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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

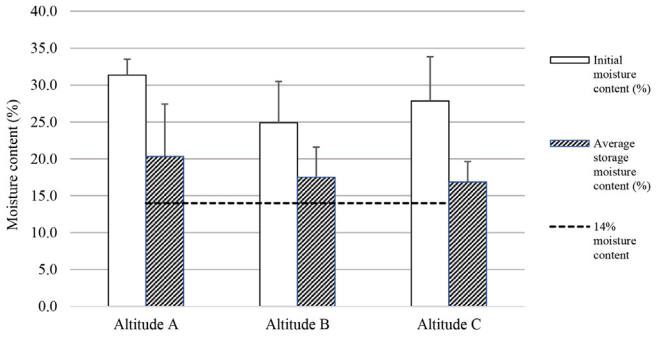

Fig. 1. Average moisture content of maize from Chiantla and Todos Santos, 2014-2015 maize season. Bars on the left (■) indicate initial moisture at harvest and bars on the right (□) indicate an average measurement of all readings during storage. There was no significant difference (post-hoc $p < 0.05$) among maize moisture data during storage. Altitude C: sea level to 1500 m above sea level (masl), altitude B: between 1500 and 2700 masl, altitude A: above 2700 masl.

during storage was 20 ± 7%, 18 ± 4% and 17 ± 3% for altitudes A, B and C respectively. No significant difference ($p > 0.05$) was observed for maize moisture data at any time point during the 90 days of storage. Farms located on altitude A, B, and C had an average temperature of 11 ± 7 °C, 16 ± 5 °C, and 20 ± 4 °C, respectively; significantly different at $p < 0.05$. For relative humidity, average values between altitudes A (62.0%), B (71.1%) and C (68.8%) were significantly different at $p < 0.05$ (data not shown).

All thirty maize samples yielded a variety of detectable fungi. Fifty two distinct operational taxonomic units (OTUs) were identified on the basis of ITS1 sequence and detected more than 10 times (range 11–295,328 reads). Even though these OTUs provide a good estimate of fungal diversity, they may mask the true taxonomic diversity of the maize samples as some groups such as *Penicillium* sp., *Cladosporium* sp., and *Fusarium graminearum-F. culmorum* cannot be satisfactorily separated to species at the ITS level. Samples contained 21 to 39 OTUs (mean, median = 32 OTUs per sample). Individual OTUs were detected in 1–30 samples (mean, median = 18 samples per OTU). The most commonly detected taxa are shown in Fig. 2. From the 52 OTUs found, 22 OTUs have been previously reported in maize (Table 1); the remaining 30 (Supplementary Table S1) are not considered primary or secondary colonizers of maize and are unlikely to play a significant ecological role, though they may lower maize quality. Alpha diversity, based on Shannon's and Simpson's indices, revealed that the community diversity was not significantly influenced by different altitudes or farms ($p > 0.05$; Supplementary Fig. 1). PCoA based on Bray-Curtis distance revealed that fungal communities from farms within altitude C clustered closer together than those of farms within altitude A and B (Supplementary Fig. 2). However, more sampling is needed to further assess the similarities between fungal communities from the farms at different altitudes. Using a sequencing depth of 3000, rarefaction analysis revealed that the entire community diversity was captured for Shannon's diversity estimator but not for the number of observed OTUs (Supplementary Fig. 3). Although this was the first study in the region employing next-generation amplicon sequencing, previous research elsewhere has successfully addressed microbial diversities in crops such as maize, soybean and wheat using MiSeq (Johnson-Monje et al., 2016; Wang et al., 2016; Liu et al., 2017).

Among the taxa identified, endophytes in the genus *Fusarium* – many of which are maize pathogens – stand out as of particular importance. Six distinct *Fusarium* taxa were detected in this study, with *F. graminearum*, *F. verticillioides*, and an unidentified *Fusarium* sp. present and abundant in all samples. In Fig. 2, *Fusarium* sp. is combined with the less abundant *F. proliferatum, F. polyphialidicum* and *F. poae* as "Other *Fusarium*". In sum, *Fusarium* species accounted for 24.4% of all sequence hits, with an average of 13,623 hits per sample (median of 8663). These pathogens survive in the soil and in infected debris as well as inside of apparently healthy seeds (Morales-Rodriguez et al., 2007), affecting the plant throughout its development. Previous reports of contaminated maize from the lowlands of Guatemala documented *F. moniliforme*, *F. oxysporum*, and *F. verticillioides*; the latter being a fumonisin producer (Jurgenson et al., 2002; Martinez et al., 1970). More recently, a study confirmed fumonisin contamination in several communities in Guatemala, favored by regional grain-handling activities (Torres et al., 2014). These findings indicate that maize from the area could represent a food safety hazard for consumers if not maintained under conditions that would control mold growth and toxin production.

