1) in preserved leaf tissue obtained from Australia ($n = 7$), New Zealand ($n = 10$), Mexico ($n = 60$), or as fresh, infected leaf tissue collected within the USA ($n = 10$) or from border interceptions from Costa Rica ($n = 5$). The preserved tissue from Australia and New Zealand had been stored in envelopes in herbaria, whereas the tissue from Mexico had been stored individually in conical polypropylene tubes at $-20\ ^\circ C$. From each preserved leaf tissue sample, DNA was extracted using a modified genomic DNA mini-prep protocol (*Lee, Milgroom & Taylor, 1988*). Briefly, multiple uredinia were scraped to

Table 1 Location and sources of *Uromyces transversalis* isolates used in this study.

Geographic origin		Original host species	Isolate identifier	Collection date	Culture Collection (Collector)
City and/or State	**Country**				
Not provided	Costa Rica	*Gladiolus* sp.	CR497224, CR498594, CR498400, CR497666, CR498457	2012	USDA-ARS; Pedley, K.
Wellington	New Zealand	*Gladiolus* sp.	NZ71109	2000	New Zealand Fungal Herbarium (NZFB); Beever, R.
Fendalton	New Zealand	*Gladiolus* sp.	NZ87970	2006	NZFB; Close, R.
Remuera	New Zealand	*Anomatheca laxa*	NZ69482	1998	NZFB; Dingley, J.M.
Remuera	New Zealand	*G. nanus*	NZ69481	1998	NZFB; Heckler, R.
Feilding	New Zealand	*Gladiolus* sp.	NZ71696	2000	NZFB; Hill, C.F.
Mount Albert	New Zealand	*G. undulatus*	NZ97335	2007	NZFB; Petley, M.
Avondale	New Zealand	*Melasphaerula*	NZ69208	1998	NZFB; Wilkie, J.P.
Mount Albert	New Zealand	*Gladiolus* sp.	NZ99990	2011	NZFB; Wilkie, J.P.
Mount Albert	New Zealand	*Tritonia*	NZ88195	2004	NZFB; Wilkie, J.P.
Mount Albert	New Zealand	*Tritonia*	NZ69483	1998	NZFB; Beever, R.
Not provided	Australia	*Gladiolus* sp.	VPRI 20841, VPRI 20858, VPRI 20881, VPRI 21344, VPRI 22299, VPRI 32661	Unknown	Victoria Plant Pathology Herbarium (VPPH)
Mont Albert, Victoria	Australia	*Gladiolus* sp.	VPRI 21238	1996	VPPH; Parbery, D.
Tlapizalco, Zumpahuacán	Mexico	*Gladiolus* sp.	TLAP1, TLAP2, TLAP3	2011	Valencia-Botín, A.
Atlixco, Puebla	Mexico	*Gladiolus* sp.	Atlix1, Atlix2, Atlix3	2011	Valencia-Botín, A.
Cuautla, Morelos	Mexico	*Gladiolus* sp.	Cua1, Cua2, Cua3	2011	Valencia-Botín, A.
Villa Guerrero, State of Mexico	Mexico	*Gladiolus* sp.	Gro1, Gro2, Gro3	2011	Valencia-Botín, A.
Tenango del Valle, State of Mexico	Mexico	*Gladiolus* sp.	Ten1, Ten2, Ten3	2011	Valencia-Botín, A.
Irimbo, Michoacán	Mexico	*Gladiolus* sp.	Iri1, Iri2, Iri3	2010	Valencia-Botín, A.
"La Finca" Villa Guerrero, State of Mexico	Mexico	*Gladiolus* sp.	LF1 1, LF1 2, LF1 3, LF2 1, LF2 2, LF2 3	2011	Valencia-Botín, A.
Cocoyoc Yautepec, Morelos	Mexico	*Gladiolus* sp.	M1 1, M1 2, M1 3	2010	Valencia-Botín, A.
Oacalco Yautepec, Morelos	Mexico	*Gladiolus* sp.	M2 1, M2 2, M2 3	2010	Valencia-Botín, A.
Yautepec Yautepec, Morelos	Mexico	*Gladiolus* sp.	M3 R1, M3 R2, M3 R3	2010	Valencia-Botín, A.
El Caracol Yautepec, Morelos	Mexico	*Gladiolus* sp.	M4 1, M4 2	2010	Valencia-Botín, A.
Villa Ayala, Morelos	Mexico	*Gladiolus* sp.	M5 R3, M6 R1, M6 R2, M6 R3, M7 1, M7 2, M7 3, M8 R1, M8 R2, M8 R3	2010	Valencia-Botín, A.

Table 1 *(continued)*

Geographic origin		Original host species	Isolate identifier	Collection date	Culture Collection (Collector)
City and/or State	**Country**				
Ejido Tlayacapan, Morelos	Mexico	*Gladiolus* sp.	M9 R1, M9 R2, M9 R3	2010	Valencia-Botín, A.
Huachinanitla Tepoztlán, Morelos	Mexico	*Gladiolus* sp.	M10 R1, M10 R2, M10 R3	2010	Valencia-Botín, A.
Villa Guerrero, State of Mexico	Mexico	*Gladiolus* sp.	JB2, JB7	2010	Valencia-Botín, A.
Cuautla, Morelos	Mexico	*Gladiolus* sp.	JB3, JB5	2010	Valencia-Botín, A.
Atlixco, Puebla	Mexico	*Gladiolus* sp.	JB1, JB4, JB6	2010	Valencia-Botin, A.
Irambo, Michoacan	Mexico	*Gladiolus* sp.	JB8	2010	Valencia-Botin, A.
California	United States	*Gladiolus* sp.	CA11-1	2011	K. Pedley
Carpenteria, California	United States	*Gladiolus* sp.	CA14-1, CA14-3, CA14-4	2014	K. Pedley
Santa Maria, California	United States	*Gladiolus* sp.	CA14-2	2014	K. Pedley
Santa Barbara, California	United States	*Gladiolus* sp.	CA14-5	2014	K. Pedley
Goleta, California	United States	*Gladiolus* sp.	CA14-6, CA14-7	2014	K. Pedley
Manatee County, Florida	United States	*Gladiolus* sp.	FL11-1	2011	K. Pedley
Hendry County, Florida	United States	*Gladiolus* sp.	FL11-2	2011	K. Pedley

