GENETIC DIVERSITY OF
Moniliophthora roreri (moniliasis disease)
IN TROPICAL AMERICA

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Economic importance of *M. roreri* in tropical America

– Moniliasis disease is a largely unresolved problem restricted to tropical America and a permanent threat for cacao cultivation worldwide.

– Historical evidence clearly demonstrates the devastating effects caused by the fungus throughout its range over almost 200 years.

– Most local reports inform about yield losses > 30%. However, losses exceed 90% in many localities.

– Thousands of cacao plantations have been abandoned/eliminated in different countries since 1832:

  - Colombia (Anon. 1832, Barros 1977)
  - Ecuador (Rorer 1918, Delgado & Suárez 1993)
  - Venezuela (Reyes & Pérez 1964)
  - Costa Rica (Enríquez et al. 1981)
  - Peru (Katip 1994; Evans et al., 1998, Krauss & Soberanis 2001)
  - Honduras (Anon. 2001)
  - México (OEDRUS 2012) 20,000 ha from 2004 to 2005
OBJECTIVES

- Determine the diversity of *M. roreri* throughout its range of dispersion using different traits:
  - Morphological
  - Phytopathological
  - Molecular
- Defining the center of origin and of genetic diversity of the species.
- Determine the geographical distribution of genetic variants and infer potential dispersal mechanisms involved.
- Explore the presence of sexual reproduction in *M. roreri*. 
HONDURAS: Department of Gracias a Dios (1)

NICARAGUA: Región Atlántico Norte (2), Departments of Río San Juan (3) and Matagalpa (4)

COSTA RICA: Provinces of Alajuela (5), Heredia (6), Limón (7), Cartago (8), Puntarenas (9)

PANAMA: Province of Colón (10)

COLOMBIA: Departments of Antioquia (11), Caldas (12), Norte de Santander (13), Santander (14), Huila (15)

VENEZUELA: States of Mérida (16), Zulia (17), Táchira (18)

ECUADOR: Provinces of Esmeraldas (19), Pichincha (20), Los Ríos (21), Guayas (22), Manabí (23), Napo (24), Imbabura (25), Carchi (26)

PERU: Departments of San Martín (27), Huánuco (28)
**Goal**: to determine phenotypic variation and to relate this information with the molecular findings.

- 88 isolates were grown in Petri dishes during 20 days at 24°C.
- 5 Petri dishes per isolate.
- 12 parameters were analyzed:
  - appearance of the colony
  - radial growth every 5 days
  - days to sporulation
  - ring intensity
  - spore production
  - spore density
  - % globose and ellipsoid spores
  - globose spore diameter
  - ellipsoid spore width and length
Results

- Significant divergence was noted between isolates for all parameters.
- There was indication of apparent morpho-physiological adaptation to specific environments (e.g., Spore size).
- This explains the ability of the fungus to thrive under a wide range of environmental conditions.
Ward's Cluster Analysis using squared Euclidian distances and five morpho-physiological variables: radial growth, days to sporulation, ring intensity, spore production and globose spore diameter.
Santander, Colombia.

Virulence of 7 isolates of *M. roreri*

- **Co1** Zulia, Norte de Santander
- **Co5** Río Negro, Santander
- **Co14** San Vicente de Chucurí, Santader
- **Co16** El Carmen de Chucurí, Santander
- **Co8** San Jerónimo, Antioquia
- **Co-13** Carepa, Antioquia
- **Co10** Palestina, Caldas

5 cacao clones: **CAP-34, ICS-1, ICS-95, SCC-61, TSH-565**
The virulence of the isolates showed statistical differences.

Five isolates were highly aggressive and two less severe (Co-1 and Co-5).

However, under appropriate conditions, all isolates are capable of inflicting considerable levels of damage.

Clone (ICS-95) displayed a significant level of resistance against all isolates, opens the possibility to select genotypes with a durable resistance.
Evaluation of cacao (Theobroma cacao) clones against seven Colombian isolates of Moniliophthora roreri from four pathogen genetic groups


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Artificial pod inoculation was used to compare the relative aggressiveness of seven Colombian isolates of Moniliophthora roreri (the causal agent of moniliasis or frosty pod disease), representing four major genetic groupings of the pathogen in cacao (cocoa), when applied to five diverse cacao genotypes (ICS-1, ICS-95, TSH-565, SCC-61 and CAP-34) at La Suiza Experimental Farm, Santander Department, Colombia. The following variables were evaluated 9 weeks after inoculation of 2- to 3-month-old pods with spore suspensions (1.2 x 10^5 spores mL^-1): (i) disease incidence (DI); (ii) external severity (ES); and (iii) internal severity (IS). IS was found to be of greatest value in classifying the reaction of the host genotype against M. roreri. Genetic variation reported between isolates and cacao genotypes was not matched by similar diversity in their aggressiveness. All isolates were generally highly aggressive against most cacao genotypes, with only two isolates showing reduced IS and ES reactions. There was considerable variation between clones in the IS and ES scores, but one cultivated clone (ICS-95) displayed a significant level of resistance against all seven isolates. This clone may be useful in cacao breeding initiatives for resistance to moniliasis of cacao.

