University of São Paulo
"Luiz de Queiroz"College of Agriculture

Molecular variability among Brazilian strains of the sugarcane smut pathogen and the genetic basis of host specialization in smut fungi

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Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

Piracicaba
2017

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Dados Internacionais de Catalogação na Publicação

DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Benevenuto, Juliana

Molecular variability among Brazilian strains of the sugarcane smut pathogen and the genetic basis of host specialization in smut fungi / Juliana Benevenuto. - - Piracicaba, 2017.

90 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Doenças do Carvão 2. Cana-de-açúcar 3. Efetores 4. Genômica 5. Adaptação ao hospedeiro I. Título

With all my love and gratitude

To my parents José Geraldo and Marta

To my brothers Ivo and Ivan

To my future husband Luis Felipe

ACKNOWLEDGEMENTS

This thesis was accomplished with the direct and indirect support of many people and institutions:

I acknowledge and thank the University of São Paulo (USP), specially the "Luiz de Queiroz College of Agriculture" campus (ESALQ). The opportunity to carry out my PhD studies at USP is the realization of a dream. From Minas Gerais, I have always admired the excellence of this institution.

I thank the Department of Genetics and the Graduate Program of Genetics and Plant Breeding, extended to professors, staff, and collegues, for the intellectual and practical support and good relationship.

I thank the São Paulo Research Foundation (FAPESP) for regular and BEPE schorlaships (grant numbers: 2014/21802-0 and 2016/03768-5). I also thank the National Council for Scientific and Technological Development (CNPq) and the Coordination for the Improvement of Higher Education Personnel (CAPES) for previous scholarships.

I sincerely thank my adviser, Prof. Claudia Barros Monteiro-Vitorello, for the support and outstanding guidance. Her combination of commitment and freedom of thought makes the lab a friendly and motivated environment to work. I am very grateful and honored to have been part of her team. "Thank you, Claudia, for your patience, all knowledge transmitted, friendly advices, and affection". I extend my gratitude to all members of the Genomics Lab for the kind and helpful environment since I joined the group: Elaine Vidotto, Patricia Schaker, Leila Peters, Natalia de Moraes, Lucas Taniguti, Giselle Carvalho, Mariana Marrafon, Suzane Saito, Daniel Longatto, Leandro de Souza, Pedro Beretta, Gustavo Crestana, Tatiane Shyton, Natalia Teixeira, Sintia Almeida, Andressa Bini, Natalia Araujo, Jackeline Borba, Marcela, Yago, Larissa. I especially thank Leila for her friendship and positivity, always with a word of encouragement; Paty and her husband Gabriel for the friendship and all good moments and trips togheter; Natalia Teixeira for all nice conversations; Lucas for being helpul and inspire me to learn bioinformatics; and Daniel for teaching me how to handle the fungus.

I thank Prof. Daniel Croll, Prof. Bruce A. McDonald, Sandra G. Weinreich and all members of the Plant Pathology Group, at ETH Zurich (Zurich, Switzerland), for receiving me and making me feel very welcome in the group. I specially thank Prof. Daniel Croll for the guidance, support with bioinformatics analyses, and all nice ideias and discussions that enriched this work. I also specially thank my dear colleagues with whom I shared the office, Norfarhan Mohd-Assaad and Fanny Hartmann. I take the opportunity to thank other very special people I met in Switzerland: Bethania Souza, Vladimir Souza, Marcela Terra, and Carolina Sardinha.

I thank Prof. Marie-Anne Van Sluys and Tatiana Corrêa for assistance in Sanger sequencing; PhD. Natalia Mielnichuk for the Argentine isolates; Prof. José Antonio Martinelli for field collection of smut pathogens; PhD. Eiko Kuramae for helping in the purchase and growing of smut pathogens from CBS bank; and the board of examiners for accepting the invitation.

I thank my lovely fiancé Luís Felipe Ventorim Ferrão for being by my side during the last five years, celebrating my achievements and giving me support in difficult moments. "Thank you for your love, confidence, and friendship. I love you!". I extend my gratitude to the Ventorim-Ferrão family for all support and affection and to Prof. Antonio Augusto Franco Garcia's lab, including here Amanda Avelar, for always receiving me so well.

