MINING THE *UROMYCES TRANSVERSALIS* GENOME FOR MOLECULAR MARKERS AND INVESTIGATING GENETIC DIVERSITY OF THE QUARANTINE-SIGNIFICANT GLADIOLUS RUST FUNGUS

by

Jeffery A. DeLong

(Under the Direction of James Buck and Marin Brewer)

ABSTRACT

Uromyces transversalis, the causal agent of Gladiolus rust, is quarantined in the EU and US because it can be devastating to *Gladiolus* spp. The aim of this research was to develop molecular markers to: (i) determine the origin of introductions of *U. transversalis* to the US, (ii) track the movement of genotypes, and (iii) understand the worldwide genetic diversity of the species. Whole genome sequencing was performed on three isolates collected in the US. Genomes were assembled *de novo* and searched for microsatellite regions. Primers were developed and tested on ten isolates from the US resulting in the identification of 24 robust polymorphic markers. Among 108 isolates collected, markers revealed polymorphism within each isolate with no diversity among isolates. The microsatellite loci and flanking regions showed high diversity and two divergent genomes within dikaryotic individuals, yet no diversity among individuals, suggesting that *U. transversalis* populations worldwide are strictly clonal.

INDEX WORDS: Gladiolus rust; *Uromyces transversalis*; Whole Genome Sequencing; Microsatellites; Genetic Diversity; Clonal Population

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DEDICATIONS

To my wife Elizabeth, seven billion plus smiles on this earth and I love yours the most. I cannot thank you enough for your selfless love, understanding and support all along the way. Walk with me my friend…

To my mother and family, who have always believed and encouraged me, even when I didn't believe in myself. Thank you.

To all my friends, who know the sacrifices I've had to make, so that I may complete this journey.

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JUSTIFICATION

The popularity of floriculture is steadily increasing across the globe, with many developing countries regarding it as a high value, export-oriented economic industry, and in some countries the economic impact exceeds traditional fruit and vegetable production (Mushtaq et al. 2013). The global ornamental plant trade for residential and commercial landscaping and cut flowers increased by $783 million from 2007 to 2011 (United Nations Comtrade Database 2014). Among the many produced cut flower species, *Gladiolus* species are a large contributor to this economic increase, due to its aesthetic beauty and use in floral arrangements. As such, it is commonly referred to as "queen of the bulbous plants". Many fresh cut flower markets in the United States seek imported flowers from foreign growers (Bonarriva et al. 2003); however, domestic production of *Gladiolus* species in the United States is well established with an estimated worth of $25.14 million (2014 Census of Horticultural Specialties, U.S. Department of Agriculture National Agricultural Statistics Services) making this an economically important floriculture crop. The majority of in-field *Gladiolus* cultivation in the United States occurs in California and Florida (U.S. Department of Agriculture National Agricultural Statistics Services 2014; Wise et al. 2004).

Gladiolus plants are susceptible to a destructive foliar rust disease caused by *Uromyces transversalis* (Martínez-de la Parte et al. 2011; Peterson and Berner 2009; Schubert et al. 2007; Valencia-Botin et al. 2013). *U. transversalis* is a biotrophic pathogen that has been shown to infect a narrow range of hosts within the family Iridaceae in arid, mediterranean, and tropical climates (Garibaldi and Aloj 1980; Hernández 2004; Peterson and Berner 2009). This fungal pathogen can be damaging on commercially grown *Gladiolus* species and cultivars, as well as

other members of the family Iridaceae, including species from *Crocosmia, Freesia, Tritonia*, and *Watsonia* (Beilharz et al. 2001; Peterson and Berner 2009). The disease reduces the aesthetic quality of the leaves and compromises the ability for photosynthesis, subsequently decreasing yield of *Gladiolus* in the field, and increasing the production costs associated with manual defoliation (Chase 1987). Due to the rapidly destructive epidemic nature of this fungus, *U. transversalis* is considered a pathogen of quarantine significance in the United States and Europe.

Despite present and historical epidemics caused by *U. transversalis*, little is known about its genetic diversity, center of origin, or dispersal mechanisms. Gaining insight into the genetic diversity of *U. transversalis* will advance our understanding of the epidemiology of Gladiolus rust, determine from where introductions of this pathogen into the United States originate, and assist in developing more efficient management strategies. The pathogen has been confirmed in many countries on multiple continents, and recently was observed in California and Florida (Brown 2005; Valencia-Botin et al. 2013). Reproduction is thought to be strictly asexual, where all spores produced are identical to the progenitor. Asexual reproduction readily occurs through urediniospore production during epidemics. Previously, only microscopic identification of the spore dimensions and morphological features have been used for confirmation of this pathogen (Hernández 2004; Rodríguez-Alvarado et al. 2006). To date, no alternate host has been identified, and only urediniospores and teliospores have been observed to occur naturally (Hernández 2004). Gaining insight into the worldwide genetic diversity of *U. transversalis* will advance our understanding of the epidemiology of Gladiolus rust, including whether sexual reproduction occurs, producing new combinations of traits, and if an alternate host is likely to exist.

The pathogen was first identified in sub-Saharan Africa (Beilharz et al. 2001; Hernández 2004) and assumed to have originated from this region. By defining the center of origin and identifying existing genetic diversity existing throughout geographic locations it is possible to reveal migration patterns and dispersal mechanisms of this pathogen. Currently, the level of genetic variation that occurs within and among populations of *U. transversalis* is unknown. Investigating and defining the global population structure will elucidate if any populations are sexually reproducing, introduced populations come from single sources, population bottlenecks are occurring, or if populations are expanding.

The purpose of this research was to develop molecular markers for the rust fungus *U. transversalis* to determine the origin of introductions to the United States, track the movement of genotypes within the United States, and understand the worldwide genetic diversity. By developing these markers to examine dispersal mechanisms of this pathogen and track introductions, an insight will be gained as to the center of origin of the pathogen, its lifecycle and worldwide genetic diversity and advance a better understanding of its biology, which may contribute to effective management strategies.

Research Objectives

In order to understand the structure and genetic diversity of *Uromyces transversalis* populations, three research objectives were established:

1) Develop microsatellite markers to genotype isolates of *U. transversalis*
2) Determine the geographic origin and track the movement of introduced genotypes of *U. transversalis* in the Unites States
3) Understand the worldwide genetic diversity of *U. transversalis* in order to elucidate centers of diversity and dispersal patterns

CHAPTER 1 – LITERATURE REVIEW

Gladiolus and Gladiolus Rust

Gladiolus is one of the largest genera within the family Iridaceae, with approximately 265 species native to sub-Saharan Africa (Goldblatt and Manning 2008; Rizvi et al. 2007). It is often known for its perennial flowering plants that grow from corms. *Gladiolus* hybrids (species and cultivars of *Gladiolus L.*), commonly referred to as sword lilies (Goldblatt and Manning 2008), serve as the major economic host for *Uromyces transversalis*. Other minor hosts include: *Crocosima aurea, Melasphaerula ramosa, Tritonia lineata, Tritonia securigera, Tritonia squalida, Watsonia angusta, Watsonia densiflora,* and *Watsonia meriana* (Beilharz et al. 2001; Farr and Rossman 2006; McKenzie 2000; Schubert et al. 2007). *Gladiolus* spp. flourish in sub-tropical areas with hot and sunny climates, but also grow in arid and temperate climate zones. Originating in Africa and naturally distributed throughout varying climatic ranges, the endemic species distribution of *Gladiolus* was described as follows: southern Africa – 170 species, tropical Africa – 82 spp., Eurasia – 12 spp., and Madagascar – 8 spp. (Goldblatt et al. 2014).

The predominant income from *Gladiolus* production is from cut flowers, as well as ornamental plantings in commercial and residential landscapes. The major *Gladiolus* producing countries are: Australia, Brazil, Bulgaria, France, Holland, India, Israel, Italy, and the United States (Mushtaq et al. 2013). The Mexican farm gate value for *Gladiolus* production in 2010 was reported to be $55.7 million (Valencia-Botin et al. 2013). In 2012, *Gladiolus* cut flower production in the United States was estimated at $25.14 million (U.S. Department of Agriculture National Agricultural Statistics Services 2014). California and Florida contributed to

approximately 40% of the total U.S. domestic floriculture production, which has an estimated worth of $4.07 billion in 2013 (U.S. Department of Agriculture National Agricultural Statistics Services 2014; Wise et al. 2004).

Despite the economic importance of cut flower production both domestically and abroad, Gladiolus rust remains an aggressive and devastating foliar disease for large, in-field *Gladiolus* production. In regions where the pathogen is established it can cause total crop losses of 10-100%, unless fungicide applications are used (Beilharz et al. 2001; Ferreira and Nevill 1989; Hernández 2004; Littlejohn and Blomerus 1996; Valencia-Botin et al. 2013). As a consequence, the pathogen is considered of quarantine significance in Europe and the United States (Peterson and Berner 2009; Rizvi et al. 2007). The growing season for *Gladiolus* before the onset of flower harvest is approximately 7-10 weeks, so the use of integrated disease management practices, including preventative fungicide applications to slow the rust disease progress, are greatly advantageous, but extremely costly. A recent study regarding fungicide efficacy for the management of Gladiolus rust observed combinations of triazole and strobilurin fungicides and rotating between products provided effective management (Valencia-Botin et al. 2013). Part of the management strategy of the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) has been to implement a national management plan (Rizvi et al. 2007). This plan focuses on the removal and destruction of infected plant material, a required host-free crop rotation zone, and enforcement of a strict fungicide application schedule. In May 2015, APHIS revised the national management response to detection of Gladiolus rust caused by *Uromyces transversalis* in the United States citing that, *Based on the current distribution of the disease, the pathogen has spread to the limit of its natural range. Given these facts APHIS will no longer take domestic action, or require others to take action, when*

Gladiolus rust is found in commercial and residential growing areas. APHIS will continue to regulate Gladiolus rust not occurring within the United States (Osama El-Lissy, APHIS Deputy Administrator, written correspondence, May 13, 2015). While this revision does change the national management strategy within the United States, the quarantine status of this pathogen with respect to import or export commodities remains intact.