remove urediniospores (0.02–0.04 g) using a sterilized scalpel and transferred into 1.5 mL reaction tubes containing 246.9 µL lysis buffer (150 µL sddH$_2$O, 25 µL 0.5 M EDTA (pH 8), 25 µL 1.0 M Tris, 43.75 µL 20% SDS solution, 3.15 µL 20 mg/L proteinase K and 0.0025 g NaHSO$_3$) (Thermo Fisher Scientific, Watham, MA). Sample tubes were vortexed for 1 min, incubated at 65 °C for 15 min, and centrifuged (13,978× g for 5 min). The precipitates were discarded and the supernatants transferred to new 1.5 µL microcentrifuge tubes. A solution of 50 µL 7.5 M NH$_4$OAc (Sigma-Aldrich, St. Louis, MO) was added to each tube, vortexed for 10 s, and tubes were chilled on ice for 15 min. Samples were then centrifuged (13,978× g for 3 min). The supernatants were again transferred to new 1.5 mL microcentrifuge tubes and 175 µL isopropanol was added, tubes were mixed and centrifuged (13,978× g for 5 min), and the supernatant was discarded. The pellets were rinsed twice with 250 µL of 70% ethanol solution, dried and re-suspended with 25 µL sddH$_2$O, then incubated at 30 °C for 10 min. All samples were stored at −20 °C until further use. DNA extractions from fresh, infected leaf tissue was performed using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (*Rodgers & Bendich, 1985*). Briefly, one to three one cm^2 excised pieces of infected leaf tissue were frozen and using a mortar and pestle, ground to a fine powder in liquid nitrogen. DNA extraction buffer (100 mM Tris, pH 7.5; 1% CTAB; 0.7 M NaCl; 10 mM EDTA; 1% 2-mercaptoethanol; 0.3 mg/ml proteinase K) was added to the ground tissue and incubated at 65 °C for 30 min, followed by two rounds of chloroform:

isoamyl alcohol (1:1) extraction, and precipitated with 2-propanol. DNA was resuspended in TE buffer containing 1 mg/ml RNase.

Three *U. transversalis* isolates collected in the USA were maintained at the USDA Agricultural Research Service, Foreign Disease-Weed Science Research Unit, biosafety level-3 plant disease containment facility at Ft. Detrick, Maryland. These samples were propagated from *U. transversalis-* infected Gladiolus plants collected from California and Florida commercial fields in 2011 and 2014 (Table 1). Prior to extraction using the modified CTAB protocol described above, urediniospores were harvested from infected Gladiolus plants with a microcyclone spore collector (*Cherry & Peet, 1966*; *Peterson & Berner, 2009*; *Tervet et al., 1951*). Spores were germinated by placing 300 mg of freshly harvested spores in a 23 cm × 33 cm glass container that contained 300 mL of sterile water with 15 μg ampicillin. A sterile wooden applicator stick was used to break up clumps of spores, so that the spores were evenly distributed across the surface of the water. The container was covered and left in the dark overnight (16–18 h). The germinated spores were then scraped from the surface of the water, blotted dry with sterile-paper towels and stored in −20 °C.

Genome sequencing and assembly

Genomic DNA obtained from three isolates (CA11-1, FL11-1, and FL11-2) was standardized to 50.0 ng/μL using a nanodrop and sent to the Georgia Genomics Facility (GGF) (University of Georgia, Athens, GA) for library preparation and sequencing using the Illumina MiSeq platform as 300-bp paired ends reads using a 600 cycle cartridge with a NGS library preparation method. The raw forward and reverse reads of each isolate was observed using FASTQC v.11.2 (Babraham Bioinformatics Institute). Quality control was performed using FASTX-Toolkit v.3.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). All reads with a phred score below $Q = 22$ were removed prior to assembly. ABySS v.1.3.6 (*Simpson et al., 2009*) was used for *de novo* assembly of forward and reverse reads into contiguous sequences (contigs) for each isolate, using an optimal K-mer value of 64 determined with multiple assembly trials. Generated contigs were then imported into Geneious v.6.1.8 (*Kearse et al., 2012*).

Microsatellite discovery and marker development

To increase the potential for successful microsatellite marker development, only contigs 200 bp or greater in size with matched pair reads were considered. Contigs and singletons were searched for at least five perfect repeats of trimeric, tetrameric, pentameric, and hexameric motifs in Geneious v.6.1.8 using Phobos v.3.3.12 (*Kearse et al., 2012*; *Mayer, 2006*). Mono and dinucleotide repeats were eliminated due to the difficulty of scoring allele differences. Contigs with microsatellites identified using our criteria were aligned by multiple sequence alignment using Geneious Align v.6.1.8. default parameters. Microsatellites shared among the three isolates were visually assessed for sequence variation. Those that showed microsatellite repeat number variation among isolates and had at least 50 bp flanking each side of the repeat were considered acceptable for primer design. Primers for amplification of microsatellite loci were designed with Primer3web v.4.0 (*Koressaar & Remm, 2007*; *Rozen & Skaletsky, 1999*; *Untergasser et al., 2012*) to produce amplicons of approximately 180–350 bp in length with an optimal annealing temperature of 59 °C.

Sixty primer pairs for candidate microsatellite loci were initially evaluated on the three sequenced isolates CA11-1, FL11-1, and FL11-2 to verify that the PCR worked with the designed primers and that the PCR amplicons were the expected size. PCR was carried out in 10 μL reactions with 1 μL of 10 × ExTaq buffer (Takara Bio Inc., Mountain View, CA), 1 μL of 2.5 mM dNTPs (Takara Bio Inc.), 0.25 μL of 10 μM forward primer, 0.25 μL of 10 μM reverse primer (Integrated DNA Technologies, Coralville, IA), 0.1 μL of ExTaq polymerase (Takara Bio Inc.), 6.9 μL of sterile distilled H_2O, and 0.5 μL of 50.0 ng/μL DNA template. Reaction conditions were 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension of 72 °C for 5 min. Amplification of PCR products within the expected size range was confirmed by electrophoresis run at 95 V (4.75 V/cm) on a 2% (wt/vol) agarose gel (Alfa Aesar, Haver Hill, MA) for 2.5 h using a 100 bp size standard (New England Biolabs Inc., Ipswich, MA).