My heartfelt thanks to my parents, José Geraldo Ferraz Benevenuto and Luzia Marta da Silva Benevenuto, and to my brothers, Ivo Benevenuto and Ivan Benevenuto, who watched me from distance. "Thank you for your endless love, encouragement, prayers, and lifelong efforts for me get here. *Eu amo vocês*!". I also express my gratitude to my entire family on behalf of my grandmothers Lili (*in memorian)* and Alzira. I also thank my friends from Visconde do Rio Branco and Viçosa for the love and friendship that remains despite the distance, specially, Fabíola (*in memorian*), Pollyana, Verônica, Fernanda, Polyanna, Andrea, Marcela, Giselle, Bárbara, David, and Iara.

I thank God for blessed my life with all such special and lovely people who have been made my walk lighter and happier.

RESUMO

Variabilidade molecular entre isolados brasileiros do agente causal do carvão da cana-de-açúcar e a base genética da especialização ao hospedeiro

Fitopatógenos apresentam a habilidade de rapidamente suplantar os mecanismos de defesas da planta e adaptar-se a um novo hospedeiro. A (re)emergência de patógenos é uma das maiores preocupações na agricultura e na conservação de populações naturais. A rápida adaptação ao hospedeiro e a novos ambientes depende da variabilidade genética nas populações de patógenos. Apesar da importância da cana-de-açúcar para o agronegócio brasileiro e da persistência do patógeno *Sporisorium scitamineum*, o agente causal do carvão da cana-de-açúcar, na maioria das áreas canavieiras, estudos de variabilidade genética ainda não foram realizados para isolados brasileiros. Nos capítulos 1 e 2, estudos de variabilidade molecular foram realizados para isolados brasileiros e argentinos de *S. scitamineum*, usando marcadores moleculares (AFLP e telRFLP) e dados de sequenciamento (ITS e um gene candidato a efetor). Nenhum polimorfismo foi encontrado usando sequências ITS. Contrariamente, o marcador telRFLP gerou quase um *fingerprint* para cada linhagem. Dois grupos geneticamente distintos foram formados pela análise conjunta dos marcadores telRFLP e AFLP. Os dois grupos também foram formados pelos haplótipos obtidos pelo sequenciamento de um candidato a efetor. A presença de polimorfismos causando mutações não-sinônimas em um candidato a efetor pode acarretar em performances distintas em diferentes genótipos de cana-de-açúcar. *S. scitamineum* pertence à classe Ustilaginomycetes, a qual também abrange vários outros agentes causais de doenças do carvão. Apesar de filogeneticamente próximos e com estilo de vida similar, espécies de carvão apresentam uma faixa distinta e estreita de hospedeiros. Portanto, outro objetivo desta tese foi identificar a base genética da especialização ao hospedeiro por fungos causadores de carvão usando análises de genômica comparativa. No capítulo 3, os *loci* envolvidos na determinação do tipo de reação sexual (*mating-type*) foram caracterizados no genoma de *S. scitamineum* e comparados com sequências de outras espécies de carvão. Tranposons foram identificados como provável mecanismo de rearranjo cromossômico entre os *loci* de *mating-type*. Polimorfismos trans-específicos nos genes codificadores de feromônios e receptores sugerem o potencial de hibridização entre espécies de carvão. No capítulo 4, análises de genômica comparativa abrangendo nove espécies de carvão infectando hospedeiros distintos foram realizadas. A base genética da especialização ao hospedeiro em fungos causadores de carvão é complexa e parece envolver processos evolutivos de ganho/perda de genes e seleção positiva. Efetores espécie-específicos e sob seleção positiva são destacados como bons candidatos para serem caracterizados quanto ao papel que estabelecem na adaptação ao hospedeiro.

