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Cell wall composition and biomass digestibility diversity in Mexican maize (*Zea mays* L) landraces and CIMMYT inbred lines

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Abstract

Maize is one of the most important crops worldwide. Historically, breeding efforts in this crop have been primarily focused on the improvement of grain yield and stability and just recently also on the potential utility of maize stover (above ground biomass excluding the grain) as a source of biomass for the production of feed, fiber and cellulosic ethanol. The International Maize and Wheat Improvement Center (CIMMYT) holds one of the largest maize germplasm collections in the world and therefore is an important source of phenotypic and genetic diversity for many traits. Our objectives were to assess the phenotypic diversity for cell wall composition and biomass digestibility in Mexican tropical, subtropical and highland maize landraces and elite maize lines (CMLs) in the CIMMYT germplasm collection, as well as to evaluate the relationship between place of origin of these materials and phenotypic expression of biomass compositional traits. The range of variation for neutral detergent fiber for three groups of landraces was from 47 to 73%. Slightly larger levels of phenotypic variation were observed for this trait in the set of CMLs evaluated (42 to 78%). Some of the inbred lines, such as CML 507, presented superior characteristics in terms of cell wall composition and digestibility. The Tuxpeño tropical-subtropical race, widely used in CIMMYT breeding programs, formed a cluster characterized by high cell wall content and low biomass digestibility. The CIMMYT germplasm collection appears to be a vast source of untapped genetic and phenotypic variation for the improvement of maize biomass composition.

Keywords: maize landraces, cell wall composition, biomass digestibility, genetic diversity, stover quality

Introduction

Mexico is the center of domestication and diversity for maize, a crop grown in a variety of environments, which plays multiple functions in farmers' livelihoods around the world, such as a source of food, income, cultural identity and social status (Bellon et al, 2011). Given the economical and social importance of maize, knowledge of genetic diversity is important for the development of conservation strategies and for its use in plant breeding as source of new, exotic and/or favorable traits. The diversity of maize found in Mexico has underpinned the breeding programs that have generated much of the higher-yielding maize used worldwide (Keleman et al, 2009). Historically, this effort has primarily focused on increasing stability and grain yield potential under abiotic and biotic stresses (Rosales et al, 2011). More recently, however, efforts have also been placed in evaluating and using the diversity of tropical and subtropical maize for animal feed and human nutrition (Harjes et al, 2008; Yan et al, 2010).

Crop residues are important sources for livestock feed in the (sub)tropics and also have other productive uses such as biofuels, construction material and mulch (Erenstein et al, 2011). Several smallholder mixed systems in developing countries rely on local crop residue to provide basal diet for livestock, resulting in an extremely low-cost feed (Romney et al, 2003; Wright et al, 2012). A recent survey of farmers in Ethiopia found that stover quantity and quality are the second most important criteria used to select maize varieties by farmers who own livestock (Blummel and Friesen, 2009). In Tanzania, where stover is used both on-farm and sold for cash, farmers ranked stover as the second most important animal feed after native pastures (Bwire et al, 2002). In India, where stover is used on-farm both for feed and fuel, exciting market opportunities are emerging for stover with improved digestibility (Erenstein et al, 2011). In Mexico, 50.6% of the area planted to landraces is used for food and feed (Lazos and Chauvet, 2011). In fact, smallholder maize-livestock production systems, in which dairy cattle predominate, are the main form of agriculture in central Mexico (Estrada-Flores et al, 2006). In some countries, stover has also become increasingly important as a potential source of feedstock for biofuels conversion (Vermerris et al, 2007; de Leon and Coors 2008, Lorenz et al, 2009).

To evaluate forage quality for animal nutrition, Van Soest (1994) developed an analytical method to as-

sess cell wall composition by estimating the neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) fractions. *In vitro* true digestibility (IVTD) is an important measurements frequently used in forage quality evaluations (Goering and Van Soest, 1970; Stern et al, 1997). IVTD combined with NDF allow the estimation of NDF digestibility or NDFD (Van Soest, 1994). Most recently, these analytical resources have been used to evaluate stover digestibility for biofuel conversion (Weimer et al, 2005; Lorenz et al, 2010). According to Lorenz et al (2009) NDF (and also ADF) can be considered structural carbohydrate concentration measurements, whereas IVTD, NDFD and ADL are convertibility measurements. These authors developed a regression model that included NDFD and NDF measurements to predict ethanol yield (determined through a simplified simultaneous saccharification and fermentation process, SSF). This regression model was able to explain 95% of the observed variation in ethanol yield for a set of 12 diverse maize varieties. This result highlights the utility of the detergent methods as well as the rumen fluid based digestibility assays, not only in the evaluation of forages for animal nutrition but also in the context of utilizing biomass for lignocellulosic biofeedstock germplasm development.