Twenty-five primer sets (Table 2) that successfully amplified the three sequenced isolates were screened for polymorphism on a panel of *U. transversalis* that included seven additional isolates from California (Table 1). A three-primer method (*Schuelke, 2000*) was used in this round of marker evaluation. The forward primer for each candidate marker had a CAG tag (5′-CAGTCGGGCGTCATCA-3′) (*Hauswaldt & Glenn, 2003*) added to the 5′ end. The third primer consisted of the CAG tag, labeled with a 6FAM fluorescent dye (Invitrogen Inc., Carlsbad, CA) on the 5′ end. PCR was carried out in 12 μL reactions with 1.2 μL of 10 × ExTaq buffer (Takara Bio Inc., Mountain View, CA), 1.2 μL of 2.5 mM dNTPs (Takara Bio Inc.), 0.1 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer (Integrated DNA Technologies, Coralville, IA), 0.5 μL of 10 μM 5′6FAM-labeled CAG tag primer (Invitrogen Inc.), 0.1 μL of ExTaq polymerase (Takara Bio Inc.), 7.9 μL of sterile distilled H_2O, and 0.5 μL of approximately 50.0 ng/μL DNA template. Reaction conditions were 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension of 72 °C for 5 min. Amplification of individual PCR products within the expected size range was confirmed by electrophoresis.

One microliter of a 1:10 dilution of PCR product was added to 0.1 μL of GeneScan 500 LIZ-labeled size standard and 9.9 μL of Hi-Di formamide (Applied Biosystems Inc., Foster City, CA). Amplicons were denatured by incubation at 95 °C for 5 min and immediately placed on ice. Fragment analysis was conducted at the GGF on an Applied Biosystems 3730xl 96-capillary DNA Analyzer. GeneMapper v.4.0 (Applied Biosystems Inc.) and Geneious v.6.1.8 (*Kearse et al., 2012*) were used to determine allele sizes from the chromatograms.

Multiplex PCR

Eight primer sets (Table 2, see loci with asterisks) that consistently produced fragments within the expected size range for the 10 isolates were optimized for multiplex PCR. Two multiplex reactions (multiplex 1 –*Ut513*, *UtCA759*, *Ut2648* and *Ut3161*; multiplex 2 –*Ut497*, *Ut1841*, *Ut1908* and *Ut2048*) were developed to increase efficiency and decrease cost for genotyping a large panel of isolates. The forward primers of the microsatellite markers selected for multiplex PCR were labeled at the 5′ end with one of the fluorescent

Table 2 Repeat motif, primer sequences, and number of alleles, allele sizes, and genotypes for 25 *U. transversalis* microsatellite markers.

Locus[a]	Repeat motif	Primer sequence (5′→3′)[b]	Number of observed alleles, allele sizes (bp), and genotypes[c]
Ut337	$(AGG)_7$	F: CGGAAGAGATGAGTGGTCAAG	2 (195, 198)
		R: TCACATCATCCCCTCCCTA	
Ut397	$(TTG)_9$	F: TTCGATTCGATTCGTTTGTTT	1 (259)
		R: GGATGTTTTGATTCTGTTAGAGAGTG	
Ut447	$(ACC)_6$	F: TGCTTCAGCTTCCCAAAACT	2 (237, 240)
		R: TGGCTGTGAATTGTGAGACC	
Ut497[*2]	$(GAA)_{15}$	F: CTTGAAGGGGATCGAGAAGA (6FAM)	2 (232, 251)
		R: TGTTCTCCGGCAGAGGTTTA	
Ut513[*1]	$(TCA)_6$	F: TCCCAAACAAATCGTGAAGA (NED)	2 (200, 203)
		R: GCTCCCGTTAATGGTCACAG	
Ut542	$(GTT)_5$	F: GTCTTCTTTGCTGCGTTTCC	2 (204, 207)
		R: TCCTGGTTTTGAACCTCCTG	
Ut568	$(ACC)_6$	F: TCCCATGGGTTTGGTTGC	2 (178, 181)
		R: TCCTTAATCTGGGTTGACATTT	
Ut575	$(TTA)_5$	F: TGACGATCCTAACGAAGGGTA	2 (241, 244)
		R: CTTGGGGTACGAGAGCACTT	
Ut697	$(AAG)_5$	F: TAGGCGAAGTGGTACGAGGT	1 (224)
		R: AGGGAAGAAGAGGGTCAACA	
Ut752	$(ATC)_6$	F: AGTCTTGTGCTGGTCTTCGTC	2 (213, 216)
		R: TTTGCCGCCTTATATTGTCA	
Ut844	$(ACT)_8$	F: CTCCGTCAGCCAGTCAGTC	1 (310)
		R: GATGAGGTTGAGGGCGAGTA	
Ut981	$(TGA)_6$	F: GGGTCAAACAGGTCTTCTGG	1 (202)
		R: CTACTGAAATGGGCCACAAA	
Ut1272	$(AAG)_5$	F: TGAAGTTTTCCACCCTGGTT	2 (253, 256)
		R: ATCTTGGGCAAACTGACCAC	
Ut1289	$(GAG)_7$	F: GGTCTTGAGAGAACGGAGGA	2 (254, 257)
		R: CTCTTCCAGATACCCCACCA	
Ut1841[*2]	$(AGG)_5$	F: GAACCCTGCCTCACACCTTA (NED)	2 (345, 348)
		R: GCGGCTACCAGAGCTTTAGA	
Ut1908[*2]	$(GAT)_6$	F: TCCTCTCAGCCAATCCAATC (PET)	2 (200, 203)
		R: CTCTTGCCCATCAATCCAAC	
Ut2035	$(TTTA)_8$	F: GGATCGAGTCGGTCGATTTA	2 (229, 232)
		R: GCCGAACAGGACTAGCATTG	
Ut2048[*2]	$(GAA)_6$	F: CGAGCGATAAATTTTTGAACA (VIC)	2 (182, 185)
		R: TGTCCGGAGAATGTGAACTG	
Ut2443	$(GAA)_8$	F: AGAATTGGATGAAACAGGGAGA	1 (188)
		R: AAGGAGGAAGCCATCACTCA	
Ut2536	$(GAG)_5$	F: AGGGCTGGTAGACGTGACTG	2 (248, 251)
		R: TCATGTCTCTGACACCACCA	