Palavras-chave: Doença do Carvão; Variabilidade; Efetores; *Mating-type*; Genoma; Adaptação ao hospedeiro; Genes orfãos; Seleção positiva

ABSTRACT

Molecular variability among Brazilian strains of the sugarcane smut pathogen and the genetic basis of host specialization in smut fungi

Plant pathogens have the ability to quickly overcome host resistance and shift to novel hosts. The (re)emergence of plant pathogens is a major concern in agriculture and in conservation of natural landscapes. The rapid adaptation to hosts and new environments depends on the genetic variability in pathogen populations. Despite of the importance of sugarcane for Brazilian agribusiness and the persistence of the smut pathogen *Sporisorium scitamineum* in most cropping areas, genetic variation studies are still missing for Brazilian isolates. In the chapters 1 and 2, molecular variability studies were performed for Brazilian and Argentine isolates of *S. scitamineum*, using molecular markers (AFLP, telRFLP) and sequencing (ITS and a candidate effector gene) strategies. No variation was found in ITS sequences. On the contrary, telRFLP marker generates almost a unique fingerprint for each strain. Two genetically distinct groups were formed by the joint analysis of the AFLP and telRFLP markers. The two groups were the same formed by haplotypes of a candidate effector gene. The presence of polymorphisms that causes non-synonymous mutations in a candidate effector gene potentially involved in the specific interaction with sugarcane may cause distinct performances on host genotypes. *S. scitamineum* is part of the highly diverse clade of Ustilaginomycetes fungi that includes several smut disease agents. Despite being phylogenetically close and present similar lifestyles, species of smut fungi have distinct and narrow host ranges. Hence, another objective in this thesis was to identify the genetic basis of host specialization in smut fungi using comparative genomics analyses. In chapter 3, the mating-type loci were described in *S. scitamineum* genome and compared among smut fungi. Transposable elements are the likely mechanism causing chromosomal rearrangements between mating-type loci. The presence of trans-specific polymorphisms at the genes encoding pheromone/receptor proteins suggests a hybridization potential among smut species. In the chapter 4, a broad comparative genomics analysis was performed among nine species of smut fungi infecting distinct hosts. The genetic basis of host specialization in smut fungi is complex and seems to involve a range of evolutionary processes, including gene gain/loss and episodic selection events. Species-specific effectors and positively selected genes will be good candidates for further characterization in regards to their role in host adaptation.

Keywords: Smut disease; Variability; Effectors; Mating-type; Genome; Host adaptation; Orphan genes; Positive selection

PREFACE

This thesis is presented as a requirement to obtain the degree of Doctor in Science at the "Genetics and Plant Breeding" Graduate Program, University of São Paulo (USP), campus "Luiz de Queiroz" College of Agriculture (ESALQ), Piracicaba, São Paulo, Brazil. The research described herein was conducted under the supervision of Professor Claudia Barros Monteiro-Vitorello between March 2013 and April 2017. The thesis was divided into four chapters that, in an overall view, aim to assess the molecular variability among Brazilian strains of *S. scitamineum* (the causal agent of sugarcane smut) and to understand the genetic basis of host specialization in smut fungi. The rapid evolution of plant pathogens seen at both inter- and intra-specific levels is what fascinates me and unifies the four chapters.

The first chapter entitled "Molecular variability and genetic relationship among Brazilian strains of the sugarcane smut fungus" was published in "FEMS Microbiology Letters" Journal. Part of the data was previously obtained by former students of the Genomics Group: Gislâine Vicente dos Reis, Daniel Prezotto Longatto, and Suzane Saito.

The second chapter is entitled "Polymorphic variant of a candidate effector potentially involved in the specific interaction between *Sporisorium scitamineum* and sugarcane". This data will be organized in a manuscript together with ongoing experiments of molecular characterization, expression time course and aggressiveness test of the two variants detected herein.

The third chapter entitled "Characterization of mating-type loci in *Sporisorium scitamineum* genome and implications in smut fungi evolution" was published as part of the paper "Complete Genome Sequence of *Sporisorium scitamineum* and Biotrophic Interaction Transcriptome with Sugarcane" in PLoS ONE Journal.

The fourth chapter entitled "The genetic basis of host specialization in smut fungi" was partially developed during my six months "Research Internship Abroad" (BEPE-FAPESP) under the supervision of Daniel Croll at ETH-Zurich, Zurich, Switzerland. We intend to submit it to "Genome Biology and Evolution" Journal.