Studies have demonstrated considerable independence between grain yield and fodder quality in maize suggesting the possibility for improvement of those traits simultaneously (Blummel et al, 2012). A first step towards the enhancement of cell wall composition and digestibility is the characterization of the phenotypic and genetic diversity of the available germplasm. The International Maize and Wheat Improvement Center (CIMMYT) has one of the largest collections of maize (*Zea mays* L) germplasm in the word, including more than 28,000 accessions and more than 500 elite maize inbred lines known as the CIMMYT Maize Lines or CMLs (Ortiz et al, 2010). The use of this rich genetic diversity is hindered by the lack of phenotypic and genetic information of each accession, and by the relatively poor agronomic performance of a large proportion of the available accessions. The identification of germplasm sources with extreme phenotypes would allow the development of mapping populations or association mapping panels that would enable the detection and localization of

favorable alleles that could be introgressed into elite cultivars through breeding. The phenotypic and genetic characterization of these genetic resources will enable plant breeders to identify germplasm resources that have superior value for traits of interest, not only in the context of enhancing biomass composition for animal production, but also to develop improved cultivars for lignocellulosic biofuel production.

Most of the research on maize digestibility has been focused on temperate germplasm, which is mainly used as a source of forage and just recently in biofuel production (Lewis et al, 2010; Barrière et al, 2010). Subtropical and tropical germplasm has been extensively used in breeding programs focusing mainly on the improvement of grain yield. Given the increasing dual-purpose use of maize, as a source of grain and stover (Erenstein et al, 2011; Estrada-Flores et al, 2006), and the scarce knowledge on genetic diversity in subtropical and tropical germplasm for cell wall composition and digestibility traits (Pena-Ramos et al, 2003), it is of main interest to study the variability for these traits in a collection of CIMMYT Mexican landraces and elite inbred lines. To this end, the objectives of this study were: 1) to assess the phenotypic diversity for cell wall composition and biomass digestibility for 150 Mexican tropical, subtropical and highland landraces as well as for 187 inbred lines of the CIMMYT germplasm collection, and 2) to study the impact of place of origin of the different germplasm resources on these compositional traits.

Materials and Methods

Germplasm and field evaluation

A set of 150 Mexican maize landrace accessions from CIMMYT's gene bank were grown in Mexico during 2010 and 2011. The accessions chosen represent 40 of the 56 Mexican races and are adapted to the three mega-environments of adaptation, which includes highland, tropical and subtropical environments. In two winters (2010 and 2011), 60 tropical landraces were grown at Agua Fría (AF), Puebla [20°32'N, 97°28'W; 110 m above sea level (masl)] and 55 subtropical landraces were grown at Tlaltizapán (TL), Morelos (18°41'N, 99°07'W; 945 masl). During two summers (2010 and 2011), 35 highland landraces were grown at El Batán (BA), Texcoco, Mexico

Table 1 - Calibration statistics for neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and in vitro true digestibility (IVTD) equations developed through modified partial least squares (MPLS) for the collection of 150 landraces and 187 elite inbred lines.

Constituent	Equation	N	Mean[†]	Min[†]	Max[†]	R²	SECV[‡]
NDF	1-4-4-1	117	62.9	34.9	80.3	0.99	1.03
ADF	1-2-2-1	114	40.2	55.0	80.3	0.99	0.86
ADL	1-4-4-1	92	8.3	3.1	19.2	0.74	0.99
IVTD	1-4-4-1	109	70.0	52.7	83.9	0.95	1.75