As of April, 2016 there are six Gladiolus rust pathogens in the order Pucciniales on the Regulated Plant Pest List for the United States: *Puccinia gladioli, P. mccleanii, Uredo gladioli-buettneri, Uromyces gladioli, U. nyikensis, and U. transversalis* (Hernández 2004; Mueller et al. 2005; U.S. Department of Agriculture APHIS 2016$_a$). *P. gladioli* is predominantly found in Asia, Africa, and Europe; and while the aecial stage of the fungus has been confirmed present in the western United States, urediniospores or telia have not been observed (Hernández 2004; Rossman et al. 2006). Both *P. mccleanii* and *Uredo gladioli-buettneri,* are not well studied and are only known to exist by type, and are considered lost according to a study evaluating the threat posed by fungi on the U.S. Regulated Plant Pest list (Rossman et al. 2006; U.S. Department of Agriculture APHIS 2016$_a$). Within the genus *Uromyces, U. gladioli*, a damaging and economically important pathogen, has only been reported in Ethiopia, Kenya, Malawi, Nigeria, Sierra Leone, South Africa, Uganda, Zambia, Argentina, and Uruguay (Centre for Agriculture and Bioscience International 2008; Hernández 2004; Martínez-de la Parte et al. 2011), and *U. nyikensis* has only been reported in Zambia and is not considered economically important. The causal agent of widespread and economically damaging Gladiolus rust is the invasive pathogen *U. transversalis* (Thum.) G. Winter, in the phylum Basidomycota. *U. transversalis* is an obligate biotrophic pathogen that grows and reproduces on members of the

family Iridaceae (Garibaldi and Aloj 1980; Hernández 2004; Peterson and Berner 2009; Rizvi et al. 2007).

Gladiolus rust primarily infects the leaves and stem of its host; however, under heavy inoculum pressure it can also infect the flowers (Ferreira and Nevill 1989). Once infection occurs, visibly infected plants reduce the economic value as an ornamental cut flower (Valencia-Botin et al. 2013). Additionally, infection by rust reduces the plant's vigor, resulting in reduced flower production (Wise et al. 2004).

As suggested by its species epithet, *U. transversalis* is named for development of sori or uredinia (urediniospore producing pustule) that transverse laterally across the leaf surface, as opposed to longitudinally along the veins of the leaf (Hernández 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 pustules. These uredinia, which develop just below the leaf epidermis and eventually rupture the host tissue, contain the urediniospores. These globose to irregular shaped spores ranging from 16-20 µm in dimension are scattered sporadically across the surface of the leaf. Spores have a pigmented wall that are bright orange to yellow-gold with an echinate surface. The uredinia coalesce to form large lesions (3-7 mm) that transverse the leaf's surface (Beilharz et al. 2001; Martínez-de la Parte et al. 2011; Rizvi et al. 2007; Rodríguez-Alvarado et al. 2006; Valencia-Botin et al. 2013). As with many rusts, the urediniospores are the dispersal and infection spores that serve as the primary and secondary inoculum.

Favorable conditions for Gladiolus rust infection are warm temperatures and high humidity, which are weather conditions common in regions of the world where its occurrence has been confirmed. Urediniospores can germinate in temperatures ranging from 5-20°C, with

optimal growth occurring within 15-20°C. Infection occurs when temperatures are between 10-20°C and the process also requires that the leaves are to remain wet for at least 12 hours (h). The incubation period requires a minimum of 10°C for approximately 22 days, followed by 8-10 days with minimum temperatures of 25°C (Garibaldi and Aloj 1980; Peterson and Berner 2009; Rizvi et al. 2007). *U. transversalis* spores may be disseminated locally by wind or water splash events. Long-distance dispersal of urediniospores (dispersal events occurring outside of the source plot) 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 et al. 2001; Wise et al. 2004).

U. transversalis was first identified in South Africa by von Thümen in 1876 and was considered harmless when observed on *Gladiolus* (Beilharz et al. 2001; Hernández 2004), until confirmed present in parts of southern Europe in 1966 (Collingwood 1970) where it was observed to be virulent. Previous studies of *U. transversalis* used spore morphology for identification (Garibaldi and Aloj 1980; Hernández 2004; McKenzie 2000; Rodríguez-Alvarado et al. 2006). The presence of *U. transversalis* has been confirmed in: Argentina in 1979 (Lindquist et al. 1979), Brazil in 1981 (Pita et al. 1981), Australia in 1994 (Beilharz et al. 2001), New Zealand in 1998 (McKenzie 2000), Mexico in 2004 (Rodríguez-Alvarado et al. 2006), the United States in 2006 (Blomquist et al. 2007), and Cuba in 2010 (Martínez-de la Parte et al. 2011).

Importation of germplasm from outside the United States increases the risk of invasive fungi infecting many floriculture and agriculture commodities grown within the United States. *Gladiolus* flowers are imported into the United States from multiple countries including Mexico, where *U. transversalis* is endemic to *Gladiolus* production areas (Valencia-Botin et al. 2013).

Shipments of *Gladiolus* flowers infected with *U. transversalis* arriving in the United States from Mexico have been repeatedly intercepted by USDA APHIS plant protection and quarantine customs agents at border stations in California and Texas (Brown 2005; Hernández 2004; Rizvi et al. 2007; Valencia-Botin et al. 2013) and a Florida border station with imports arriving from Mexico and Brazil (Schubert et al. 2007). More recently, *U. transversalis* was confirmed present in Florida and California in April and May 2006, respectively (Brown 2005; Rizvi et al. 2007; Valencia-Botin et al. 2013). Detection of this pathogen in the United States resulted in a quarantine and national management plan strategy by both federal and state quarantine officials to contain and manage *U. transversalis* (Rizvi et al. 2007; Valencia-Botin et al. 2013). Despite quarantine measures, outbreaks of Gladiolus rust have still occurred as recently as 2014 (U.S. Department of Agriculture APHIS 2016$_b$). States that produce large quantities of *Gladiolus* where the pathogen has not been reported include: Arizona, Illinois, Michigan, Minnesota, New Jersey and New York (Rizvi et al. 2007). In these states, a large percentage of production occurs in covered greenhouses (U.S. Department of Agriculture National Agricultural Statistics Services 2014).

Biology of Rust Fungi

Rust fungi are highly specialized obligate plant parasites in the order Pucciniales that require a living host for growth and sexual reproduction. They are considered one of the largest groups (approximately 7000 species) of phytopathogens (Kirk et al. 2008; Tavares et al. 2014). Genomic features related to their obligate biotrophic lifestyle and consequently narrow host ranges includes: expanded lineage-specific gene families, families of membrane transporters, and a repertoire of effector-like, small secreted proteins (Duplessis et al. 2011; Guttman et al. 2014). Upregulation of transcripts for these small-secreted proteins, secreted hydrolytic enzymes and

transporters suggests a role in host infection and nutrient acquisition (Duplessis et al. 2011). It has been hypothesized that expansion of these gene families are reflected in the large genome sizes of sequenced rusts (>305 Mbp; compared to an average fungal genome size of 37.7 Mbp), as evidence of close co-evolutionary history with their host, and an increase in suppressing plant innate immunity to evade host immune responses (Duplessis et al. 2011; Eilam et al. 1994; Tavares et al. 2014). This variation in the genome size may directly impact pathogenicity, as relationships between the genome size and biological parameters have been speculated to assist the organism overcome selection pressures (D'hondt et al. 2011).

Life cycles of rust fungi are diverse in regards to both the number of spore types produced and whether an unrelated, alternate host is required to complete the cycle (Hiratsuka and Cummins 1983). These complex life cycles can have as many as five (macrocyclic) or three or fewer distinct spore stages (Kolmer et al. 2009). Rusts that require two unrelated hosts are known as heteroecious as opposed to autoecious, which require no alternate host thus, complete the lifecycle on a single host (Kirk et al. 2008; Kolmer et al. 2009).

An example of a complex (heteroecious macrocyclic) life cycle, is that of *Puccinia graminis* f. sp. *tritici*, the causal agent of wheat stem rust. Diploid teliospores are the overwintering spore stage and are produced on the primary cereal host, wheat, in structures known as telia. The nuclei in teliospores undergo meiosis (upon germination) producing haploid basidiospores in a basidium. Basidiospores are dispersed by wind and can only infect the alternate host, barberry. Once a basidiospore germinates and the fungus successfully invades the alternate host, a haploid pycnium is formed on the upper leaf surface and haploid pycniospores are produced. Pycniospores are insect dispersed and if a spore of a different mating type comes into contact with the receptive hyphae on pycnium of a compatible mating type plasmogamy can

occur. The dikaryotic hyphae produce an aecium on the underside of the leaf where dikaryotic aeciospores are produced. Aeciospores are wind or rain dispersed and can only infect the primary host species, wheat. Aeciospore infection of the primary host results in the production of uredinia that produce dikaryotic urediniospores. Each uredinium produces thousands of urediniospores, which can continue to infect the primary host plants multiple times within a single growing season resulting in disease epidemics. In response to physiological changes in the host and a decline in temperature toward the end of the growing season, the uredinia develop into a telia with dikaryotic teliospores that rapidly undergo karyogamy and form diploid teliospores completing the full life cycle (Kolmer et al. 2009; Schumann and Leonard 2000). Rust fungi may exist in a haploid or dikaryotic state, but the diploid teliospores are the site for recombination (Aime 2006). Recombinant genotypes are only successful if the basidiospores infect an alternate host and mating occurs in the pycnia.