INTRODUCTION

Rapid evolution of fungal plant pathogens

Fungal plant pathogens have the ability to quickly overcome host resistance and shift to novel hosts. The (re)emergence of plant pathogens is a major concern in agricultural and natural landscapes (Fisher *et al*., 2012; McDonald and Stukenbrock, 2016). Pathogenic fungi usually have short generation time and produce billions of spores during an infection (Giraud *et al*., 2010). Such features allow the rapid creation of genetic variation by mutations (Giraud *et al*., 2010). High mutation rates increase the probability that a mutation affects virulence and pathogenicity genes, leading to higher fitness on resistant genotypes or novel hosts (McDonald and Linde, 2002).

Genomic features of fungal plant pathogens also appears as drivers of their rapid evolution (Croll and McDonald, 2012; Rafaelle and Kamoun, 2012; Dong *et al*., 2015). Plant pathogens genomes harbor large repertoires of effector genes potentially associated with virulence and pathogenecity. In multiple species, effectors are frequently found in the proximity of transposable elements (Raffaele and Kamoun, 2012; Dong *et al*., 2015). Such non-random association between effector genes and repetitive elements probably serve as cradle for adaptive evolution in fungal plant pathogens, allowing uneven patterns of evolution across the genome (Dong *et al*., 2015). Active transposable elements can accelerate the evolution by causing duplications, losses and disruptions of genes, generating new proteins by exon shuffling, regulating gene expression, or facilitating horizontal gene transfer (Wöstemeyer and Kreibich, 2002; Castanera *et al*., 2016).

Studying the genetic diversity at intra- and inter-specific levels can reveal the adaptive potential of pathogens to overcome host resistance and also provide insights into evolutionary mechanisms leading to specialization into novel hosts.

Co-evolution and host specialization

The evolutionary trajectory of pathogens is intimately associated with their hosts due to strong reciprocal selection pressures (Woolhouse *et al*., 2002). Pathogens have a negative effect on host fitness, triggering selection for enhanced defense mechanisms. Likewise, host defenses impose selection on pathogens for overcoming host immune responses. This antagonism governs coevolution, in which gene frequencies in one species determine the fitness of genotypes of the other species (Brown & Tellier, 2011).

The genetic basis of host-pathogen interactions was first proposed to be governed by gene-for-gene (GFG) relationships (Flor, 1956). The GFG hypothesis envisages that for each host resistance gene (R) there is a corresponding pathogen avirulence gene (Avr). In a classical receptor-ligand model, the protein products of R and Avr genes interact with each other triggering resistance response via hypersensitive cell death pathway. Currently, others mechanisms have also been associated with virulence, including fungal toxins, modulation of the host defense response, and transcriptional regulation (Poland *et al*., 2009). The precision of GFG interaction is considered the ultimate extreme of an evolutionary arms race (Thrall, 2001). To overcome the complex network of plant defense mechanisms and complete its life cycle, a successful pathogen probably accumulated a continuum of genetic changes throughout evolution (Kirzinger & Stavrinides, 2012).

The first line of plant defenses (basal immune system) involves the recognition of broadly conserved molecules in a wide range of microorganisms, named PAMPs (pathogen-associated molecular patterns). Well-known examples of fungal PAMPs are chitin and glucan residues that are detected by plant membrane-localized receptors, known as pattern recognition receptors (PRRs). The perception of cell wall damage by plants also activates basal defense signaling pathways (Bellincampi *et al*., 2014). The recognition of pathogen residues by PRRs induce "PAMP triggered immunity" (PTI) through the secretion of antifungal compounds, production of reactive oxygen species (ROS), callose deposition, protease inhibitors, inhibitors of plant cell wall degrading enzymes, chitinases and glucanases. PRRs are considered conserved and heritable defense mechanisms, allowing early detection of all potential pathogens. However, basal defenses are only partially effective at restricting pathogens (Bent and Mackey, 2007). Adapted pathogens are able to counteract PTI by secreting effector molecules into plant cells. Effectors can act either as an escape of host recognition, suppressor of host immune response, manipulator of host cell physiology, or a toxin that kill the host cell. However, effectors can activate a second layer of plant immune response, termed "effector triggered immunity" (ETI). Effectors that activate ETI are known as an avirulence (Avr) factors and are recognized by specific plant resistance proteins (R proteins) as proposed in the GFG concept. PTI and ETI triggered similar defense responses, although in ETI the defense responses are enhanced in timing and amplitude. The ETI leads to hypersensitive cell death response (HR) that arrests the pathogen growth at the infection site, resulting in an incompatible interaction or resistance. The HR is effective against biotrophic pathogens by restricting pathogen access to nutrients and activating salicylic acid-dependent signaling. Necrotrophic pathogens promote HR-like cell death and jasmonic acid and