The aflatoxin-producer *Aspergillus flavus*, previously reported in lowland areas of the country (Martinez et al., 1970; Torres et al., 2015), is another significant mycotoxin-producing fungus. *A. flavus* was detected in 18 samples, collected from all farms but with a higher prevalence at the high and medium altitude farms (farms 1, 3, and 4). This organism is one of the most common species associated with agricultural products mainly due to its stability in soil (Perrone et al., 2007), being likely to affect future harvests when the land is not properly treated between seasons, i.e., tilled. Although multiple benefits have been reported in association with not tilling, fungal biomass is enhanced in the topsoil under no-till conditions (Jansa et al., 2003) further aggravating the problem after each harvest. Tilling practices were not commonly observed in the region of study (Mendoza et al., 2016).

The amount of aflatoxin-producing fungi associated with crops varies with climate. *A. flavus* is more common in warm areas, while it decreases in presence in colder areas (Cotty and Jaime-García, 2007). This pattern was not observed for the analyzed farms as the ones located in the coldest temperature had a higher presence of this fungi compared to some lower-altitude farms; however, none of our study sites met the definition of "warm" used by Cotty and Jaime-García (temperature minima >25 °C). Additionally, our findings could be influenced by sample size ($n = 6$), erratic weather conditions, the maize handling process (e.g. kernel exposure during shelling) which may have promoted wounds, or because affected farms did not have proper pest control, which may have allowed for entry points for this organism.

Stenocarpella maydis, detected in all samples (mean of 5,503, and median of 374 hits per sample) is the causative agent of dry-rot of maize ears. It has been associated with a neuromycotoxicosis in cattle when animals graze harvested maize fields in southern Africa and Argentina, an activity which is also common in Guatemala (Wicklow et al., 2011). In the same genus, *S. macrospora* was detected at much lower prevalence. This plant pathogen is commonly widespread in humid subtropical and tropical zones where plants exhibiting dry-ear rot and stalk rot may also display symptoms of leaf and husk striping (Wicklow et al., 2011). This plant disease may affect alternate uses of additional maize parts (e.g. husks to make *chuchitos*), a practice frequently observed in the country.

Cladosporium as well as *Nigrospora* (present in all samples) have been reported previously in Guatemala in association with maize (Martinez et al., 1970). *Cladosporium* can produce undesirable effects on maize quality such as discoloration, reduced germination, mustiness, sour odors, chemical changes, and loss of weight (Katab, 2012). *Nigrospora* affects the proper development of the maize plant, as it is frequently involved in ear-rots (Blaney et al., 1986).

On the positive side, some endophytes, such as Pleosporales species (including *Phoma*) have been found to benefit the plant by