U. transversalis is a rust that produces urediniospores and teliospores, but has no known alternate host (Centre for Agriculture and Bioscience International 2005; Hernández 2004). It is probable that the alternate host is extinct or no longer in proximity to *Gladiolus.* The pycnia, aecia, and the respective spore-producing structures are also unknown (Hernández 2004; Rizvi et al. 2007). Lack of an alternate host and other spore types that suggests sexual reproduction and consequently, recombinant genotypes are absent due to an incomplete life cycle. Asexual reproduction readily occurs during the repeating urediniospore stage where *U. transversalis* exists in a dikaryotic state with two haploid nuclei of compatible mating types.

Lack of an alternate host and, thus, sexual recombination does not limit the success of many species in the Pucciniales. Since rust fungi are highly specialized with narrow host ranges and coevolve rapidly with their hosts, rusts are able to occupy a niche that allows them to survive

and flourish by asexual reproduction (Tavares et al. 2014). *Puccinia horiana* (chrysanthemum white rust) is an autoecious microcyclic pathogen that produces only teliospores and basidiospores. The observed genotypic diversity is larger than expected for *P. horiana*, indicating recombination from a possible parasexual cycle (De Backer et al. 2013). Other notable rusts for which only urediniospore and teliospore production have been observed include: *Hemileia vastatrix* (coffee rust), *Phakopsora pachyrhizi* (soybean rust), *Uromyces betae* (beet rust), and *Puccinia thaliae* (Canna lily rust). *H. vastatrix*, which also exists primarily in a dikaryotic state, only producing urediniospores and developing telia, relies on asexual reproduction (Arneson 2000). Breeding of coffee resistant genotypes to different *H. vastatrix* races from across the world has been successful, but as a consequence of the adaptive potential of the pathogen, emergence of new pathotypes and breakdowns of resistance have been observed in many coffee varieties. Thus, coffee leaf rust still stands as the major constraint to Arabica coffee production (Talhinhas et al. 2014). *P. pachyrhizi* is similar to both *H. vastatrix* and *U. transversalis*, where the lack of sexual reproduction does not limit the variability with respect to virulence (Rupe and Sconyers 2008). It is possible that for many of these rusts, a single mutation in a gene conferring pathogenicity is enough to overcome resistance on hosts with new dominant resistance genes, as in the case for *H. vastatrix* and *P. pachyrhizi*. These select examples of reduced spore stage rusts are able to demonstrate that lack of sexual recombination does not confer lack of pathogenicity. On the contrary, *Uromyces appendiculatus* (dry bean rust) is autoecious and macrocyclic, completing its life cycle with all five spore stages on a single host; however, the economic impact of this pathogen is of less concern than those with reduced spore stage life cycles (Chung et al. 2003; McMillan et al. 2003).

Microsatellite Markers to Study Genetic Diversity and Population Structure

It is important to study the genetic structure of pathogen populations to reveal their dispersal mechanisms, gaining insight into their evolutionary potential to overcome fungicide sensitivity or increase in pathogenicity. From a management perspective, this is critical for selecting the correct host breeding strategy for disease resistance. To better understand the genetic diversity and dispersal mechanisms of *U. transversalis,* required the development of molecular markers to genotype isolates. Genetic markers have been used to study genetic variation and population structure in many organisms including fungi. Genotyping individuals, produces the ability to estimate migration rates and population sizes, and identify population structure and bottlenecks (Selkoe and Toonen 2006). Population genetic studies rely on polymorphic molecular markers because they can be used to make inferences about population structure (Frenkel et al. 2012). Markers can be developed from genomic sequence data and used to estimate variation occurring within and among populations of a species of interest.

While different types of molecular markers are available such as random amplification polymorphic DNA (RAPD) and amplified fragments length polymorphism (AFLP) (Milgroom 2015), microsatellites have become the primary choice for population genetic studies (Dutech et al. 2007; Frenkel et al. 2012; Santana et al. 2009; Selkoe and Toonen 2006). Microsatellites are near perfect, tandem motifs of short sequence repeats (SSR) that are common throughout the eukaryotic genome (Ellegren 2004). Microsatellites are highly mutable genomic sequences, comprised of short 1-6 nucleotide repeats, which vary in length from 5-40 repeats and are commonly localized to intergenic regions (Leclercq et al. 2007; Santana et al. 2009; Selkoe and Toonen 2006). Microsatellite loci may vary in sequence length because of insertions or deletions of one or more repeats, which may result from mechanisms such as polymerase slippage. High

mutation rates in these regions result in polymorphisms within populations, and are therefore advantageous in the identification of multiple unique alleles that aid in genotyping unique individuals (Frenkel et al. 2012; Leclercq et al. 2007). These markers contribute to a substantial amount of genomic variation, with polymorphisms being derived from variability in length, rather than sequence as opposed to many other molecular markers (Ellegren 2004).

Historically, the identification and development of microsatellite markers was limited by the sequence data available for the organism being studied. For organisms with a sequenced genome it often required the generation of enriched libraries, cloning and Sanger sequencing (Santana et al. 2009). However, with massively parallel sequencing technologies becoming increasing affordable and the development of comprehensive bioinformatics tools which use intensive statistical approaches, some of the difficulties in marker development are reduced and make the process affordable and efficient (Santana et al. 2009; Selkoe and Toonen 2006). 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 et al. 2007), microsatellites are the ideal marker choice for determining the genetic diversity and population structure of *U. transversalis*. For dikaryotic organisms like *U. transversalis,* codominant markers such as microsatellites, are more informative in revealing genetic variation when compared to dominant markers, due to high levels of polymorphism observed at each locus.

CHAPTER 2 – DEVELOPMENT OF MICROSATELLITE MARKERS TO GENOTYPE ISOLATES OF *UROMYCES TRANSVERSALIS*

Overview

Three *U. transversalis* isolates from the United States (CA11-1, FL11-1, and FL11-2) were sequenced for the purposes of microsatellite marker development. Genomic sequence data for each isolate was assembled *de novo* and mined for microsatellite repeats. Primers were designed to the flanking regions of each target microsatellite sequence, and each locus was tested for amplification via the polymerase chain reaction (PCR). Fragment analysis was used to genotype isolates and identify polymorphic markers. Markers that produced both PCR products of the expected size and observable alleles in the electropherograms were then tested on a panel of seven additional isolates from the United States.

Materials and Methods

Samples of DNA from three isolates of *U. transversalis,* CA11-1, FL11-1, and FL11-2, were obtained from the USDA Agricultural Research Services, 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 by the USDA from California and Florida commercial fields in 2011. Prior to extraction, urediniospores were harvested from infected *Gladiolus* plants, with sporulating uredinia, in a greenhouse (24ºC±3ºC) with a microcyclone spore collector (Peterson and Berner 2009; Tervet et al. 1951). Collected urediniospores were germinated in a 50 mL conical tube by suspending spores in 25 mL of a sterilized double-deionized water (sddH_2O)/Tween-20 solution (Sigma Inc. St. Louise,

MO), prepared by adding one drop of Tween-20 per 100 mL sddH_2O. The tube was then vortexed for approximately 3 minutes (min). The resulting mixture was poured into a tray and covered with cellophane and aluminum foil for 24 hours (h). Spores were scraped from the surface and germination rate was obtained using a haemocytometer under a compound microscope at magnification $_X$ 200. DNA was extracted from germinated urediniospores using a cetyl trimethyl ammonium bromide (CTAB) extraction protocol (DiGuistini et al. 2009; Zhang et al. 2010). First, germinated spores were frozen in liquid nitrogen, then ground into a powder with a mortar and pestle. A total of 500 µL of CTAB extraction buffer (18.25 mL sddH_2O, 0.25 g CTAB, 3.5 mL 5M NaCl, 2.5 mL 1M Tris, 0.5 mL 0.5M 7.5pH EDTA 250 µL 2-Mercaptoethanol, 7.5 mg proteinase K) was added to pulverized sample and vortexed. The tube was incubated for 30 min at 65ºC. A total of 500 µL of chloroform was added to the mixture and the tube was again briefly vortexed and spun in a centrifuge for 10 min at 13,978 x *g.* The aqueous layer was removed and placed into new sterile tube along with 300 µL of CTAB extraction buffer and 500 µL of chloroform, after which the new tube was vortexed for 1 min and spun for 10 min at 13,978 x *g.* Aqueous phase was again removed and placed into a new sterile tube along with equal volume of isopropanol and spun for 15 min at 13,978 x *g*. The supernatant was discarded and 500 µL of 70% EtOH was added to the tube with pellet. Samples were spun for 20 min at 4ºC at 13,978 x *g*. Excess EtOH was carefully poured into a waste container and tubes placed in a speed vacuum until all liquid was evaporated. Approximately 50 µL of TE buffer (10mM Tris at pH 8.0, 1mM EDTA) and RNase (1 mg/mL) were added to tubes. Tubes were incubated in a 65ºC water bath and agitated every 5 min until DNA was dissolved. Extracted DNA was stored at -20ºC until further use.