ethylene-dependent pathways are major regulators of defense responses against necrotrophics (Glazebrook, 2005). In contrast to the conserved molecules involved in PTI, effectors and R proteins are highly variable, waging an arms race (Jones and Dangl, 2006). Fast evolutionary changes in effectors (Avr) genes make them unrecognizable by the host R genes, resulting in a compatible interaction, or disease (Sonah *et al*., 2016). Conventional mutations, pseudogenization, expansion/contraction of gene families, gene gain/loss, and also genetic and epigenetic changes in controlling the effector gene expression state can promote gain of virulence (Gijzen *et al*., 2014).

The current cost-effectiveness of sequencing technologies has opened the way for the whole-genome sequencing of many organisms. Comparative genomics tools have been applied widely across the fungal pathogens, providing insights into mechanisms of pathogenicity, lifestyle, and genome plasticity (Plissonneau *et al*., 2017). A particular focus has been the repertoire of genes encoding effectors. Effectors genes are predicted as encoding small, cysteine-rich and secreted proteins. Besides their role in pathogenicity and virulence, effectors are also associated with the host range of pathogens (Djamei *et al*., 2011; Feldbrügge *et al*., 2013).

Due to coevolution, pathogens are expected to evolve to higher degrees of host specialization (Antonovics *et al*., 2012) and, therefore, phylogenetically constrained host ranges (Gilbert and Webb, 2007). As a consequence of the intimate interaction established with their hosts, in general, biotrophic pathogens have a narrow host range (Oliver and Ipcho, 2004). Specialist pathogens evolved host-specific adaptations that enable them to infect, defeat of host defenses, uptake nutrients, multiply and reproduce within the host (Haueisen and Stukenbrock, 2016). Hence, specialization onto hosts probably involve different genetic and evolutionary basis in each fungal pathogen.

Smut fungi are suitable pathogens to investigate host specialization mechanisms. Most of smut species infect only a single or a small number of susceptible hosts (Begerow *et al*., 2004). The host specificity seen in the field are so striking that, for over 200 years, host associations were used as an "Ecological Species Criterion" for smut classification (Cai *et al*., 2011).

Smut diseases

True smut fungi belong to Ustilaginomycetes class into the Basidiomycota phylum and comprise more than 1,650 smut species (Toh and Perlin, 2016; Cai *et al*., 2011). Smut hosts are distributed over many angiosperm clades, although most occurs on monocots,

especially on Poaceae family members (Begerow *et al*., 2004). Many agronomically important crops such as cereals, sugarcane, and forage grasses are affected (Martínez-Espinoza *et al*., 2002). Losses range from negligible to significant proportions (Christensen, 1963; Quijano *et al*., 2016; Sundar *et al*., 2012). In general, smut pathogens are found along the geographic distribution of their hosts (Begerow *et al*., 2014). The dispersion mainly occurs through the teliospores carried by wind, water or animal vectors.