(continued on next page)

Table 2 (*continued*)

Locus[a]	Repeat motif	Primer sequence (5′→3′)[b]	Number of observed alleles, allele sizes (bp), and genotypes[c]
Ut2648[*1]	$(CAG)_6$	F: GAACTGGTGCAACCGATACA (VIC)	2 (266, 269)
		R: CACAGCCTTGGCTCTTGAGT	
Ut3161[*1]	$(TCC)_6$	F: GAGTCTGGCCCAGCTGTTT (6FAM)	2 (192, 195)
		R: TCTGATCTTGCAGGGGATTC	
UtCA759[*1]	$(CAT)_7$	F: GATGGCCAGAAGAAAGATGC (PET)	1 (296)
		R: TTAACCAGCGCGAGAGTCTT	
UtCA809	$(TTA)_7$	F: GCCACTTCTCCAAACGCTTA	1 (258)
		R: TCGCAAGATCAAGAAACAACC	
UtCA950	$(GTT)_9$	F: GGCAGAGGATGAGTCGTGTA	2 (272, 287)
		R: TCATCTCATCCCCACAATCA	

Notes.

[a]Asterisks indicate loci that were used for the multiplex reactions and the 1 or 2 indicate multiplex 1 or 2, respectively.

[b]The fluorescent dye used for multiplex reactions is listed in parentheses to the right of the forward primer.

[c]Genotype of all 10 isolates from the United States. Allele sizes are listed based on the results of the multiplex reactions or what the length of the alleles would be without the 16 nucleotide CAGTAG.

dyes from the DS-33 dye set: 6-FAM (Integrated DNA Technologies), VIC, PET, or NED (Applied Biosystems Inc.). Multiplex reactions were optimized so that loci with alleles of similar size ranges were labeled with different dyes. All 92 samples were genotyped with the eight markers in the multiplex reactions.

Multiplex PCR was conducted using a modified protocol of the Type-it Microsatellite PCR kit (Qiagen, Hilden, Germany) in 10 μL reactions with 5 μL of 2 × Type-it Master Mix buffer, 1 μL of 10 × primer mix (2 μM of each primer in the multiplex), 3 μL of sterile distilled H_2O, and 1 μL of approximately 50.0 ng/μL DNA template. Reaction conditions were 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension of 72 °C for 5 min. Amplification of PCR products within the expected size range was confirmed by electrophoresis. The PCR products were prepared as described above and sent to GGF for fragment analysis. Geneious v.6.1.8 (*Kearse et al., 2012*) was used to determine allele sizes from the chromatograms. Loci were distinguished by fluorescent dye. Only peaks above the relative intensity cutoff threshold of 500 relative fluorescence units (RFU) were scored.

RESULTS

Whole genome sequencing and assembly

The Illumina MiSeq PE 300 sequencing platform generated 32,461,282 reads with an average insert size of 575 bp and read lengths of 301 bp. Sequence quality was assessed by phred score and signal purity filter values resulting in a total of 25,452,493 reads with a P F of 99.26%, which corresponds to 6.02 to 9.98 million reads for CA11-1, FL11-1, and FL11-2 (Table 3).

The *de novo* draft assemblies resulted in 5,706,372 (94.7% assembled out of the total filtered reads), 4,305,978 (43.2%), and 7,444,849 (80.4%) total assembled reads for CA11, FL11-1, and FL11-2, respectively. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accessions PTJR00000000, PTJQ00000000,

Table 3   Genome assembly and microsatellite statistics.

Isolates	Total reads	Reads after quality filtering[a]	Reads that assembled	# Contigs > 200 bp	Contigs w/ microsatellites[b]	% Microsatellites per assembly
CA11-1	7,762,942	6,023,634	5,706,372	466,181	4,599	0.98%
FL11-1	12,804,893	9,976,981	4,305,978	548,017	5,685	1.03%
FL11-2	11,893,445	9,262,312	7,444,849	645,533	8,666	1.34%

Notes.

[a]Based upon purity filter value of 99.26%.

[b]Contigs with identified microsatellites based on the annotation criteria: repeat unit length = min: 3 max: 6, min. length of 15. Mono and dinucleotide repeats not considered due to the difficulty of scoring alleles during fragment analysis.

and PTJP00000000 for CA11, FL11-1, and FL11-2, respectively. Using Geneious v.6.1.8 (*Kearse et al., 2012*), contigs for each isolate were filtered to select only those 200 bp in length or greater. This resulted in 466,181, 548,017, and 645,533 contigs for CA11, FL11-1, and FL11-2, respectively, which were subsequently used to search for microsatellite repeats (Table 3).

Microsatellite marker development

An alignment of the 18,950 contigs produced 4,296 contigs with potentially informative microsatellite loci shared among the three isolates. Microsatellite loci shared by at least two of the three isolates were observed in 2,754 of the aligned contigs. Microsatellites were identified in 0.98%, 1.03%, and 1.34% of the contigs, showing that the discovery rate of microsatellites was consistent among sequenced isolates.