Sample concentrations were determined using a Qubit fluorometer v.2.0 (Life Technologies Inc., Carlsbad, CA) and standardized to 50.0 ng/µL. Genomic DNA obtained from each isolate was sent to the Georgia Genomics Facility (GGF) (University of Georgia, Athens, GA) for sequencing, using the Illumina MiSeq platform and PE300 protocol with a NGS library preparation method. Files were uploaded to the University of Georgia's Zcluster networking computer cluster using FileZilla v.3.8.1 (GNU Free Software Foundation) and analyzed using a UNIX-based, command script interface. All scripts were written and executed using text editor ViM v.7.0.237 (Gregoire 2007). Quality control for the raw forward and reverse reads of each isolate was observed using FASTQC v.11.2 (Babraham Bioinformatics Institute). All reads with a phred score below Q=22 (base call accuracy of 99.26%) were discarded. Reads were trimmed and adaptors removed prior to assembly, using FASTX-Toolkit v.3.0.13 (Hannon Lab). ABySS v.1.3.6 (Simpson et al. 2009) was used for *de novo* assembly of trimmed forward and reverse reads into contigs for each isolate, using an optimal K-mer value (substrings of length *k* in DNA sequence data) of 64 determined with multiple assembly trials. Generated contigs were then imported into Geneious v.6.1.8 (Kearse et al. 2012).

To increase the potential for successful microsatellite marker development, only contigs 200 bp or greater in size with matched pair reads were considered. Microsatellite motifs were identified using a combined tandem repeat search tool and next generation sequencing approach. Contigs and singletons were searched for at least five perfect repeats of trimeric, tetrameric, pentameric, and hexameric microsatellites 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 alleles based on a difference of one or two nucleotides based on fragment analysis. Contigs with identified microsatellites were aligned using Geneious Align v.6.1.8.

Microsatellites shared among the three isolates were visually assessed for sequence variation and manually sorted according to potential origin of the variation (e.g. whether it was the result of a poor alignment or possible polymerase slippage). Sequences with 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 and Remm 2007; Rozen and Skaletsky 1999; Untergasser et al. 2012) to produce amplicons of approximately 180-350 bp in length with an optimal annealing temperature of 59ºC.

Candidate microsatellite loci were initially evaluated on the three sequenced isolates CA11-1, FL11-1, and FL11-2 (Table 1) to verify that the PCR worked with the designed primers and that the PCR products were the expected size. A three-primer method (Schuelke 2000) was used in the first round of the marker examination. The forward primer for each candidate marker had a 16-base long sequence, known as a CAG tag (5′-CAGTCGGGCGTCATCA-3′) (Hauswaldt and Glenn 2003) attached to the 5′ end. The reverse primer contained a 'pigtail' sequence (5′-GTTT-3′) on the 5' end, which was added to promote adenylation and reduce stuttering. 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 10x 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 sddH_20, and 0.5 µL of approximately 50.0 ng/µL DNA template. Reaction conditions were 94ºC for 2 min followed by 35 cycles of denaturation at 94ºC for 30 seconds (s), annealing at 55ºC for 30 s and extension at 72ºC for 30 s, followed by a final extension of 72ºC for 5 min. Amplification of individual

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). Primer sets that successfully amplified the three tested isolate DNA were screened for polymorphism on a panel that included seven additional isolates, CA14-1 through CA14-7 (Table 1), and PCR was conducted as described above. A 1:10 dilution of PCR product (1 µL) 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). Products were denatured by incubation at 95ºC 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) software were used to determine allele sizes based on electropherograms. Only primer sets that produced single peaks within the expected size range for the 10 isolates were optimized for multiplex PCR.

Results

The Illumina MiSeq PE 300 sequencing platform produced 32,461,280 total unassembled reads with an average insertion size of 575 bp and 301 bp read lengths. Sequence quality was assessed by phred score (percent of purity filter reads greater than a phred value of 30) and signal purity filter (PF) values (second lowest chastity score in the first 25 cycles greater than 0.6) resulting in a total of 25,452,492 unassembled reads with a PF of 99.26%, which corresponds to 6,023,634, 9,976,981, and 9,262,312 unassembled reads for CA11-1, FL11-1, and FL11-2 respectively.

The *de novo* draft assemblies produced 5,706,372, 4,305,978, and 7,444,849 total assembled reads (contigs) for CA11, FL11-1, and FL11-2, respectively. Using Geneious v.6.1.8

(Kearse et al. 2012), contigs for each isolate were filtered based on size, selecting only contigs 200 bp or greater in length resulting in 470,330, 553,331, and 652,337 for CA11, FL11-1, and FL11-2, respectively which, were subsequently used to search for microsatellite repeats. Overall, microsatellites were identified in 0.98%, 1.03%, and 1.34% of the contigs, showing that the discovery rate of microsatellites was consistent among isolates. Microsatellite motifs fitting our criteria were identified in 4,599, 5,685, and 8,666 of the contigs from CA11, FL11-1, and FL11-2, respectively containing microsatellites. Of the total 18,950 contigs, I was able to align 13,143 among the three isolates to produce 4,296 contigs with potentially informative microsatellites.

From the 4,296 aligned contigs 2,754 contained microsatellite loci shared by at least two of the three isolates. Visual assessment of the aligned sequences revealed that there were two distinct sets of sequences or haplotypes for each of the three isolates occurring at nearly all loci (Figure 1). The flanking sequences were slightly divergent based upon single nucleotide polymorphisms, with approximately 97% sequence similarity (Table 3).

Sixty sets of primers were developed and screened by PCR on the panel of ten isolates from the United States (Table 1). Isolate CA14-7 consistently failed to produce strong PCR products if any, for all putative markers and was speculated to result from a low DNA concentration (37.6 ng/µL). Fragment analysis did reveal peaks at these alleles, however, the allele sizes were below our scoring criteria. Of the 60 putative markers 24 were successfully amplified by PCR and genotyped by fragment analysis (Table 4). Eight of these markers were monomorphic producing one allele and 16 were observed to be polymorphic producing two alleles. However, the polymorphism was identified within each isolate for this dikaryotic fungus, rather than among individuals. Overall, the microsatellite markers showed no genotypic diversity among the nine isolates; however, there was a high heterozygosity within individuals.

Discussion

Prior to this research, there had been no studies on the genetic diversity of *U. transversalis*. Since *U. transversalis* urediniospores are dikaryotic, I hypothesized that the 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; Zhong et al. 2009). 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 shared among the three isolates of *Uromyces transversalis*, but also supplied genomic sequencing, which could potentially be used for gene annotation and other genomic analyses at a substantially lower cost than traditional capillary sequencing.

While there are many sequencing technologies available, the Illumina MiSeq PE 300 platform for whole genome sequencing was selected. This was primarily due to the fact that the genome size and structural complexity of *U. transversalis* had not yet been established and this sequencing platform addressed these concerns by producing short reads, which increased the depth of coverage. While various strategies have been proposed to take advantage of different sequencing technologies to gain greater sequencing depth and coverage (DiGuistini et al. 2009), the Illumina MiSeq platform was economically affordable in contrast to the proposed approaches and still gave us sufficient coverage to manage our research questions. This platform produced short reads 50-300 nucleotides in length. Sequencing short reads of the ends of target-sized insert

fragments of 575 bp (Insert fragment sizes are dependent of sequencing platform used) (Seemann 2013), can be used to bridge data over genomic regions that might otherwise be problematic for sequencing (Haridas et al. 2011). The phred score of 99.26% indicated that the probability of an incorrect base call during assembly was 1 in 400 during the sequencing process (Illumina Inc.). Often the accuracy of the quality scores varies depending on the library preparation method and selection of sequencing primers, which can cause a bias towards certain motifs during sequencing (Schirmer et al. 2015). While a higher phred score would equate a lower sequencing error rate, this coverage was sufficient for creating *de novo* genome assemblies for each of the isolates.

Contigs produced for each isolate during genome assembly were then filtered based on size, with a selection of contigs 200 bp or greater, as this increased the chances of identifying successful microsatellite motifs fitting the predefined criteria. Of the 13,143 contigs containing identified microsatellite regions, 5,807 contigs were not aligned since these were observed to occur in only singleton contigs further reducing the volume of data for analysis. The distribution of microsatellites, whether introns, exons or intergenic regions, varies greatly based on repeat criteria and taxa examined (Oliveira et al. 2006; Tóth et al. 2000). Furthermore, microsatellite density particularly mono-, di-, and tri-repeat motifs tends to be positively correlated to genome size (Ellegren 2004). Average microsatellite distribution identified in the total genome size in plants is estimated at 0.85% while the human genome contains approximately 1.07% (Oliveira et al. 2006), supporting our finding of 0.98%, 1.03%, and 1.34% microsatellite identification in our assembled genomes.

Sixty primer sets were designed with the intention of identifying a set of 20 polymorphic markers that would successfully amplify across multiple isolates. Primer set selection from the

2,754 contigs was based on the content of microsatellite and flanking regions. The microsatellite regions had to meet the previously discussed criteria, whereas the flanking regions were assessed on the absence of excessive SNPs or complex variation patterns; which, resulted from poor quality alignments, in any of the shared isolate sequences for at least 50 nucleotides in either direction, spanning the repeat region. Out of the 60 sets of primers developed only 24 sets were selected for future genotyping, as more than half of the original sixty either failed during PCR or because they were problematic with producing consistent and acceptable peaks during fragment analysis. It was speculated that lack of amplification may have resulted due to issues with annealing temperature and variables of concentration in master mix solutions. Developed primers were designed to anneal at 59^0C, however, to accommodate amplification across multiple isolates, the reaction conditions while optimized by lowering the temperature to 55^0C. This was because initial reaction conditions consistently resulted in a low PCR product yields. To increase our amplicon yields, various cycling conditions with isolates (n = 3) from the United States were examined and observed that 55^0C was the optimal annealing temperature. In general, altering variables, such as annealing temperature or master mix concentration ratios in PCR reactions can cause either a favoring of specificity, while potentially reducing yield, or an increase in yield at the expense of target site specificity (Cha and Thilly 1993; Hecker and Roux 1996). During our primer optimization, changes in cycling condition temperature favoring yield may have reduced specificity and may have resulted in failed fragment analysis. Regardless, for the purposes of answering our questions on genetic diversity, both fidelity and yield were the primary factors in determining optimal conditions, as specificity could be examined via fragment analysis.