Keywords: cacao, cocoa, disease resistance, frosty pod of cocoa, moniliasis, resistance breeding
94 isolates were analysed to determine the origin, biogeography and molecular variation of *M. roreri* in tropical America.

3 techniques were used:

- AFLP (Amplified Fragment Length polymorphism)
- ISSR (Inter Simple Sequence Repeat)
- ITS sequence analyses
Biodiversity and biogeography of the cacao (Theobroma cacao) pathogen Monilophthora roreroi in tropical America

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Monilophthora roreroi, the cause of moniliasis or frosty pod rot, occurs on the neotropical rainforest genera Theobroma and Herrera. While this basidiomycete has had devastating effects on the cacao tree (T. cacao) in tropical America, where it is confined, little is known of its biogeography and intraspecific genetic variability. Here, AFLP and ISSR profiles of 94 isolates of M. roreroi from across its geographic range in Central/South America were analyzed. The study provided limited evidence to support the hypothesis that M. roreroi is capable of sexual reproduction. The highest levels of genetic diversity occurred in Colombia and not in Ecuador as originally believed. The fungus was broadly divided into five genetic groups. Two of these have a wide geographic range: Bolivar group (north of Santander in Colombia, eastern Venezuela, peripheral Ecuador, Peru), and Co-West group (western Colombia, central Ecuador, Central America). The other groups are all apparently endemic to Colombia (Co-East and Co-Central groups) or north-western Ecuador (Gileri group). We speculate that central/north-eastern Colombia may represent the centre of origin for M. roreroi. Sequence data from the internal transcribed spacer region of the nuclear rDNA repeat were congruent with the AFLP/ISSR results, dividing M. roreroi into two broad groups: the Orientalis group, comprising most isolates from the Co-East, Co-Central and Bolivar groups, and the Occidentalis group, comprising isolates from the Co-West and Gileri groups. The spread of M. roreroi into new areas and countries mediated by human activity is discussed.

Keywords: AFLP, cocoa, frosty pod rot, ISSR, ITS, moniliasis

Introduction

Monilophthora roreroi is a parasitic basidiomycete belonging to the Marasmiaceae (Aime & Phillips-Mora, 2003) with a host range limited to apparently all species of the closely related genera Herrera and Theobroma (Evans, 1981). The fungus attacks only the fruits of these species causing internal and external pod damage that results in total pod loss. The presence of the disease on the cacao tree (Theobroma cacao) has had such devastating effects on yield that long-term economic viability of the crop has been compromised (Evans, 2002). In different Latin American countries losses attributable to the disease have been so severe that cacao cultivation has been abandoned in entire areas (Rorer, 1918; Parsons, 1949; Enriquez et al., 1981).

Shaded agro-ecosystems such as cacao provide a promising means of addressing the challenges of creating a forest-like habitat for tropical biodiversity in a rapidly deforested landscape, while simultaneously providing a lucrative crop for local agricultural communities (Perfecto et al., 1996). The conservation value of the crop is heavily dependent on its local economic viability. However, the economic and therefore ecological significance of this crop is currently in flux as M. roreroi has progressively moved from its native range in N.E. South America to invade northwards through Central America and Mexico, and westwards across the Andes and towards the Amazonian forests.

The genetic diversity of fungi can affect all aspects of their biology including the relationship between a pathogen and its host. Genetic variation allows pathogens to adapt readily to changing environmental conditions and evolve new pathogenic types quickly in response to enhanced resistance in crops, such as those brought about by advances in crop breeding (Carlile & Watkinson, 1994; Wang & Szmidt, 1998). It follows that an improved understanding of the mechanisms of pathogenicity and sources of genetic variation in plant pathogens is likely to be critical for the future control of fungal disease (Talbot,
Considerable genetic variation was observed for *M. roreri*.

However, genetic uniformity was also identified in some geographical areas (e.g. Central America, Central Ecuador, Peru).

Information collected is helpful to define new strategies for disease control including local and regional quarantine measures.
Genetic diversity of *M. roreri* in Colombia, Ecuador and Central America
The highest level of genetic diversity was found in the middle Magdalena Region (north-eastern Colombia).

The pattern of variation outside this region suggest that it also represents the probable center of origin of the fungus.

The presence of wild species of *Theobroma* and *Herrania* and the existence of the older records of moniliasis in this area support this hypothesis.

This will permit to target the search for new sources of cacao resistance materials and antagonists for biocontrol.
Five major genetic groups of *M. roreri* were identified.

- Two groups are widely dispersed:
  - **Bolivar**: Easter Colombia, Peripheric Ecuador, Venezuela and Peru
  - **Co-West**: Western Colombia, Central Ecuador and Central America.