Sixty sets of primers were developed and screened by PCR on the three isolates of *U. transversalis*. Of the 60 putative markers, 25 were successfully amplified by PCR and evaluated for polymorphism on the panel of ten isolates from the United States (Tables 1, 2). To determine if contigs used for marker development were sequences of *U. transversalis* and not contaminant sequence, we used blastn at NCBI (*Altschul et al., 1990*) to identify the sequences in the database that were most similar with high coverage. Most often the top hits were *Puccinia graminis*, another rust species, or another fungus, with percent identity always <90%. Sometimes the top hit was another eukaryote, but it was an unlikely contaminant, such as mouse, sheep or fish. The percent identity for these was always <90% and the e-values were usually high (>0.01) or the query coverage was low (<10%). We concluded that the contigs selected for marker development were sequences of *U. transversalis* and not contaminants. Isolate CA14-7 consistently produced peaks below our cutoff of 500 RFU for reliable data; however, there were fragments at the expected allele size ranges. It is possible that there were PCR inhibitors in the DNA extract or that the DNA was of lower quality than the other samples. Of the 25 markers, seven were monomorphic, with only one allele each, and 18 were polymorphic, with two alleles each (Table 2). All 10 isolates from the United States were the same genotype based on the 25 markers. The polymorphism was identified among alleles within each locus, rather than among individuals. Overall, the microsatellite markers showed allelic diversity, but no genotypic diversity among the isolates from the United States. There was a high heterozygosity within individuals with each isolate having both alleles for the polymorphic loci (Table 2).

Genotypic analyses

Eight primer sets that consistently produced fragments within the expected size range for the 10 isolates were optimized for multiplex PCR. We selected markers that were polymorphic and did not produce alleles in overlapping size ranges. Unfortunately, after subsequent genotyping runs one of the markers *UtCA759* was determined to be monomorphic. Even though the four markers within each multiplex were run with different fluorescent dyes, we minimized size overlap to prevent pull-up effects or bleed through from the fragments run with the other dyes. When using the eight microsatellite markers in two multiplex reactions, samples from the USA, Costa Rica and Mexico consistently produced PCR products of the expected size. Samples from New Zealand and Australia produced inconsistently sized PCR products despite duplicate reactions. Using the same eight markers, we attempted to genotype 16 leaf samples from South Africa, but these repeatedly failed to produce PCR products. Only one isolate, PREM 57128, which was sampled in 1998 produced a faint PCR product; however, no fragments were detected in the analysis.

Fragment analysis showed allelic variation within individuals, but no genotypic variation was observed among the isolates from Australia, Costa Rica, Mexico, New Zealand, and the USA. In all cases where fragments were observed and were above the relative intensity cutoff of 500 RFU, the genotypes were identical to each other and to all isolates from the USA (Table 2). In some cases where the peaks were below the relative intensity cutoff of 500 RFU, there was a fragment for only one of the alleles or the alleles were a slightly different size; however, these results were not reliable (Table S1). Marker *Ut497* consistently failed to produce fragments or peaks above the relative intensity cutoff for almost all isolates where DNA was obtained from preserved leaf tissue. The six remaining markers (*Ut513*, *Ut1841*, *Ut1908*, *Ut2048*, *Ut2648* and *Ut3161*) were polymorphic with only two allele sizes observed for each marker, while one marker (*UtCA759*) was monomorphic, producing only one allele size.

Sequence divergence

Visual assessment of the aligned contigs for the three sequenced isolates revealed that there were two distinct haplotypes (nucleotide sequence patterns) for each isolate occurring at nearly all microsatellite loci (Table 4, Fig. 2). The variation in repeat number occurred between alleles or haplotypes of the same isolate. Additionally, there were numerous single nucleotide polymorphisms (SNPs) detected in the regions flanking the microsatellite repeats. The two haplotypes within each of the three isolates sequenced were identical to the haplotypes among all three isolates for the loci compared, including microsatellite loci that were monomorphic based on sequence length. Variation in the microsatellite-flanking sequences between haplotypes was estimated for loci with complete data sets for the three isolates. The two alleles differed in nucleotide sequence by 1.6% to 6.9% (Table 4).

DISCUSSION

There was no genotypic diversity observed among the *U. transversalis* isolates from Australia, Costa Rica, New Zealand, Mexico, and the USA based on the eight microsatellite loci developed in the present study. However, there was missing data for most of the

Table 4 Variation between alleles within sequenced genomes of *U. transversalis*.

Microsatellite	No. Single nucleotide polymorphisms	No. Nucleotides in flanking regions	% Difference
Ut337	3	180	1.7
Ut513	7	291	2.4
Ut568	13	187	6.9
Ut575	3	187	1.6
Ut752	9	263	3.4
Ut1908	10	264	3.8

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CA11-1   CAAGACAATCAAAGTTCTAAGCAACCCGGGCCGCTAGGAAACTTGGGGCAAATTTCTTAAATATCTCCACCTACCTATGCATCAGCTGTTTCCAGAATAGCCCC

FL11-1   CAAGACAATCAAAGTTCTAAGCAACCCGGGCCGCTAGGAAACTTGGGGCAAATTTCTTAAATATCTCCACCTACCTATGCATCAGCTGTTTCCAGAATAGCCCC

FL11-2   CAAGACAATCAAAGTTCTAAGCAACCCGGGCCGCTAGGAAACTTGGGGCAAATTTCTTAAATATCTCCACCTACCTATGCATCAGCTGTTTCCAGAATAGCCCC

CA11-1   CAAGACAGTCAAAGTTGAAAGCAACCCGGGCCGCTAGGAAACATGGGGCAAATTTCTTAAATATCTCCACCTACCTATGCATCACCTGTTTCCAGAATAGCTCC

FL11-1   CAAGACAGTCAAAGTTGAAAGCAACCCGGGCCGCTAGGAAACATGGGGCAAATTTCTTAAATATCTCCACCTACCTATGCATCACCTGTTTCCAGAATAGCTCC

FL11-2   CAAGACAGTCAAAGTTGAAAGCAACCCGGGCCGCTAGGAAACATGGGGCAAATTTCTTAAATATCTCCACCTACCTATGCATCACCTGTTTCCAGAATAGCTCC

Coord.(bp)   235                              285                                                              338
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Figure 2 Comparison of partial sequences of the locus *Ut789* showing two distinct alleles for each of the three sequenced isolates. While the image represents a single locus, a similar pattern was observed for the sequences of most microsatellite flanking regions. The sequences shown correspond with GenBank accession numbers PTJR01079144.1, PTJQ01464835.1, and PTJP01109568.1 for CA11-1, FL11-1, FL11-2, respectively, for the top genomes, and accession numbers PTJR01079145.1, PTJQ01463031.1, and PTJP01109569.1, for CA11-1, FL11-1, FL11-2, respectively, for the bottom genomes of the dikaryon. The coordinates are based on accession number PTJR01079144.1.