In our panel of ten isolates genotyped using 24 loci, I observed that eight of the markers were monomorphic and 16 were polymorphic, with all polymorphism observed within each of

the isolates. There was no observable genotypic diversity among the 10 isolates. Lack of genotypic diversity drastically reduces the ability to track introductions of a genotype from a separate population (McDonald and Linde 2002; Milgroom and Peever 2003).

While assessing the aligned sequences containing identified microsatellites for putative markers meeting our criteria, two distinct haplotypes were observed for most of the loci. The extracted genomic DNA that was sequenced and analyzed was from dikaryotic urediniospores of *U. transversalis*. I hypothesized that these distinct sequences were alleles from the two nuclei. The two nuclei contain divergent genomes with 97% similarity at the observed loci. These genomes within the same individual are at the threshold of acceptable divergence for what is usually observed within a single fungal species (Hibbett 2016), and to our knowledge, this occurrence was a novel observation. Evidence of the lack of genotypic diversity observed among the isolates as inferred from; the frequency of multilocus genotypes, 100% of isolates revealing the same genotype, and the diverging allelic pattern on the flanking regions of the microsatellites, together suggest that *U. transversalis* populations in the United States are not sexually recombining and thus, they are asexually reproducing, clonal populations (Milgroom 1996). Furthermore, in dikaryotic organisms, the absence of sexual reproduction will increase the divergence between alleles at a given locus and stochastic mutations will occur over time (Balloux et al. 2003; Birky 1996). Heterozygosity increases under strict clonal propagation. This heterozygosity was observed in 16 of the 24 loci examined for isolates from the United States. These results provide evidence of a high rate of clonal reproduction, which is consistent with the established research on the reproductive biology of *U. transversalis*. However, since these two genomes in each nucleus for the dikaryon are so distinct, it is likely that there has been no sexual reproduction or recombination in this species for a very long period of time.

TABLE 1. Location and sources of *U. transversalis* isolates used in the development of microsatellite markers

Location	Isolate name [a]
N/A, CA	CA11-1
Carpenteria, CA	CA14-1
Santa Maria, CA	CA14-2
Carpenteria, CA	CA14-3
Carpenteria, CA	CA14-4
Santa Barbara, CA	CA14-5
Goleta, CA	CA14-6
Goleta, CA	CA14-7
Manatee County, FL	FL11-1
Hendry County, FL	FL11-2

[a] Isolates DNA provided by Dr. Kerry Pedley (K.P.), Foreign Disease-Weed Science Research Unit, ARS- United States Department of Agriculture, Fort Deterick, MD.

TABLE 2. Genome assembly and microsatellite statistics

Isolates	Unassembled reads[a]	Assembled reads	# Contigs > 200 bp	Contigs w/ microsatellites[b]	% Microsatellites per assembly
CA11-1	6,023,634	5,706,372	470,330	4,599	0.98%
FL11-1	9,976,981	4,305,978	553,331	5,685	1.03%
FL11-2	9,262,312	7,444,849	652,337	8,666	1.34%

[a] Unassembled reads 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.

TABLE 3. Variation between alleles within sequenced genomes of *U. transversalis*

Microsatellite	# Single nucleotide polymorphisms	# Nucleotides in flanking regions	% Similarity
Ut337	3	180	98.3
Ut513	7	291	97.6
Ut568	13	187	93.1
Ut575	3	187	98.4
Ut752	9	263	96.6
Ut1908	10	264	96.2

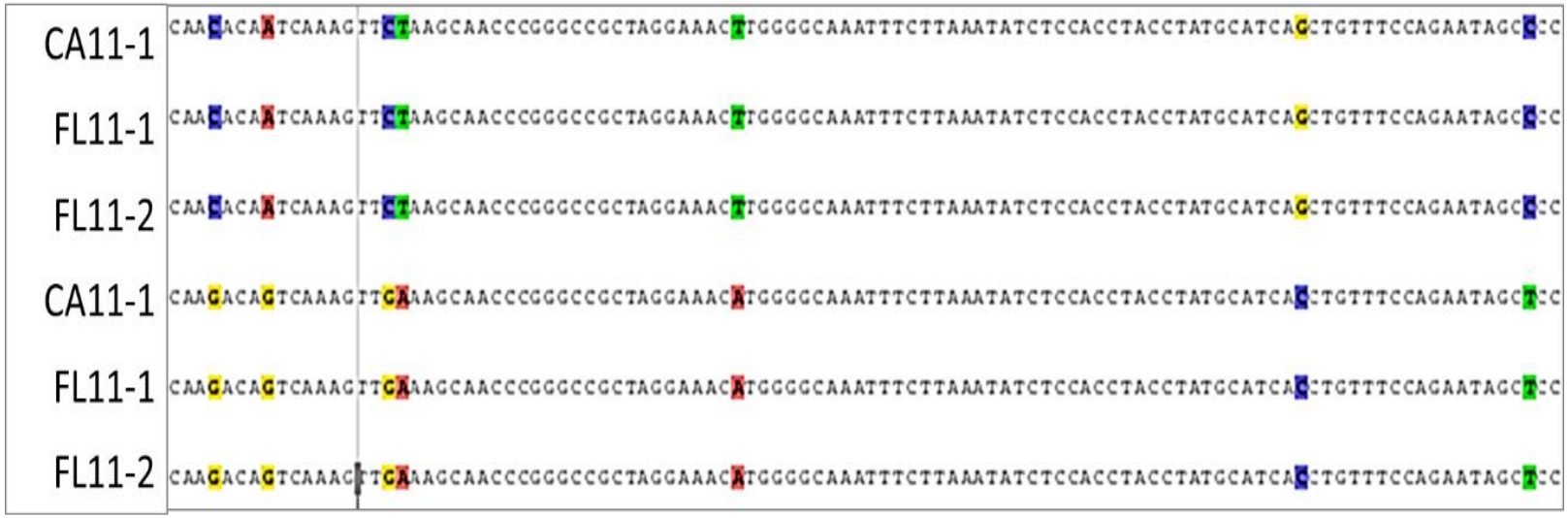
TABLE 4. Repeat motif, primer sequences, number and allele sizes for *U. transversalis* microsatellite loci

Locus	Repeat motif	Primer sequence (5′→3′)	Number of observed alleles and sizes (bp)
Ut337	$(AGG)_7$	F:CGGAAGAGATGAGTGGTCAAG R: TCACATCATCCCCTCCCTA	2 (211, 214)
Ut397	$(TTG)_9$	F: TTCGATTCGATTCGTTTGTTT R:GGATGTTTTGATTCTGTTAGAGAGTG	1 (275)
Ut447	$(ACC)_6$	F: TGCTTCAGCTTCCCAAAACT R: TGGCTGTGAATTGTGAGACC	2 (253, 256)
Ut497	$(GAA)_{15}$	F: CTTGAAGGGGATCGAGAAGA R: TGTTCTCCGGCAGAGGTTTA	2 (250, 268)
Ut513	$(TCA)_6$	F: TCCCAAACAAATCGTGAAGA R: GCTCCCGTTAATGGTCACAG	2 (217, 220)
Ut542	$(GTT)_5$	F: GTCTTCTTTGCTGCGTTTCC R: TCCTGGTTTTGAACCTCCTG	2 (220, 223)
Ut568	$(ACC)_6$	F: TCCCATGGGTTTGGTTGC R: TCCTTAATCTGGGTTGACATTT	2 (194, 197)
Ut575	$(TTA)_5$	F: TGACGATCCTAACGAAGGGTA R: CTTGGGGTACGAGAGCACTT	2 (257, 260)
Ut697	$(AAG)_5$	F: TAGGCGAAGTGGTACGAGGT R: AGGGAAGAAGAGGGTCAACA	1 (240)
Ut752	$(ATC)_6$	F: AGTCTTGTGCTGGTCTTCGTC R: TTTGCCGCCTTATATTGTCA	2 (229, 232)
Ut844	$(ACT)_8$	F: CTCCGTCAGCCAGTCAGTC R: GATGAGGTTGAGGGCGAGTA	1 (326)
Ut981	$(TGA)_6$	F: GGGTCAAACAGGTCTTCTGG R: CTACTGAAATGGGCCACAAA	1 (218)
Ut1272	$(AAG)_5$	F: TGAAGTTTTCCACCCTGGTT R: ATCTTGGGCAAACTGACCAC	2 (269, 272)
Ut1289	$(GAG)_7$	F: GGTCTTGAGAGAACGGAGGA R: CTCTTCCAGATACCCCACCA	2 (270, 273)
Ut1841	$(AGG)_5$	F: GAACCCTGCCTCACACCTTA R: GCGGCTACCAGAGCTTTAGA	2 (360, 363)
Ut1908	$(GAT)_6$	F: TCCTCTCAGCCAATCCAATC R: CTCTTGCCCATCAATCCAAC	2 (214, 217)
Ut2035	$(TTTA)_8$	F: GGATCGAGTCGGTCGATTTA R: GCCGAACAGGACTAGCATTG	2 (245, 248)
Ut2048	$(GAA)_6$	F: CGAGCGATAAATTTTTGAACA R: TGTCCGGAGAATGTGAACTG	2 (198, 201)
Ut2443	$(GAA)_8$	F: AGAATTGGATGAAACAGGGAGA R: AAGGAGGAAGCCATCACTCA	1 (204)
Ut2536	$(GAG)_5$	F: AGGGCTGGTAGACGTGACTG R: TCATGTCTCTGACACCACCA	2 (264, 267)
Ut2648	$(CAG)_6$	F: GAACTGGTGCAACCGATACA	2 (283, 286)

		R: CACAGCCTTGGCTCTTGAGT	
Ut3161	$(TCC)_6$	F: GAGTCTGGCCCAGCTGTTT	2 (209, 212)
		R: TCTGATCTTGCAGGGGATTC	
UtCA759	$(CAT)_7$	F: GATGGCCAGAAGAAAGATGC	1 (310)
		R: TTAACCAGCGCGAGAGTCTT	
UtCA809	$(TTA)_7$	F: GCCACTTCTCCAAACGCTTA	1 (274)
		R:TCGCAAGATCAAGAAACAACC	
UtCA950	$(GTT)_9$	F: GGCAGAGGATGAGTCGTGTA	2 (288, 303)
		R: TCATCTCATCCCCACAATCA	

[a] Fluorescent primers allowed for visualization on ABI3730 DNA Analyzer and cost-effective, efficient polymerase chain reaction (PCR) multiplexing.

