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Short communication

Understanding the mycobiota of maize from the highlands of Guatemala, and implications for maize quality and safety

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ABSTRACT

Maize is a staple crop in Guatemala, especially in the rural regions where it is consumed in high amounts. Given that traditional pre- and post-harvest practices lead to exposure to the environmental surroundings where pests and microorganisms may be present, maize quality and safety can be compromised severely. In order to assess the potential degree of risk, an exploratory study involving maize mycobiota from six farms from Huehuetenango, Guatemala was conducted. DNA was extracted from the maize samples, and the ITS1 region was subjected to Illumina sequencing. This survey identified 52 fungal taxa in the 90-day maize storage period. For the samples where the maize moisture content exceeded 20%, a high yeast content was observed which can reflect spoilage during storage. A significant amount of *Fusarium* and *Aspergillus* – mycotoxin-producing molds – was found, representing a potential for mycotoxin contamination. This indicates a plausible health risk in a region where maize represents a significant portion of the diet. Potential maize pathogens in the genera *Acremonium* and *Cladosproium*, and *Stenocarpella maydis*, were also common. Results from this study can help better understand the potential health-risk scenario in the Highlands of Guatemala if poor grain handling practices are adopted.

1. Introduction

In tropical developing countries, such as Guatemala, environmental conditions coupled with poor grain handling practices are conducive to microbial growth and mycotoxin production, exposing inhabitants to staples that are often contaminated (Cotty and Jaime-García, 2007). Moreover, maize consumption in rural communities of Guatemala, where people have limited economic resources, is considerably higher than the worldwide average (Torres et al., 2007). Consequently even small levels of mycotoxin contamination could pose a health risk to this population. Mycotoxin effects are dose-dependent, producing a variety of symptoms in the consumer. Furthermore, mycotoxicosis cases have increased during the past two decades in Latin America and worldwide, and at-risk populations have risen dramatically. Examples of these populations include pregnant women, organ transplant recipients, HIV positive individuals (Romanelli et al., 2014; Sifuentes-Osornio et al., 2012), or people suffering from certain medical conditions such as hepatitis (Kew, 2003) where exposure to specific mycotoxins may have a synergistic effect.

Aflatoxins and fumonisins are recurrently implicated in mycotoxin contamination of maize. Health effects due to consuming aflatoxins, synthesized by some *Aspergillus* species, include liver necrosis and tumors, reduced growth, and depressed immune response (Cornell University, 2015; Perrone et al., 2007; Wild and Gong, 2009). Fumonisin, a mycotoxin produced by some species within the *Fusarium fujikuroi* species complex, is correlated with esophageal cancer, stunting, neural tube defect, and other symptoms (Bryła et al., 2013).

Fungi are frequently encountered in agricultural products at different stages including pre-harvest, harvest, processing and handling (Perrone et al., 2007), thus there is a risk of fungal growth in every step of the maize production chain. This may lead to decrease in yield, spoilage, and development of mycotoxins. Factors promoting mold proliferation and mycotoxin production include oxygen availability, heat, high moisture levels, or insect damage (Richard et al., 2007). Changes due to fungal spoilage include sensorial (discoloration), nutritional, and qualitative (rot, off odors)

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damage (Perrone et al., 2007). Yeasts colonize maize when moisture levels are high (Glewen et al., 2013), and are often associated with quality issues. Overall, fungi can pose food safety, quality, economic, and food security risks, especially to communities heavily reliant on a single staple food.

Morphology (i.e. spores, hyphae, etc.) of cultures grown on defined media have been used for traditional fungal identification. However a significant proportion of microorganisms, including fungi, cannot be cultivated in axenic conditions. Moreover, such conditions of analysis are laborious and entail isolation and purification of each microbial species prior to their identification (Richard et al., 2009). In addition, molds may not always produce spores in culture and thus are not distinguishable by classic mycological methods (Romanelli et al., 2014).

Molecular-based approaches are becoming more commonly used for fungal identification because they allow for a more rapid and objective identification, and provide insight into microbial occurrence, relative abundance and microbial niches (Romanelli et al., 2014; Tedersoo et al., 2010). Nuclear ribosomal genes and markers, notably the internal transcribed spacer (ITS) region for fungi, are widely used for barcoding microorganisms (Nilsson et al., 2006; Tedersoo et al., 2010). The ITS is a variable region located between conserved genes encoding the 18S and 28S, and encompassing the 5.8S, ribosomal subunits (Romanelli et al., 2014).

The aim of this research was to investigate the mycoflora diversity of maize from the Western-highlands of Guatemala during a 3-month period, from harvest through storage, in order to assess the safety and quality of this regional staple commodity utilizing Illumina amplicon sequencing of the ITS 1 region. This would represent the first study describing the mycobiota of maize in this part of Guatemala.