Full-size 🖼 DOI: 10.7717/peerj.7986/fig-2

isolates from New Zealand and Australia, which could either be due to poor amplification of the low-quality DNA obtained from these dried herbarium samples or null alleles from poor primer binding as a result of genetic differences in these populations that could be biologically meaningful. For the ten isolates from the USA genotyped with all 25 microsatellite loci, eight of the markers were monomorphic and 16 were polymorphic, with all polymorphism observed within each of the isolates. Additionally, we genotyped some of the other isolates (five isolates from Costa Rica, one isolate from New Zealand, and three isolates from Mexico) at all 25 loci (Table 2) and still found no genotypic diversity. Since no genetic differences were observed among isolates with complete or nearly complete data, it is not possible to track individuals or introductions of individuals from other populations that may have occurred (*McDonald & Linde, 2002*; *Milgroom & Peever, 2003*). It may be possible to detect genotypic diversity within *U. transversalis* using different genotyping methods, such as whole genome sequencing (WGS) or genotyping-by-sequencing (GBS) for SNP detection; however, the large genome size of rust fungi or high heterozygosity present within individuals may obscure the detection of genotypic diversity among individuals (*Elshire et al., 2011*). Additionally, levels of genetic diversity and patterns of population genetic structure detected with microsatellite markers and SNPs derived from GBS have been found to be in agreement, although greater resolution of genotypes is usually possible

with the increased number of SNP loci generated by WGS or GBS, or similar methods such as RAD-seq (*Rafiei et al., 2018*; *Maurice et al., 2019*). A different member of the Pucciniales, *Phakopsora pachyrhizi*, for which only clonal reproduction has been observed, has some genotypic diversity both in the USA where it has been introduced and in Asia, where it is native (*Zhang et al., 2012*). Nonetheless, the microsatellite markers developed for *U. transversalis* may be useful for diagnostic purposes or detection of *U. transversalis* in asymptomatic plant material.

In this study, all of the genetic diversity identified in *U. transversalis* occurred as allelic diversity within individuals. Most of the microsatellite markers and all of the repeat-flanking sequence that were compared showed two alleles or two distinct haplotypes at each locus, indicative of very high heterozygosity. It is not entirely surprising that loci were polymorphic, since this fungus is a dikaryon, but it was surprising that all genotyped individuals shared the same genotype with fixed differences. Additionally, one would expect some of the loci to be homozygous following Hardy-Weinberg equilibrium in some individuals if the population were undergoing sexual reproduction. This level of allelic variation within each individual is suggestive of divergent genomes between the nuclei of the dikaryon across the invasive population. The flanking sequences from each locus showed approximately 97% sequence similarity (Table 4). In some cases, the two genomes of the dikaryon are more divergent than what is usually observed within a single fungal species (*Hibbett, 2016*). The lack of genotypic diversity among isolates and the distinct sequences and microsatellite alleles within individuals suggests that *U. transversalis* samples from Costa Rica, Mexico, and the USA are asexually reproducing populations that are not recombining through sexual reproduction (*Milgroom, 1996*). Clonal invasions are common among plants pathogens (*Milgroom et al., 2009*; *Goss, Carbone & Grünwald, 2009*). In dikaryotic organisms and diploids, the absence of sexual reproduction will increase the divergence between sequences in each genome as random mutations will occur over time (*Birky, 1996*). Thus, low genotypic diversity combined with high allelic diversity within individuals is suggestive of strict clonal reproduction for an extensive period of time (*Balloux, Lehmann & De Meeûs, 2003*; *Birky, 1996*). Our results provide support for clonal reproduction of *U. transversalis* in the USA, Mexico, and Costa Rica, which is consistent with the observed research on reproductive biology of *U. transversalis*.

To our knowledge this is the first study on the genetic diversity of *U. transversalis*. Since *U. transversalis* urediniospores are dikaryotic, development of codominant, sequence-specific microsatellite markers would be appropriate in addressing our questions of genetic diversity, origin, and sources of introductions for this rust fungus. Traditionally, microsatellite development required the construction of a genomic library enriched for repeated motifs, isolation, and sequencing clones; primer design and optimization; and testing for polymorphism on a few unrelated individuals (*Abdelkrim et al., 2009*; *Frenkel et al., 2012*; *Santana et al., 2009*; *Zhang et al., 2012*). As an alternate approach, the microsatellite markers in the present study were developed using whole genome sequencing of multiple isolates. This approach not only increased our chances of identifying polymorphic alleles within *U. transversalis*, but also supplied genomic sequence, which could be used for comparative analyses or other purposes. However, the main focus of this study was not

the whole genome sequencing and assembly, but to use these data to develop markers. Although useful for marker development, the three draft genomes were highly fragmented (4.3–7.4 million assembled reads or contigs compared to 6.0–9.9 million unassembled), which could be the result of repetitive DNA and a large genome, which are common among rust fungi (*Ramos et al., 2015*; *Tavares et al., 2014*).

Although we were unable to detect genetic diversity among isolates of *U. transversalis* across a wide geographic range, the genome sequences will serve as a resource for further studies on this destructive fungal pathogen of Gladiolus. Since all isolates sampled exhibited limited diversity and were genetically similar, this demonstrates that disease management strategies both current and future, should work for all locations and current hosts for *Uromyces transversalis*. However, there could be undetected variation in regions of the genome associated with virulence, host specificity, or fungicide resistance that are not linked to the microsatellite loci studied here. Future studies will help us to determine the usefulness of the microsatellite markers in diagnosis and detection.