FIGURE 1. Two distinct alleles



The locus UT789 showing two distinct alleles. While the image represents a single locus, a similar pattern was observed for the sequences of most microsatellite motifs and associated flanking regions.

CHAPTER 3 - DETERMINING THE GEOGRAPHIC ORIGIN AND TRACKING THE MOVEMENT OF INTRODUCED GENOTYPES OF *U. TRANSVERSALIS* IN THE UNITED STATES

Overview

Since the majority of *Gladiolus* cultivars originated from South Africa, and initial reports of *Uromyces transversalis* infecting *Gladiolus* flowers were described to be from this region, I hypothesized that the center of origin and genetic diversity of this pathogen is South Africa.

U. transversalis is prevalent in many of the commercial fields in Mexico where *Gladiolus* production occurs (Valencia-Botin et al. 2013). All reported interceptions of infected *Gladiolus* flowers at ports entering the United States have come from Mexico (Brown 2005; Hernández 2004; Rizvi et al. 2007; Valencia-Botin et al. 2013). Isolates from both the United States and Mexico were genotyped and compared for genetic similarities using our previously developed markers (refer to Chapter 2). I hypothesized that the source of *U. transversalis* isolates in the United States is Mexico.

Based on these hypotheses, a larger number of alleles and a greater genetic diversity in isolates from South Africa than isolates from other regions are expected. I also expect that a subset of these genotypes would be detected in isolates from the United States, Mexico, and the other countries examined due to reductions in genetic diversity that accompany populations bottlenecks following introductions.

Materials and Methods

A total of 93 samples of preserved leaf tissue infected with *U. transversalis* representative of the global distribution of the pathogen were obtained from Australia (*n* = 7), New Zealand (*n* = 10), Mexico (*n* = 60), South Africa (*n* = 16), and five samples of extracted DNA from Costa Rica (*n* = 5) (Table 5). The collection dates obtained for Australia (*n* = 7) were unknown, while the samples from New Zealand (*n* = 10) ranged from 1998-2007, Mexico (*n* = 60) 2010-2011, and South Africa (*n* = 16) from 1915-1998 (Table 5). Three trials were conducted to select the most effective DNA extraction method for use on preserved leaf tissue, which included 5% Chelex extraction (Walsh et al. 1991), heated-water extraction and a genomic DNA mini-prep protocol (Lee et al. 1988). All extraction techniques were tested using Mexico isolate M8R2. Each sample of extracted DNA was PCR-screened twice with marker UT844, along with two positive DNA controls (CA11-1, FL11-1; previously extracted using the CTAB extraction protocol (DiGuistini et al. 2009; Zhang et al. 2010)), and a negative control (sddH_2O).

The 5% Chelex extraction was conducted by preparing a 5% by weight slurry of Chelex-100 resin (1 g of Chelex; 5 mL of sddH_2O) (Bio-Rad, Richmond, CA). 500 µL of 5% Chelex solution was aliquoted into a 1.5 mL microcentrifuge tube. The Chelex solution was vortexed for 2 min prior to usage to ensure Chelex bead suspension was evenly distributed throughout sample tubes. Using aseptic technique, urediniospores (approximately 0.02-0.04 g) were scraped with a sterilized scalpel blade from infected leaf tissue into the tubes containing the 5% Chelex solution. The mixture was vortexed for 10 s, briefly spun in a micro-centrifuge, and incubated in a dry bath at 95ºC for 10 min. Following incubation, tubes were vortexed for 5-10 s, and spun in a centrifuge at 13,978 x *g* for 2 min. Samples were then stored at -20ºC until further use. Aliquots of the lysed cell elutions were used for PCR.

The heated-water DNA extraction protocol was performed in 1.5 mL microcentrifuge tubes using sddH_2O. Similar to the Chelex-100 protocol, aseptic techniques were used. Approximately 0.02-0.04 g of urediniospores was scraped with a sterilized scalpel from the infected *Gladiolus* leaf tissue into tubes containing 500 µL of sddH_2O. The tubes were vortexed for 5-10 s and incubated in a dry bath at 95ºC for 10 min. The aliquots of the lysed cell elutions were stored in a freezer at -20ºC.

A genomic DNA mini-prep protocol (Lee et al. 1988) was used with a modified 246.9 µL lysis buffer consisting of: 150 µL of sddH_2O, 25 µL of 0.5 M EDTA (pH 8), 25 µL of 1.0 M Tris, 43.75 µL of 20% SDS solution, 3.15 µL of 20 mg/L proteinase K and 0.0025 g of sodium bisulfite. Similar to the other two protocols, urediniospores (0.02-0.04 g) from each tissue sample were scraped using a sterilized scalpel from the leaf tissue into tubes containing the buffer solution. Sample tubes were vortexed for 1 min, incubated in a dry bath at 65ºC for 15 min, and centrifuged (13,978 x *g* for 5 min). The precipitates were discarded and the supernatants were transferred to new 1.5 µL microcentrifuge tubes. A solution of 50 µL of 7.5 M NH_4OAc was added to each tube, vortexed for 10 s, and tubes were chilled on ice for 15 min. Samples were then centrifuged (13,978 x *g* for 3 min). The supernatants were again transferred to new 1.5 mL microcentrifuge tubes and 175 µL isopropanol was added, tubes were rack mixed 20 times and centrifuged (13,978 x *g* for 5 min), and the supernatant was discarded. The pellets were rinsed twice with 250 µL of 70% ETOH solution, dried and re-suspended with 25 µL sddH_2O, then incubated at 30ºC for 10 min. All samples were stored at -20ºC until further use.

PCR with the microsatellite marker UT844 was performed on M8R2 DNA from all three extraction methods. DNA from isolates CA11-1 and FL11-1 were used as positive controls and sddH_2O was used as the negative control. PCR amplification was carried out in 10.0 µL

reactions. Mastermix preparation for each sample included 1.0 µL of 10X PCR buffer (Takara Bio Inc.), 1.0 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), 0.1 µL of Taq polymerase (Takara Bio Inc.), 6.9 µL of sddH_20, and 0.5 µL DNA template. Thermal cycling conditions were the same as previously described ("Microsatellite Marker Development") in Chapter 2. Amplification of individual PCR products was confirmed by gel electrophoresis on a 1% (wt/vol) D1-LE agarose (Alfa Aesar) gel at 85V for 1.5 h.

Additionally, trials were conducted to identify the most effective means of sample preparation for DNA extractions. Using aseptic technique, five uredinia were either (i) scraped to remove urediniospores or (ii) cut directly from the sample. Three isolates from Mexico (ATLIX1, CUA1, and TLAP1), a negative control (sddH_2O), and a positive control (CA11-1) were tested in replicate using the genomic mini-prep protocol for extraction and imaged by electrophoresis for PCR products. DNA concentrations for each successful amplicon were quantified using a Qubit fluorometer v.2.0 (Life Technologies).

Using the modified mini-prep protocol and scraping of the uredinia sampling method, DNA was extracted for all isolates from Australia, Mexico, New Zealand, and South Africa (Table 5). A total of sixty isolates from Mexico and five isolates from Costa Rica were first screened for variation using 6 of the 24 robust microsatellite markers previously developed. Two multiplex reactions (MP1 and MP2) containing the eight most robust markers (MP1 – UT513, UTCA759, UT2648 and UT3161; MP2 – UT497, UT1841, UT1908 and UT2048) were developed from the 24 markers. Multiplex PCR reactions 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 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 range sizes were labeled with different dyes. Range sizes were identified based on the singleplex reactions established from the original panel of isolates from the United States during marker development. Ten isolates from the United States were run with the 65 isolates from Costa Rica and Mexico, and the remaining 33 isolates from Australia, New Zealand, and South Africa were genotyped with the eight markers in the same two multiplex reactions (MP1 and MP2) to verify the results.

PCR amplification was performed using the same parameters and cycling conditions used during the preliminary development of these microsatellite markers (refer to Chapter 2). Amplification of PCR products within the expected size range was again confirmed by gel electrophoresis, run at 95 V (4.75 V/cm) on a 2% (wt/vol) agarose gel (Alfa Aesar) for 2.5 h, using a 100 bp size standard (New England Biolabs Inc.). For successful products, a 1:10 dilution of PCR product (1 µL) was added to a mastermix solution of 0.1 µL of GeneScan 500 LIZ dye Size Standard and 9.9 µL of Hi-Di formamide (Applied Biosystems Inc.) in preparation for fragment analysis. Amplicons in mastermix solution were denatured by incubation at 95ºC for 5 min, then immediately placed on ice. Fragment analysis was conducted at the GGF. Fragment analysis files in FASTA format (FSA) were downloaded from GGF, and Geneious v.6.1.8 (Kearse et al. 2012) software was used to determine allele sizes based on electropherograms.