Smuts are characterized by the biotrophic growth *in planta* culminating with the production of a sooty dark brown mass of teliospores (Bakkeren *et al*., 2008; Morrow and Fraser, 2009). Under proper environmental conditions, the diploid teliospores germinate and, thereafter, entail meiosis forming haploid sporidia, which grow as saprophytic budding-like yeasts (Figure 1). In the smut life cycle occurs a switch between the haploid saprophytic yeast form to the dikaryotic infective hyphae in a process triggered by mating reaction between two compatible sporidia. This morphogenetic transition and sexual reproduction are required to smut pathogenesis, since outside the host, sporogenesis does not occur (Bakkeren *et al*., 2008). After penetrating the plant surface, most of smut species systemically colonize the host plant and intense proliferation occurs close to meristematic tissue (Piepenbring, 2009). In general, the plants remain asymptomatic until the fungal sporogenesis (Martínez-Espinoza *et al*., 2002; Brefort *et al*., 2009; Morrow and Fraser, 2009). Sporogenesis mainly ocurrs in ovaries, flowers, or in entire inflorescences where there is massive fungal proliferation, followed by karyogamy, hyphae fragmentation, pigment deposition and formation of billions of diploid teliospores that reinitiate the cycle (Figure 1). In addition to the host range, smut species also differ in secondary symptoms, local of infection and local of sporogenesis.

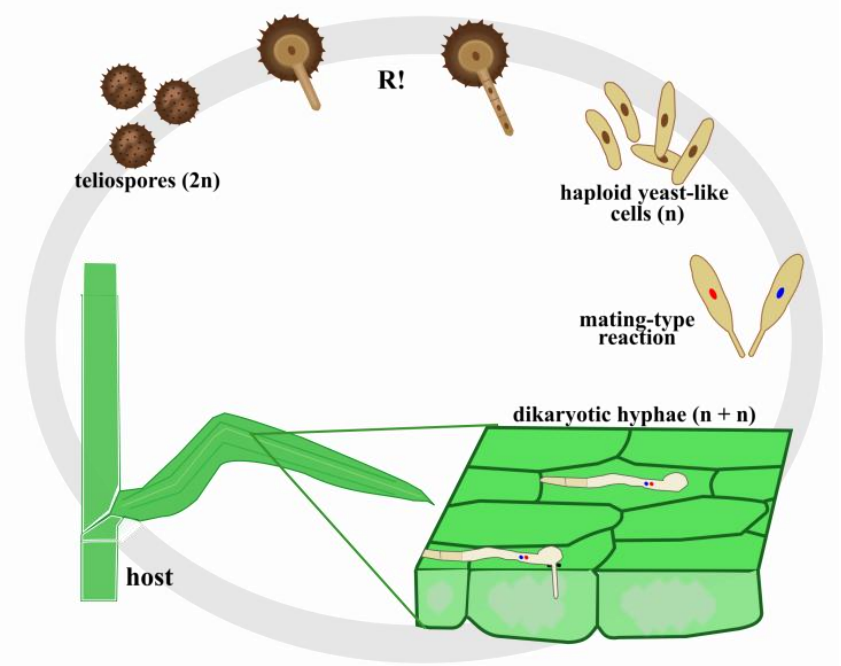

Figure 1. General representation of smut fungi lifecycle. The three phases are shown: diploid teliospores, haploid yeast-like cells, and dikaryotic infective hyphae. Meiosis is shown as R!. Opposite mating-type cells are indicated by red and blue nuclei.

Sugarcane smut disease

Sugarcane (*Saccharum spp.*) is an important agricultural crop mostly due its high sucrose accumulation capacity. Sugarcane has been responsible for 70% of the world's sugar production and is an increasingly source of alcohol-based fuel (McCormick *et al*., 2009). Sugarcane belongs to Poaceae family and modern varieties are interspecific hybrids from natural and artificial crosses between species of the genus *Saccharum*, mainly involving crosses between *S. officinarum* and *S. spontaneum* (Souza *et al*., 2011). Sugarcane has a complex aneu-polyploid genome (2n = 100-130) (D'Hont, 2005; Souza *et al*., 2011), hindering the understanding of the genetic architectures of agronomically important traits (Cheavegatti-Gianotto *et al*., 2011). Due to its genome complexity, sugarcane commercial varieties are vegetatively propagated.

Sugarcane is widely cultivated in tropical and subtropical regions of the world. In 2014, the world production of sugarcane was about 1.8 billion tons on a harvest area of 27 million hectares (FAOSTAT, 2014). Brazil is the largest sugarcane producer, being responsible for around 40% of the world's production (FAOSTAT, 2014). The State of São Paulo is the largest national producer, responsible for 55% of Brazil's production (UNICA, 2016).