CONCLUSIONS

There was no genotypic diversity observed among the invasive *U. transversalis* populations from Australia, Costa Rica, New Zealand, Mexico, and the USA based on the eight microsatellite loci developed in the present study. However, there was missing data for most of the isolates from New Zealand and Australia, which could either be due to poor amplification of the low-quality DNA obtained from these dried herbarium samples or null alleles from poor primer binding as a result of genetic differences in these populations that could be biologically meaningful. The lack of genotypic diversity among isolates from North America and the distinct sequences and microsatellite alleles within individuals suggests that *U. transversalis* samples in introduced ranges are asexually reproducing populations that are not recombining through sexual reproduction. Our results provide support for clonal reproduction of *U. transversalis* in the USA, Mexico, and Costa Rica, which is consistent with the observed research on reproductive biology of *U. transversalis* and other invasive plant pathogens.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Jeffery A. DeLong conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Jane E. Stewart conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Alberto Valencia-Botín performed the experiments, contributed reagents/materials/-analysis tools, prepared figures and/or tables, approved the final draft.
- Kerry F. Pedley conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- James W. Buck and Marin T. Brewer conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

This Whole Genome Shotgun project is available at DDBJ/ENA/GenBank: PTJR00000000, PTJQ00000000, and PTJP00000000 for CA11, FL11-1, and FL11-2.

Data Availability

The following information was supplied regarding data availability:

The raw genotype data is available in Table S1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7986#supplemental-information.

REFERENCES

Abdelkrim J, Robertson BC, Stanton JAL, Gemmell NJ. 2009. Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. *Biotechniques* **46**:185–191 DOI 10.2144/000113084.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**:403–410 DOI 10.1016/S0022-2836(05)80360-2.

Balloux F, Lehmann L, De Meeûs T. 2003. The population genetics of clonal and partially clonal diploids. *Genetics* **164**:1635–1644.

Beilharz V, Parbery DG, Pascoe IG. 2001. Gladiolus rust (caused by *Uromyces transversalis*) in eastern Australia. Australas. *Plant Pathology* **30**:267–270.

Birky CW. 1996. Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. *Genetics* **144**:427–437.

Blomquist CL, Thomas SL, McKemy JM, Nolan PA, Luque-Williams M. 2007. First report of *Uromyces transversalis*, causal agent of Gladiolus rust, in San Diego County, California. *Plant Disease* **91**:1202–1202.

Brown L. 2005. *Uromyces transversalis* Assessment of the Risk of Introduction Recommendations for risk mitigation for *Gladiolus* spp. Cut Flowers and Propagative Material from México. online USDA Plant Protection and Quarantine.

Cherry E, Peet CE. 1966. An efficient device for the rapid collection of fungal spores from infected plants. *Phytopathology* **56**:1102–1103.

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLOS ONE* **6**:e19379 DOI 10.1371/journal.pone.0019379.

Ferreira JF, Nevill WG. 1989. Evaluation of bitertanol and triadimefon for the control of Gladiolus rust caused by *Uromyces transversalis*. *Plant Disease* **73**:987–990 DOI 10.1094/PD-73-0987.

Frenkel O, Portillo I, Brewer MT, Peros JP, Cadle-Davidson L, Milgroom MG. 2012. Development of microsatellite markers from the transcriptome of *Erysiphe necator* for analysing population structure in North America and Europe. *Plant Pathology* **61**:106–119 DOI 10.1111/j.1365-3059.2011.02502.x.

Garibaldi A, Aloj B. 1980. Observations on biology and control of *Uromyces transversalis* (Thum.) Winter on Gladiolus in southern Italy. *International Symposium on Flower Bulbs* **109**:409–414.

Goss EM, Carbone I, Grünwald NJ. 2009. Ancient isolation and independent evolution of the three clonal lineages of the exotic sudden oak death pathogen *Phytophthora ramorum*. *Molecular Ecology* **18**:1161–1174 DOI 10.1111/j.1365-294X.2009.04089.x.

Hauswaldt JS, Glenn TC. 2003. Microsatellite DNA loci from the diamondback terrapin (*Malaclemys terrapin*). *Molecular Ecology Notes* **3**:174–176 DOI 10.1046/j.1471-8286.2003.00388.x.

Hernández J. 2004. Systematic Mycology and Microbiology Laboratory, ARS, USDA. Invasive Fungi: Gladiolus Rust. (accessed on 21 April 2009).

Hibbett D. 2016. The invisible dimension of fungal diversity. *Science* **351**:1150–1151 DOI 10.1126/science.aae0380.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious basic: an integrated and extendable desktop software platform

for the organization and analysis of sequence data. *Bioinformatics* **28**:1647–1649 DOI 10.1093/bioinformatics/bts199.

Koressaar T, Remm M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* **23**:1289–1291 DOI 10.1093/bioinformatics/btm091.

Leclercq S, Rivals E, Jarne P. 2007. Detecting microsatellites within genomes: significant variation among algorithms. *BMC Bioinformatics* **8**:125 DOI 10.1186/1471-2105-8-125.

Lee SB, Milgroom MG, Taylor J. 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genetics Newsletter* **35**:23–24 DOI 10.4148/1941-4765.1531.

Lindquist J, Alippi H, Medera C. 1979. A serious epiphytotic of Gladiolus rust (*Uromyces gladioli*) in Santa Fe province (Republic of Argentina). *III Jornada Fitosanitaria Argentina* **2**:717–724.

Littlejohn G, Blomerus L. 1997. Studies on Gladiolus resistance to transverse rust. *Acta Horticulturae* **430**:509–516.

Maurice S, Montes MS, Nielsen BJ, Bødker L, Martin MD, Jønck CG, Kjøller R, Rosendahl S. 2019. Population genomics of an outbreak of the potato late blight pathogen, *Phytophthora infestans* reveals both clonality and high genotypic diversity. *Molecular Plant Pathology* **20(8)**:1134–1146.

Mayer C. 2006. Phobos. *Available at http://www.rub.de/spezzoo/cm/cm/_phobos.htm.*

McDonald BA, Linde C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* **40**:349–379 DOI 10.1146/annurev.phyto.40.120501.101443.