2. Materials and methods

Hand-shelled maize samples from the 2014-2015 harvest season from six farms in Todos Santos (n = 3) and Chiantla (n = 3), townships of Huehuetenango, Guatemala, were analyzed in this study. The farms were distributed among three different altitudes: Type C: from sea level to 1500 m above sea level (masl), type B: between 1500 and 2700 masl, and type A: above 2700 masl. Sampling time points included harvest ("H"), as well as 0, 30, 60 and 90 days of storage. Temperature and relative humidity (RH) of the storage area were monitored for each farm every 60 min with a data logger HOBO-ONSET UX100 and Sony CR2032 HRB-TEMP-1. Approximately 4000 readings were obtained per farm. Moisture was quantified in maize immediately after sampling at each farm by employing a John Deere Grain Moisture Tester (SW08120, US), according to manufacturer's instructions. The sampling process involved selecting 55 maize cobs from the traditional storage conditions used on the farm (attic [= *tapancos*], hanging in bundles from the rafters [= *mancuernas*] or in wooden boxes [= *cajones*]) at the appropriate time point, which were husked and then shelled. Approximately 4.5 kg kernels were placed in a clean plastic container and mixed thoroughly. A subsample of approximately 1.0 kg was placed in a sterile plastic bag and stored at -20 °C until shipment to the US. Both symptomless and visibly contaminated kernels, randomly selected, were used for the analysis.

DNA analyses, extraction, and PCR were performed at the University of Nebraska-Lincoln. Samples were shipped on ice and kept at -20 °C until analysis. Prior to examination, samples were homogenized, and a subsample of 45 g was used. DNA extraction from maize was performed according to the CTAB-chloroform procedure established by the European Commission's Community Reference Laboratory for GM Food and Feed CRL-GMFF (Directorate General-Joint Research Centre of the European Commission, 2007). PCR was performed in 50 μL reactions using the QIAGEN *Taq* PCR Master Mix Kit (QIAGEN, Germantown, MD, USA), with forward primer ITS1-F (Gardes and Bruns, 1993) and reverse primer TW13 or ITS 4 (White et al. 1990) on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Conditions included an initial denaturation at 94 °C for 4 min; 40 amplification cycles which included denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 3 min; and final extension of 10 min at 72 °C. Ten μL of each amplicon was run on a 0.7% agarose gel with 0.5 μg mL^{-1} ethidium bromide and visualized on a GelDoc XR+ (Bio-Rad) to confirm amplification.

PCR amplicons were sequenced by MrDNA (Shallowater, TX) using barcoded and adaptor-modified ITS1F:ITS2 primers for Illumina MiSeq sequencing. Nested PCR was performed since fungal DNA was presumed to be present in relatively low proportions. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were joined, depleted of barcodes then sequences <150bp removed, sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI. Results were expressed as proportion of identified taxa per time-point (harvest, days 0, 30, 60 and 90 of storage). Organisms receiving unclear identification from MrDNA were run on BLAST (National Center for Biotechnology Information, NCBI). Samples from which ITS1 sequence could not be identified to species were clustered at genus level when necessary. In those cases, fungal identification was made based on maximum identities (>98%). Differences in the fungal community were reflected in the relative abundance at each time-point. QIIME (ver 1.9) was used to perform community diversity analyses (Caporaso et al., 2010). To standardize sequencing depth, all samples were subsampled to 3000 reads and rarefaction analysis was performed on the samples based on observed OTUs and Shannon's index to assess community coverage. Alpha diversity of the different altitudes and farms was assessed based on Simpson and Shannon's index and beta diversity was assessed between altitudes using principal coordinate analysis (PCoA) on Bray-Curtis distance. Raw sequences have been deposited in GenBank under BioProject PRJNA391691.

R version 3.2.3 (R Core Team, 2013) was used to perform the statistical analysis. The three different altitudes were compared using an ANOVA test with the objective of evaluating any significant differences of the average temperature and relative humidity during storage. After confirming that the ANOVA was significant (data not shown, $p < 2e^{-16}$), pairwise comparisons of altitudes (AB, AC, BC) using t tests with pooled SD were done. Bonferroni was used as the p value adjustment method to evaluate significant differences. Instead of using all of the temperature values (n > 4000), 6 representative values were used to summarize the distribution: mean, 25 percentile, median, min, max and 50 percentile. Maize moisture content was evaluated using Wilcoxon rank sum test pairwise comparisons between altitudes (AB, AC, BC).

3. Results and discussion

Maize is naturally high in moisture after harvest. On average, moisture at harvest was 31 ± 2%, 25 ± 6% and 28 ± 6% for altitudes A, B and C respectively; data shown in Fig. 1. Therefore, prior to storage and consumption, farmers from the region dry their maize via diverse methods, delaying germination and subsequent microbial growth. Consequently, to compare variations of moisture during storage between altitudes, point of harvest data were not taken into account for the statistical analysis. On average, moisture