Sporisorium scitamineum (Syd.) [Piepenbring *et al*. (2002) (Syn: *Ustilago scitaminea* H. and P. Sydow)] is the causal agent of sugarcane smut and one of the major threats to the culture. The disease is present in almost all sugarcane growing area (Comstock, 2000; Croft & Braithwaite, 2006). The main symptom of the disease is the emergence of a long black whip-like structure (*sori* containing teliospores) in the shoot apical meristem of the plant. When the infection occurs in young plants, abundant tillering and narrow leaves are reported, which makes the cane appears ‘‘grass-like” (Sundar *et al*., 2012). The disease leads to increased fiber and reduced sucrose content, causing significant losses in cane tonnage and juice quality (Tokeshi and Rago, 2005; Wad *et al*., 2016).

Yield losses vary widely, depending on the environmental conditions, the pathogen races, and the tolerance level of the sugarcane varieties (Sundar *et al*., 2012).Sugarcane varieties resistant to smut disease appear to be sustainable, but there is no smut immunity in sugarcane genotypes (CanaOnline, 2014). In addition, the complexity of sugarcane genome makes it difficult to identify disease specific resistance genes (Rott *et al*., 2013). Few breakdowns have been reported for smut diseases and the presence of pathogen races is still controversial (Ferreira and Comstock,1989). Differences in sugarcane genotypes responses to distinct fungal populations was found in many localities, but strong evidence for distinct races was observed only in Taiwan (Grisham, 2001).

A major concern in sugarcane breeding is the highly homogenous environment provided by sugarcane fields with clonal varieties, long-term monoculture and uniform agronomic practices. If a disease outbreak occurs due to new virulent isolate, these homogenous environments will be conducive for quickly pathogen dispersal, leading to the devastation of large areas. Another raising concern is the introgression of *S. spontaneum* germoplasm in breeding programs for energy cane. The wild species *S. spontaneum* is not resistant to smut and is considered a collateral host and a major reservoir of inoculum in India (Braithwaite *et al*., 2004; Jose *et al*., 2016; Srinivasan and Chenulu, 1953). It is also important to point out that climate changes and the prohibition of burn-before-harvesting management of the crop in Brazil can also have large effects on the disease epidemiology.

Monitoring the genetic variability in pathogen populations is very important for optimize breeding strategies and screening cultivar genotypes for resistance, and, thus, stay ahead of pathogens.

CONCLUSION

In the first chapter, new clues about informative regions of the *S. scitamineum* genome were provided, revealing genetic variability among Brazilian strains that so far were not described. The fifty-three strains were clustered into two genetically distinct major groups that do not reflect their geographical origins. A mixture of isolates from these two major genetic backgrounds should be used in breeding programs to cover different adaptive potentials of Brazilian isolates.

In the second chapter, the presence of two polymorphic sites was identifiyed in a candidate effector gene potentially involved in the specific interaction between *S. scitamineum* and sugarcane. Additional experiments are needed to characterize the function this gene and verify whether the two haplotypes imply variations in pathogen aggressiveness degree.

In the third chapter, the mating-type genes of *S. scitamineum* were annotated and the intergenic region between the two mating-type loci was firstly shown for smut fungi, confirming the bipolar system of *S. scitamineum* and revealing the presence of transposable elements as potential drivers of chromosomal rearrangements. The degree of divergence between mating-type alleles among and within species was also assessed. The *a* locus showed an interesting pattern of divergence between alleles, suggesting that interspecific sex could occurs. The presence of a third pheromone allele in *S. scitamineum* remains to be investigated by functional and populational experiments.

In the fourth chapter, the comparative genomic study provided insights on smut pathogen biology and symptoms development. Complex genetic basis underlies host specialization in smut fungi and may involve series of episodic selection events at shared genes and gene loss/gain events generating species-specific genes. The acquisition and maintenance of an optimal effector repertoire may are the main determinants of host specificity. Further functional studies are required to test this hypothesis.

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