- The remaining groups are endemic to Colombia (Co-East 2 Co-Central 1) or Ecuador (Gileri 4).
Intraspecific variation of *M. roreri* from the analysis of the ITS region

- Only 10 mutations were detected among the 95 isolates of *M. roreri*.
- Five rare mutations were observed only in one or two isolates.
- The remaining 5 mutations separated the set of 95 isolates into two apparent subspecific groups that are congruent with the AFLP/ISSR findings:
  - **Orientalis**: Co-Eastern, Co-Central and Bolívar genetic groups
  - **Occidentalis**: Co-West and Gileri groups
- Highly significant differences were also obtained between these two groups for 10 morpho-physiological variables analyzed.
Current geographic distribution of the ITS groups
Moniliophthora roreri:
Phylogenetic Analysis of isolates
Maximum Parsimony of ITS data

Crinipellis perniciosa

Moniliophthora roreri

Orientalis

Moniliophthora roreri:
Phylogenetic Analysis of isolates
Maximum Parsimony of ITS data

Crinipellis perniciosa

Dis43: T. Bicolor, Peru
Dis 71: T. cacao, Ecuador
Dis 70: Liana sp, Ecuador
Geographical division

Axis 1: 49%

Axis 2: 21%

Andean Cordillera

Temporal division
Very few strands of evidence supporting recombination were found.

Only two genotypes showed some bands typically present in other genetic group so they could represent hybrids between two groups.

Point mutations were evident and possibly constitutes the main source of genetic differentiation.
COLLECTION AND ISOLATION OF NEW SAMPLES
Combination of RNAseq and SNP nanofluidic array reveals the center of genetic diversity of cacao pathogen *Moniliophthora roreri* in the upper Magdalena Valley of Colombia and its clonality

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**Methods:**

*Moniliophthora roreri* is the fungal pathogen that causes frosty pod rot (FPR) disease of *Theobroma cacao* L., the source of chocolate. FPR occurs in most of the cacao producing countries in the Western Hemisphere, causing yield losses up to 80%. Genetic diversity within the FPR pathogen population may allow the population to adapt to changing environmental conditions and adapt to enhanced resistance in the host plant. The present study developed single nucleotide polymorphism (SNP) markers from RNAseq results for 13 *M. roreri* isolates and validated the markers for their ability to reveal genetic diversity in an international *M. roreri* collection. The SNP resources reported herein represent the first study of RNA sequencing (RNAseq)-derived SNP validation in *M. roreri* and demonstrates the utility of RNAseq as an approach for de novo SNP identification in *M. roreri*. A total of 88 polymorphic SNPs were used to evaluate the genetic diversity of 172 *M. roreri* cacao isolates resulting in 37 distinct genotypes (including 14 synonymous groups). Absence of heterozygosity for the 88 SNP markers indicates reproduction in *M. roreri* is clonal and likely due to a homothallic life style. The upper Magdalena Valley of Colombia showed the highest levels of genetic diversity with 20 distinct genotypes of which 13 were limited to this region, and indicates this region as the possible center of origin for *M. roreri*.

**Keywords:** RNAseq, SNP, monilia pod rot, genotyping, biodiversity, homothallism

**Introduction**

*Moniliophthora roreri* H. C. Evans, Stalpers, Samson, and Benny is the causal agent of frosty pod rot (FPR) of *Theobroma cacao* L. (Evans et al., 1978), a major cash crop in the tropics and the source of chocolate. FPR occurs in most major cacao producing countries in the Western Hemisphere, other than Brazil (Phillips-Mora et al., 2007), causing yield losses up to 80% (Hidalgo et al., 2003). *M. roreri* is a hemibiotrophic fungus. During the biotrophic phase the fungus slowly colonizes the

- 88 (RNASeq)-derived SNPs
- 172 *M. roreri* isolates
RESULTS

• 37 distinct genotypes including 14 synonymous groups.

• Absence of heterozygosity for the 88 SNP markers indicates reproduction in *M. roreri* is clonal and likely due to a homothallic life style.

• The upper Magdalena Valley of Colombia showed the highest levels of genetic diversity with 20 distinct genotypes of which 13 were limited to this region, and indicates this region as the possible center of origin for *M. roreri*.

• Peruvian and Bolivian isolates belong to the same groups indicating that *M. roreri* was introduced to Bolivia from Perú.
FIGURE 4 | Geographical distributions of *M. rorei* isolates collected from frosty pod rot affected areas of South and Central America and their phylogenetic relation. Isolates were collected from infected cacao pods from 1999 to 2013. Approximate geographical locations were indicated using Google map application software. See Figure 3 for phylogenetic relationships based on the color code and Table 2 for synonymous group codes and isolates with unique genotypes.
Remarks on the studies of the pathogen

- A significant amount of novel information on different aspects of the biology of *M. roreri* was obtained by studying a group of isolates representing the entire geographic range of the fungus.

- The possible origin of the fungus, the presence and distribution of genetic variants, the virulence and morpho-physiology of the isolates were revealed by using a multidisciplinary approach.

- This information will be very useful to:
  - improve the strategies for control of moniliasis
  - determine more effective quarantine measures
  - target the search for new sources of cacao resistant materials
  - define the extension of the results of the breeding program.
THANK YOU