Results

Gel-imaging revealed that DNA extracted from each sample using the genomic DNA mini-prep protocol produced PCR products of the expected size, whereas the Chelex extraction

and heated water extraction techniques failed to produce observable bands. Therefore, it was determined that the genomic DNA mini-prep protocol was the most effective DNA extraction method from *U. transversalis* infected *Gladiolus* leaf tissue samples. The 5% Chelex and heated-water extractions did not result in PCR products of expected size for the targeted isolate M8R2; however, both positive controls (previously CTAB extracted isolates, CA11-1 and FL11-1) were successful, suggesting that there were no issues in the preparation of reagents for PCR amplification resulting in these negative results.

Sample preparation trials to identify the most effective method of spore collection revealed that scraping the uredinia was the most effective in comparison to cutting off or inclusion of the entire uredinia. For the two sample preparation methods, the three isolates tested produced PCR products with the expected size of 306 bp. DNA concentrations were observably higher for the three samples used for the uredinia excision method, with 326.6, 45.6, and 242.8 (ng/µL) compared to the scraping method, which resulted in readings of 279.4, 36.5, and 175.7 (ng/µL) for ATLIX, CUA1, and TLAP1 respectively. A different result was observed for 2 out of 3 of the isolates for the 260/280 ratio of absorbance with 1.96, 1.97, and 1.79 (nm) compared to 1.90, 1.83, and 1.85 (nm) for ATLIX, CUA1, and TLAP1 respectively.

Six markers were tested for amplification on the panel of 65 isolates. Only 9 isolates (CR497224, CR498594, CR497666, CR498457, GRO1, IRI1, LF2-2, M3R3, and TLAP3) successfully produced PCR products for two or more of the markers. The 9 isolates were used to test the remaining markers to select the eight most robust markers (UT497, UT513, UTCA759, UT1841, UT1908, UT2048, UT2648 and UT3161) for use in multiplex reactions. When using these eight microsatellite markers in two multiplex reactions isolates from Costa Rica and New Zealand consistently produced PCR products of the expected size whereas, isolates from

Australia and Mexico inconsistently produced PCR products despite duplicate reactions. Fifteen out of the 16 isolates from South Africa repeatedly failed to produce PCR products. Only one isolate, SA57128, sampled in 1998 (Table 5) was successfully amplified.

Fragment analysis of PCR amplicons from the eight markers in the two multiplex reactions, MP1 and MP2, showed genotypic variation within individual isolates but no genotypic variation among all 108 from Australia, Costa Rica, Mexico, New Zealand, and South Africa, or the United States. The genotypes of all 108 isolates were identical. Observation of electropherograms revealed that marker UT497 consistently failed to produce peaks above the lower acceptable threshold for 92 out of 108 isolates and consequently is no longer considered a reliable marker. Six of the remaining markers (UT513, UT1841, UT1908, UT2048, UT2648 and UT3161) were polymorphic with only two allele sizes observed for each marker and one marker (UTCA759) was observed to be monomorphic, producing only one allele size. All six polymorphic markers only differed by three nucleotides between allele peaks or one additional repeat of microsatellite sequence for each marker. The overall size range for all alleles was 194-363 bp (Table 4).

Discussion

High quality DNA is an essential pre-requisite for many downstream applications such as PCR amplification. Since 93 out of the 108 samples for this project were obtained as leaf tissue infected with *Uromyces transversalis*, it was essential to find a DNA extraction method to effectively produce a sufficient amount of quality DNA for use, regardless of the means of preservation and age of sample received. Collection dates and storage methods varied for the samples with some samples from South Africa collected over 100 years ago. Samples from Australia, New Zealand and Mexico were received in individually sealed 15 mL and 50 mL

polypropylene conical tubes, with approximately half containing some solution of alcohol, either isopropyl or ethyl; while, the other half of the tubes contained no solution at all. The alcohol serves to preserve the integrity of the DNA as, (i) DNA is insoluble in ethanol or versions of alcohol and, (ii) the solution also serves to prevent DNA molecules from oxidizing and thus increasing the potential degradation of the hydrogen bonds. The isolates from South Africa were received completely desiccated in individually wrapped envelopes, exposed to air which also leads to DNA degradation.

The presence of high amounts of phenolic compounds, polysaccharides, and secondary metabolites found within the leaf tissue can impede the DNA extraction procedure. Polyphenolic compounds interact irreversibly with nucleic acids, resulting in the inability of different modifying enzymes including Taq polymerase to manipulate the DNA (Manoj et al. 2007). Polysaccharides are also problematic as they can hinder the activity of polymerases, ligases, and restriction endonucleases (Azmat et al. 2012). All of these factors affect the DNA template which will be used for PCR amplification. The CTAB DNA extraction protocol (DiGuistini et al. 2009; Zhang et al. 2010) often results in better preservation of extracted DNA, and was used for the isolates from Costa Rica and the United States. Due to the large number of samples, the various storage conditions, and the cost and potential hazards inherited with using compounds associated with CTAB extraction methods (chloroform and hexadecyltrimethylammonium bromid) finding different methods for DNA isolation were elected that were not only safer and quicker, but would still be effective despite the factors which reduced the quality of DNA as well as inhibited PCR amplification. Additionally, CTAB methods require abundant tissue, which usually needs to be propagated for rust fungi. There were very small amounts of spores to work

with and propagation of *U. transversalis* in the United States requires a biosafety level-3 plant disease containment facility, so using a CTAB extraction protocol was not feasible.

Since the DNA in our samples may have been degraded during storage, the extraction methods were selected based on the rational of avoiding overly aggressive treatments such as high temperatures or strong detergents. While treatments with aggressive compounds such as these may increase DNA quantity release, they potentially could decrease quality yield by further damaging the DNA molecules (Drábková 2014). The extraction methods tested (heated-water, Chelex, and the modified mini-prep) are similar in their basis of the approach in that they do not include harsh treatments. The Chelex extraction prevents DNAses from degrading the DNA (Bio-Rad Inc., Berkley, CA). I speculate that one of the reasons these extraction methods failed to produce PCR products was because PCR inhibitors such as phenolic compounds and polysaccharides that interfere with DNA amplification were present, and possibly limited the efficiency of the *Taq* polymerase (Takara Bio Inc.). The genomic DNA mini-prep protocol includes a modified concentration of proteinase K (20 mg/mL) and a diluted detergent concentration of SDS (5%), supplying an enzymatic degradation of DNase as well as the detergent assisting in the dissolution of the lipid layer of the urediniospore cells. These effects are clear when examining isolates from South Africa. The only isolate to successfully produce PCR products was isolate SA57128. This isolate was relatively collected more recently in 1998 while the others were collected much earlier, suggesting the age of the samples likely impacted the DNA extraction.

All genetic diversity identified in the isolates of *Uromyces transversalis* tested, occurred within individual isolates. There was no genotypic diversity observed among the 108 isolates from Australia, Costa Rica, New Zealand, Mexico, South Africa, or the United States based on

the seven loci. No genetic differences among isolates prevents us from being able to track individuals or introductions of individuals from other populations (Milgroom and Peever 2003). Nonetheless, low genotypic diversity combined with genetic diversity within individuals is suggestive of strict clonal reproduction for an extensive period of time (Balloux et al. 2003; Birky 1996).

The variation within each of the isolates suggests diverging dikaryotic genomes. While assessing the sequences containing microsatellites, two distinct sequences for many of the loci were observed. The sequenced genomic DNA was from dikaryotic urediniospores of *U. transversalis*, so I hypothesized that these distinct sequences at each locus were from each of the unique nuclei. The sequences from each locus showed approximately 97% sequence similarity (Table 3). The lack of genotypic diversity among isolates and the distinct sequences and microsatellite alleles within individuals suggests that *U. transversalis* samples from Australia, Costa Rica, Mexico, New Zealand, South Africa and the United States, are asexually reproducing, clonal populations that are not recombining through sexual reproduction (Milgroom 1996). Furthermore, in dikaryotic organisms, the absence of sexual reproduction will increase the divergence between gene sequences at a given locus and random mutations will occur over time (Birky 1996). This suggests that variation within an individual increases with clonal reproduction. Individual variation in 100% of all variable genomic locations examined was observed. These results provide evidence of clonal reproduction of *U. transversalis* in the USA, Mexico, and elsewhere, which is consistent with the observed research on the species reproductive biology (Hernández 2004). However, the extent of sequence divergence indicates that these populations have been clonal for hundreds to thousands of years.

Future approaches to this research may include examining the diagnostic potential of our microsatellite markers and genotyping by sequencing. Testing the developed microsatellites for the purposes of diagnostic testing on symptomatic as well as asymptomatic tissue would be informative for management purposes and could be used for rapid identification in order to limit epidemics associated with this pathogen. The developed markers identified variation within, but not among isolates, thereby limiting the ability to track individuals or their introductions. Genotyping by sequencing may address this concern by identifying variability among isolates, as the restriction enzyme methods used with this technique are advantageous for use on species with no reference genome and limited available resources. This would require high quality DNA yielded from the propagation of additional isolates. Propagating new isolates would also eliminate issues associated with DNA extraction methods of aged and poorly preserved tissue samples. It is likely that this approach would increase the ability to identify SNPs among individuals.