McKenzie E. 2000. *Uromyces transversalis*, rust fungus found infecting Iridaceae in New Zealand. *New Zealand Journal of Crop and Horticultural Science* **28**:289–291 DOI 10.1080/01140671.1997.9514151.

Milgroom MG. 1996. Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**:457–477 DOI 10.1146/annurev.phyto.34.1.457.

Milgroom MG, Peever TL. 2003. Population biology of plant pathogens: the synthesis of plant disease epidemiology and population genetics. *Plant Disease* **87**:608–617 DOI 10.1094/PDIS.2003.87.6.608.

Milgroom MG, Sotirovski K, Risteski M, Brewer MT. 2009. Heterokaryons and parasexual recombinants of *Cryphonectria parasitica* in two clonal populations in southeastern Europe. *Fungal Genetics and Biology* **46**:849–854 DOI 10.1016/j.fgb.2009.07.007.

Mohali RS, Aime MC. 2018. First report of Gladiolus rust caused by Uromyces transversalis in Merida, Venezuela. *Plant Disease* **102**:444–445.

Martínez-de la Parte E, Pérez-Vicente L, Cantillo-Pérez T, Guerrero-Barriel D, Ramos A. 2011. First report of Gladiolus rust caused by Uromyces transversalis in Cuba. *New Disease Reports* **23**:20 DOI 10.5197/j.2044-0588.2011.023.020.

Peterson GL, Berner DK. 2009. Effects of temperature and humidity on the survival of urediniospores of Gladiolus rust (*Uromyces transversalis*). *European Journal of Plant Pathology* **125**:509–513 DOI 10.1007/s10658-009-9492-5.

Pita G, Figueiredo M, Cardoso R, Hennen J. 1981. Ferrugem (*Uromyces transversalis* (Thuemen) Winter, uma nova doenca do gladiolo (*Gladiolus* spp). no Brasil). *Biologico* **47**:323–328.

Rafiei V, Banihashemi Z, Jiménez-Díaz RM, Navas-Cortés JA, Landa BB, Jiménez-Gasco MM, Turgeon BG, Milgroom MG. 2018. Comparison of genotyping by sequencing and microsatellite markers for unravelling population structure in the clonal fungus *Verticillium dahliae*. *Plant Pathology* **67**:76–86 DOI 10.1111/ppa.12713.

Ramos AP, Tavares S, Tavares D, Silva MDC, Loureiro J, Talhinhas P. 2015. Flow cytometry reveals that the rust fungus, Uromyces Bidentis (Pucciniales), posseses the largest fungal genome reported - 2489Mbp. *Molecular Plant Pathology* **16**:1006–1010 DOI 10.1111/mpp.12255.

Rizvi A, Man-Son-Hing A, Jackson A, Parra G, Schartzburg K, Brown L, Sullivan M, Duffie L, Kosta K, Clark R. 2007. Gladiolus rust (*Uromyces transversalis*): a national management plan for exclusion and eradication. USDA APHIS PPQ. (accessed on 10 February 2009).

Rodgers SO, Bendich AJ. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* **5(2)**:69–76 DOI 10.1007/BF00020088.

Rodríguez-Alvarado G, Fernández-Pavía S, Valenzuela-Vázquez M, Loya-Ramírez J. 2006. First report of Gladiolus rust caused by *Uromyces transversalis* in Michoacán, México. *Plant Disease* **90**:687–687.

Rozen S, Skaletsky H. 1999. Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols* **132**:365–386 DOI 10.1385/1-59259-192-2:365.

Santana QC, Coetzee MP, Steenkamp ET, Mlonyeni OX, Hammond GN, Wingfield MJ, Wingfield BD. 2009. Microsatellite discovery by deep sequencing of enriched genomic libraries. *Biotechniques* **46**:217–223 DOI 10.2144/000113085.

Schubert TS, Leahy RM, Davison DA, Silagyi AJ, Killgore EM. 2007. Gladiolus rust caused by *Uromyces transversalis* makes first nearctic appearance in Florida. *Plant Disease* **91**:1202.

Schuelke M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* **18**:233–234 DOI 10.1038/72708.

Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I. 2009. ABySS: a parallel assembler for short read sequence data. *Genome Research* **19**:1117–1123 DOI 10.1101/gr.089532.108.

Tavares S, Ramos AP, Pires AS, Azinheira HG, Caldeirinha P, Link T, Abranches R, Silva MC, Voegele RT, Loureiro J, Talhinhas P. 2014. Genome size analyses of Pucciniales reveal the largest fungal genomes. *Frontiers in Plant Science* **5**:422 DOI 10.3389/fpls.2014.00422.

Tervet I, Rawson A, Cherry E, Saxon R. 1951. A method for the collection of microscopic particles. *Phytopathology* **41**:282–285.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Research* **40**:e115 DOI 10.1093/nar/gks596.

US Department of Agriculture APHIS. 2015. *Available at https://www.aphis.usda.gov/plant_health/plant_pest_info/gladiolus_rust/downloads/DA-2015-20.pdf* .

Valencia-Botin A, Jeffers S, Palmer C, Buck JW. 2013. Fungicides used alone, in combinations, and in rotations for managing Gladiolus rust in Mexico. *Plant Disease* **97**:1491–1496 DOI 10.1094/PDIS-03-13-0272-RE.

Wise K, Mueller D, Buck JW. 2004. *Quarantines and ornamental rusts. Online publication.* Saint Paul: APSnet Features DOI 10.1094/APSnetFeature-2004-0204.

Zhang X, Freire M, Le M, Hartman GL, Upchurch RG, Pedley KF, Stacey G. 2012. Genetic diversity and origins of *Phakopsora pachyrhizi* isolates in the United States. *Asian Journal of Plant Pathology* **6(3)**:52–65 DOI 10.3923/ajppaj.2012.52.65.

Zhang Y, Zhang S, Liu X, Wen H, Wang M. 2010. A simple method of genomic DNA extraction suitable for analysis of bulk fungal strains. *Letters in Applied Microbiology* **51**:114–118.