[†]Mean, minimum and maximum concentrations (% of dry matter) of each constituent in the calibration set of sample size N.
[‡]SECV, standard error of cross validation.
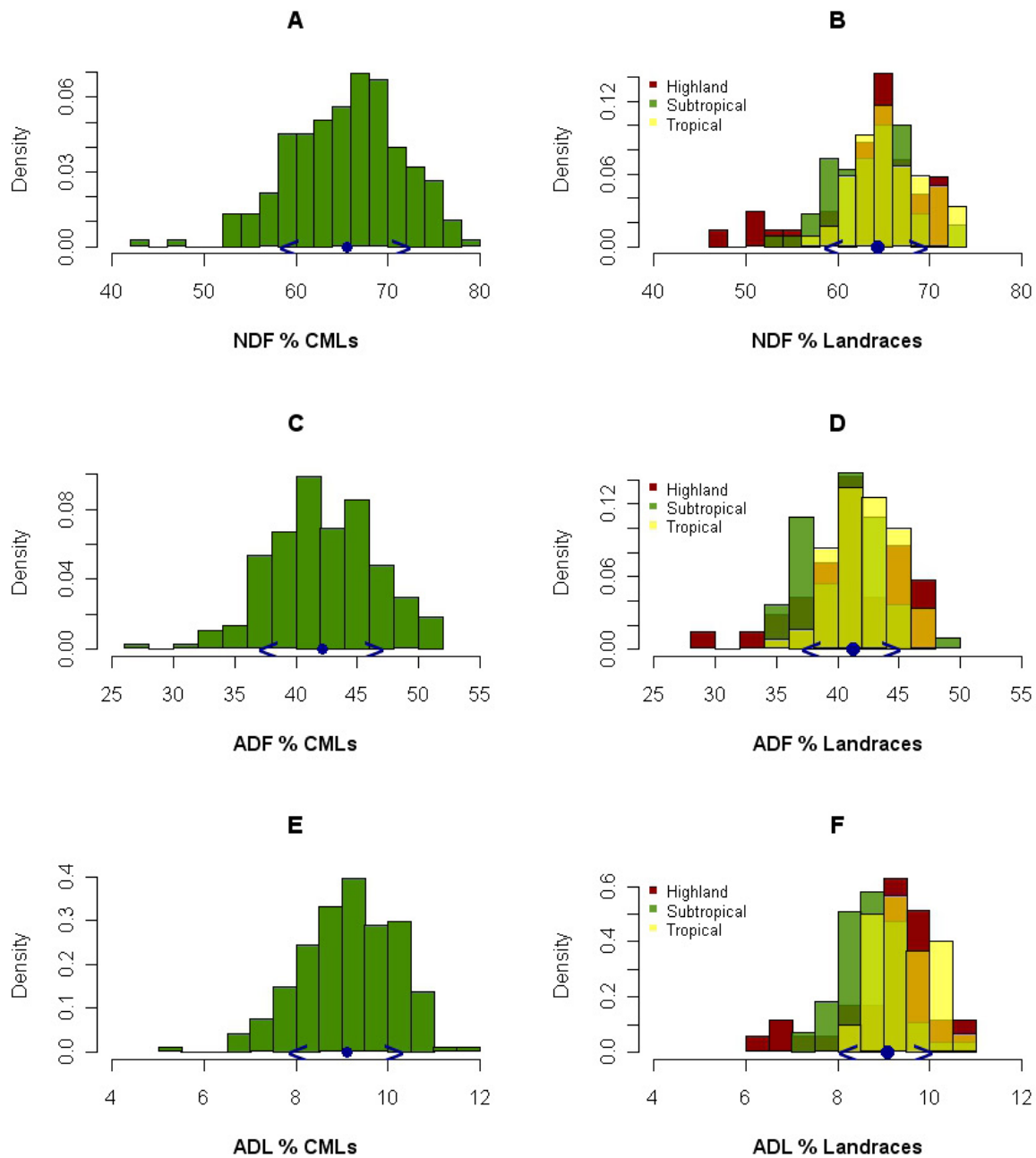
Figure 1 - Variability of cell wall constituents in the 187 CMLs (A, C and E) and 150 landraces (B, D and F). The distribution of each of the three groups of landraces evaluated (highland, tropical and subtropical) is shown. The blue dot and brackets indicate the overall mean and ± one standard deviation from the mean, respectively. NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.

(19°N, 99°03'W; 2,250 masl). Each landrace accession was grown in a single replication using four-row plots, each 5 m long with a target planting density of approximately 53,000 plants ha^{-1}. Additionally, a set of 187 CIMMYT elite inbred lines (CMLs) were grown at AF and TL in 2010 and at TL in 2011. Each CML was grown in a single replication plot using two row-plots of 2.5 m long.