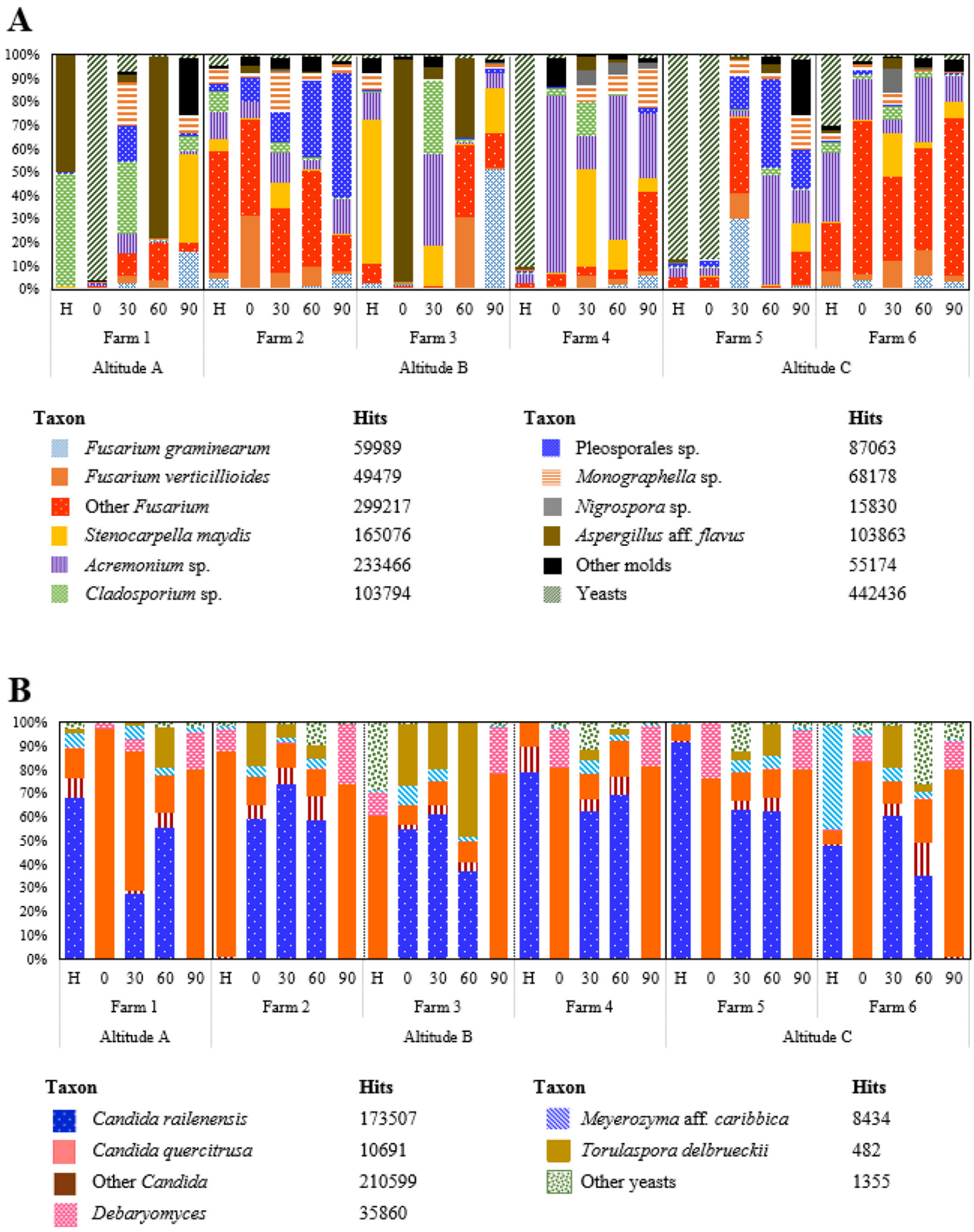

Fig. 2. Distribution of fungi (**A**) and yeasts (**B**) in maize farms from Chiantla and Todos Santos, Huehuetenango, Guatemala. Maize season 2014-2015. Altitude C: sea level to 1500 m above sea level (masl), altitude B: between 1500 and 2700 masl, altitude A: above 2700 masl.

Table 1
Fungal taxa with a documented association with maize, isolated from maize from Chiantla and Todos Santos, Huehuetenango, Guatemala. Growing season 2014-2015.