TABLE 5. Location and sources of Gladiolus rust isolates outside of the United States

Location	Original host species	Isolate name	Collection date	Source
Costa Rica	*Gladiolus* sp.	CR497224	2015	Valencia-Botin, A.
Costa Rica	*Gladiolus* sp.	CR498594	2015	Valencia-Botin, A.
Costa Rica	*Gladiolus* sp.	CR498400	2015	Valencia-Botin, A.
Costa Rica	*Gladiolus* sp.	CR497666	2015	Valencia-Botin, A.
Costa Rica	*Gladiolus* sp.	CR498457	2015	Valencia-Botin, A.
Wellington, New Zealand	*Gladiolus* sp.	NZ71109	2000	New Zealand Fungal Herbarium; Beever, R.
Findelton, New Zealand	*Gladiolus* sp.	NZ87970	2006	New Zealand Fungal Herbarium; Close, R.
Remuera, New Zealand	*A. laxa*	NZ69482	1998	New Zealand Fungal Herbarium; Dingley, JM.
Remuera, New Zealand	*G. nanus*	NZ69481	1998	New Zealand Fungal Herbarium; Dingley, JM.
Feilding, New Zealand	*Gladiolus* sp.	NZ71696	2000	New Zealand Fungal Herbarium; Heckler, R.
Mt. Albert, New Zealand	*G. undulatus*	NZ97335	2007	New Zealand Fungal Herbarium; Hill, CF.
Avondale, New Zealand	*Melasphaerula*	NZ69208	1998	New Zealand Fungal Herbarium; Petley, M.
Mt. Albert, New Zealand	*Gladiolus* sp.	NZ99990	2011	New Zealand Fungal Herbarium; Wilkie, JP.
Mt. Albert, New Zealand	*Tritonia*	NZ88195	2004	New Zealand Fungal Herbarium; Wilkie, JP.
Mt. Albert, New Zealand	*Tritonia*	NZ69483	1998	New Zealand Fungal Herbarium; Wilkie, JP.
Australia	*Gladiolus* sp.	VPRI 32661	Unknown	Victoria Plant Pathology Herbarium; Irvine, G.
Australia	*Gladiolus* sp.	VPRI 21344	Unknown	Victoria Plant Pathology Herbarium
Mont Albert, Australia	*Gladiolus* sp.	VPRI 21238	1996	Victoria Plant Pathology Herbarium; Parbery, DG.
Australia	*Gladiolus* sp.	VPRI 22299	Unknown	Victoria Plant Pathology Herbarium
Australia	*Gladiolus* sp.	VPRI 20858	Unknown	Victoria Plant Pathology Herbarium
Australia	*Gladiolus* sp.	VPRI 20881	Unknown	Victoria Plant Pathology Herbarium
Australia	*Gladiolus* sp.	VPRI 20841	Unknown	Victoria Plant Pathology Herbarium
Tlapizalco, Zumpahuacán, MX	*Gladiolus* sp.	TLAP1	2011	Valencia-Botin, A.
Tlapizalco, Zumpahuacán, MX	*Gladiolus* sp.	TLAP2	2011	Valencia-Botin, A.
Tlapizalco, Zumpahuacán, MX	*Gladiolus* sp.	TLAP3	2011	Valencia-Botin, A.
Atlixco, Puebla, MX	*Gladiolus* sp.	Atlix1	2011	Valencia-Botin, A.
Atlixco, Puebla, MX	*Gladiolus* sp.	Atlix2	2011	Valencia-Botin, A.
Atlixco, Puebla, MX	*Gladiolus* sp.	Atlix3	2011	Valencia-Botin, A.
Cuautla, Morelos, MX	*Gladiolus* sp.	Cua1	2011	Valencia-Botin, A.
Cuautla, Morelos, MX	*Gladiolus* sp.	Cua2	2011	Valencia-Botin, A.
Cuautla, Morelos, MX	*Gladiolus* sp.	Cua3	2011	Valencia-Botin, A.
Villa Guerrero, MX	*Gladiolus* sp.	Gro1	2011	Valencia-Botin, A.

Villa Guerrero, MX	*Gladiolus* sp.	Gro2	2011	Valencia-Botin, A.
Villa Guerrero, MX	*Gladiolus* sp.	Gro3	2011	Valencia-Botin, A.
Atlatlahuacán, MX	*Gladiolus* sp.	Ten1	2011	Valencia-Botin, A.
Atlatlahuacán, MX	*Gladiolus* sp.	Ten2	2011	Valencia-Botin, A.
Atlatlahuacán, MX	*Gladiolus* sp.	Ten3	2011	Valencia-Botin, A.
Irimbo, Michoacán, MX	*Gladiolus* sp.	Iri1	2010	Valencia-Botin, A.
Irimbo, Michoacán, MX	*Gladiolus* sp.	Iri2	2010	Valencia-Botin, A.
Irimbo, Michoacán, MX	*Gladiolus* sp.	LF1 1	2011	Valencia-Botin, A.
“La Finca” Villa Guerrero, MX	*Gladiolus* sp.	LF1 2	2011	Valencia-Botin, A.
“La Finca” Villa Guerrero, MX	*Gladiolus* sp.	LF1 3	2011	Valencia-Botin, A.
“La Finca” Villa Guerrero, MX	*Gladiolus* sp.	LF2 1	2010	Valencia-Botin, A.
“La Finca” Villa Guerrero, MX	*Gladiolus* sp.	LF2 2	2010	Valencia-Botin, A.
“La Finca” Villa Guerrero, MX	*Gladiolus* sp.	LF2 3	2010	Valencia-Botin, A.
“La Finca” Villa Guerrero, MX	*Gladiolus* sp.	M1 R1	2010	Valencia-Botin, A.
Cocoyoc Yautepec, Morelos, MX	*Gladiolus* sp.	M1 R2	2010	Valencia-Botin, A.
Cocoyoc Yautepec, Morelos, MX	*Gladiolus* sp.	M1 R3	2010	Valencia-Botin, A.
Cocoyoc Yautepec, Morelos, MX	*Gladiolus* sp.	M2 R1	2010	Valencia-Botin, A.
Oacalco Yautepec, Morelos, MX	*Gladiolus* sp.	M2 R2	2010	Valencia-Botin, A.
Oacalco Yautepec, Morelos, MX	*Gladiolus* sp.	M2 R3	2010	Valencia-Botin, A.
Oacalco Yautepec, Morelos, MX	*Gladiolus* sp.	M3 R1	2010	Valencia-Botin, A.
Yautepec Yautepec, Morelos, MX	*Gladiolus* sp.	M3 R2	2010	Valencia-Botin, A.
Yautepec Yautepec, Morelos, MX	*Gladiolus* sp.	M3 R3	2010	Valencia-Botin, A.
Yautepec Yautepec, Morelos, MX	*Gladiolus* sp.	M4 1	2010	Valencia-Botin, A.
El Caracol Yautepec, Morelos, MX	*Gladiolus* sp.	M4 2	2010	Valencia-Botin, A.
El Caracol Yautepec, Morelos, MX	*Gladiolus* sp.	M5 R1	2010	Valencia-Botin, A.
El Caracol Yautepec, Morelos, MX	*Gladiolus* sp.	M5 R2	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M5 R3	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M6 R1	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M6 R2	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M6 R3	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M7 1	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M7 2	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M7 3	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M8 R1	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M8 R2	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M8 R3	2010	Valencia-Botin, A.

Villa Ayala, Morelos , MX	*Gladiolus* sp.	M9 R1	2010	Valencia-Botin, A.
Villa Ayala, Morelos , MX	*Gladiolus* sp.	M9 R2	2010	Valencia-Botin, A.
Ejido Tlayacapan, Morelos, MX	*Gladiolus* sp.	M9 R3	2010	Valencia-Botin, A.
Ejido Tlayacapan, Morelos, MX	*Gladiolus* sp.	M10 R1	2010	Valencia-Botin, A.
Ejido Tlayacapan, Morelos, MX	*Gladiolus* sp.	M10 R2	2010	Valencia-Botin, A.
Tepoztlán, Morelos, MX	*Gladiolus* sp.	M10 R3	2010	Valencia-Botin, A.
Tepoztlán, Morelos, MX	*Gladiolus* sp.	JB1	2010	Valencia-Botin, A.
Tepoztlán, Morelos, MX	*Gladiolus* sp.	JB2	2010	Valencia-Botin, A.
6.M. Texmel, Pue, MX	*Gladiolus* sp.	JB3	2010	Valencia-Botin, A.
Villa Guerrero, , MX	*Gladiolus* sp.	JB4	2010	Valencia-Botin, A.
Cuautla, Morelos, MX	*Gladiolus* sp.	JB5	2010	Valencia-Botin, A.
Atlixco, Puebla, MX	*Gladiolus* sp.	JB6	2010	Valencia-Botin, A.
TurpamMick, MX	*Gladiolus* sp.	JB7	2010	Valencia-Botin, A.
Atlixco, Puebla, MX	*Gladiolus* sp.	JB8	2010	Valencia-Botin, A.
South Africa	*Watsonia*	SA32142	1940	South African National Collection of Fungi
South Africa	*Watsonia*	SA9165	1915	South African National Collection of Fungi
South Africa	*W. angusta*	SA9192	1915	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA10987	1917	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA10134	1917	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA11676	Unknown	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA14181	1916	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA14145	Unknown	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA44660	1972	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA47693	1985	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA57128	1998	South African National Collection of Fungi
South Africa	*Watsonia*	SA42694	1963	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA34421	1939	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA32310	1939	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA30981	1938	South African National Collection of Fungi
South Africa	*Gladiolus* sp.	SA29853	1928	South African National Collection of Fungi

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