Taxa			Reference[a]
Yeasts	Ascomycota, Saccharomycetes	*Candida quercitrusa*	Nguyen et al., 2007, Su-lin, 2012, Xiao et al., 2014
		Candida sake	Mansfield, 2005
		Candida sp.	Lumi-Abe et al., 2015
		Debaryomyces sp.	Nout et al., 1997[b]
	Basidiomycota, Tremellomycetes	*Cryptococcus* sp.	Kurtzmann, 1973, Mansfield, 2005, Kohl et al., 2015
		Hannaella zeae	Global Catalogue of Microorganisms, (2008)[c]
Molds	Ascomycota, Dothidiomycetes	*Cladosporium* sp.	Lumi-Abe et al., 2015
		Pleosporales sp.	Remesova et al., 2007[d], Orole and Adejumo, 2009[e], Peterson and Kurtzmann, 2015
	Ascomycota, Eurotiomycetes	*Aspergillus flavus*	Payne et al., 2006, de Lange et al., 2014, Torres et al., 2015
		Penicillium sp.	Marin et al., 1998, Mansfield et al., 2008
	Ascomycota, Leotiomycetes	*Botrytis cinerea*	ten Have et al., 2001, Shurtleff et al., 2016
	Ascomycota, Sordariomycetes	*Stenocarpella macrospora*	European and Med. Plant Prot. Org., 2016
		Stenocarpella maydis	Wicklow et al., 2011, European and Med. Plant Prot. Org., 2016
		Acremonium sp.	Tagne et al., 2002[f], Wicklow et al., 2005[g], Poling et al., 2008
		Hypocrea rufa	Hou et al., 1972[h]
		Trichoderma koningii	McAllister et al., 1994[i]
		Fusarium graminearum	Reid et al., 1999, Broders et al., 2007
		Fusarium proliferatum	Logrieco et al., 1995, Scarpino et al., 2015
		Fusarium verticillioides	Jurgenson et al., 2002, Maschietto et al., 2015
		Nigrospora sp.	Standen, 1941, Blaney et al., 1986, Wicklow et al., 2014
		Monographella sp.	Müller and Samuels, 1984
	Zygomycota, Mucormycotina	*Mucor fragilis*	Lumi-Abe et al., 2015

[a] References to work where specific taxa was isolated from maize.
[b] *Debaryomyces hansenii*.
[c] Isolated from guts of *Liabrotica virgifera* (corn rootworm).
[d] *Epicoccum nigrum* causes leaf spot.
[e] *Phoma* species have been isolated from roots.
[f] *A. strictum*.
[g] *A. zeae*.
[h] Isolated from plants infected with southern corn leaf blight.
[i] Affects maize growth in conjunction with *Fusarium solani*.

promoting health, improving growth potential and acting as biological control agents against fungal (e.g. *Fusarium*) and bacterial diseases of plants (Orole and Adejumo, 2009). In some cases, the abundance of Pleosporales in maize samples increased during storage (Fig. 2, farms 2 and 5).

Yeasts were detected in all samples, but were in low abundance (less than 3%) at 30 days of storage or later (Fig. 2), likely reflecting the decrease in moisture during storage. *Candida* was the dominant yeast genus in all samples but farm 3 at 60 days (50%). Overall, *Candida* yeast abundance was highest at farm 1 (altitude A) and farm 5 (altitude C). *Candida railenensis* was the most abundant named species. *C. railenensis* has been previously reported in oak trees, a cold-resistant tree found in the region of study (Terradas, 1999; Tzuk, 2011), hence its presence is understandable since all the farmers in the region perform traditional agricultural practices, such as exposing the maize to the environment for sun drying. Another common traditional practice is shelling the maize cobs by hand, an activity which may have contributed to the low-level presence of *C. zeylanoides, Malassezia restricta,* and *Malassezia sympodialis*, frequently reported on skin (Dawson, 2007; Khosravi et al., 2013).

In summary, farm-to-farm variance was observed for many taxa. As previously discussed, yeasts were more prevalent at earlier time points, while Pleosporales, when present, appeared to increase in abundance during storage. Overall, results revealed a high presence of both field fungi (e.g. *Alternaria, Cladosporium*, and *Fusarium*) and storage fungi (e.g. *Aspergillus* and *Penicillium*), with some degree of succession across farms. These outcomes were most likely the result of elevated moisture during harvesting and throughout storage, as well as possible pest-associated wounds (Hirst Sole, 1994). Regardless of the cause, the presence of such organisms associated with poor practices throughout the maize chain in this rural region of the country could compromise the quality and safety of the maize consumed. Therefore, efforts should be made to dry the maize to safe moisture levels along with the use of appropriate storage structures. Furthermore, the association of maize with such spoilage organisms may also compromise the food security in the region. Fungi are ubiquitous, and controlling their presence presents a degree of difficulty, however, proper pre- and post-harvest practices may help attain conditions that delay fungal growth and toxin production.

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Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.cropro.2017.07.009.

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