Biomass composition and digestibility analyses

Biomass samples were taken at grain physiological maturity (13-15% grain moisture) (Ritchie et al, 2008) using ten representative plants for each genotype. Samples were gathered by cutting a stalk (i.e. main stem) segment from mid-internode to mid-internode, immediately below the uppermost-ear. This specific tissue was selected based on previously
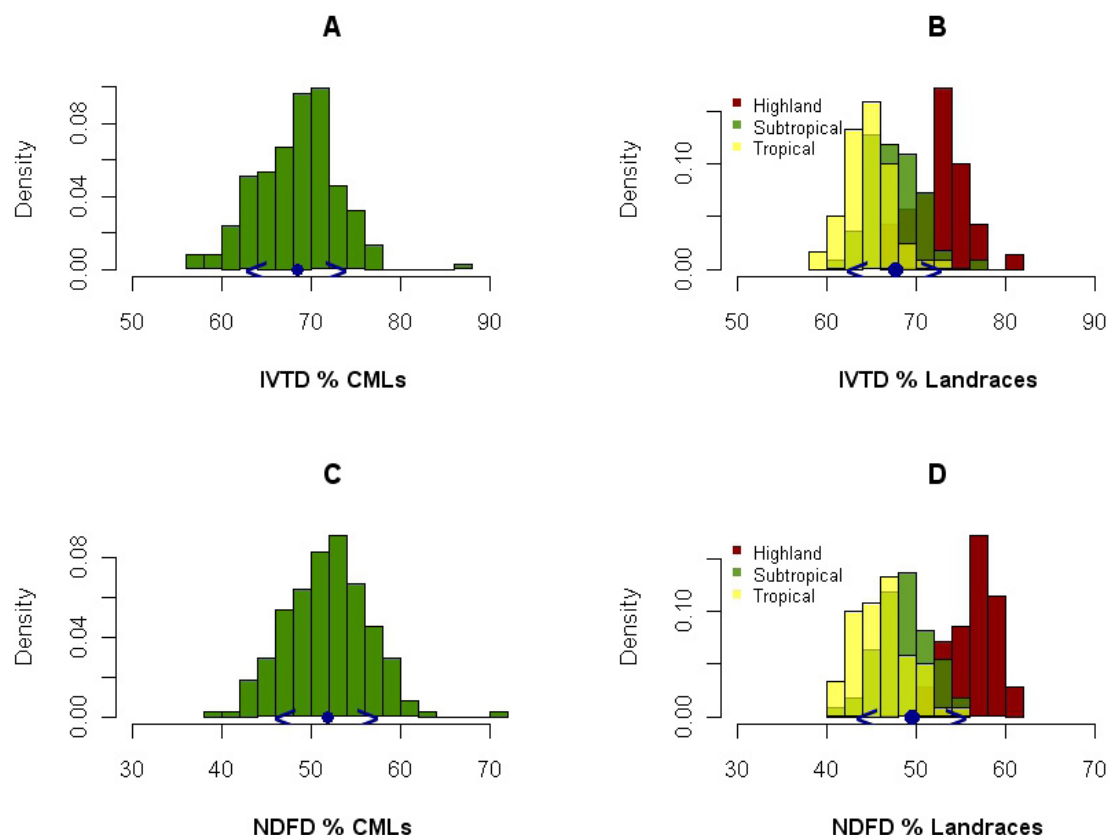
Figure 2 - Variability of digestibility measurements in the 187 CMLs (A and C) and 150 Mexican landraces (B and D). The distribution of each of the three groups of landraces evaluated (highland, tropical and subtropical) is shown. The blue dot and brackets indicate the overall mean and ± one standard deviation from the mean, respectively. IVTD, in vitro true digestibility; NDFD, NDF digestibility.

reported results that show that the maize stalk represents 46-53% of the total stover dry biomass at grain physiological maturity and this tissue component has a significant correlation with whole plant composition at maturity (Hansey et al, 2010; Pordesimo et al, 2005; Garlock et al, 2009; Tolera and Sundstøl, 1999). Biomass samples were dried during seven days at 70°C and ground to pass through a 1 mm screen. Biomass samples were scanned with a NIRSystems 6500 (FOSS, Eden Prairie, MN) near-infrared reflectance spectroscopy (NIRS) unit to develop a calibration set for wet-lab analysis using the SELECT procedure (Shenk and Westerhaus, 1991) of the Infrasoft International software (v. 3.11). A standardized H value (Mahalanobis distance) of 1.0 was used to develop the calibration set. Van Soest detergent analysis (Van Soest, 1994) was used to determine sequentially neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) with the ANKOM-200 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY). Five hundred mg of ground biomass were placed in ANKOM F57 filter bags, which were heat-sealed. The samples were treated with neutral detergent and the dried residue

was weighed to determine the percent NDF. The NDF residue was then treated with acid detergent solution and the dried residue was weighed to determine the percent ADF. The acid detergent lignin (ADL) was determined treating a sample of five hundred milligrams (placed on ANKOM F57 filter bags) with acid detergent followed by extraction with 72% sulfuric acid (H_2SO_4). The dried residue was weighed to determine the percent of ADL. Detailed protocols for these analytical assays can be found at www.ankom.com/analytical-procedures.aspx. *In vitro* true digestibility (IVTD) (Stern et al, 1997) was determined using the ANKOM F57 filter bags filled with 250 mg of ground biomass. The samples were incubated in a mix of rumen fluid from lactating Holstein cows (which were fed on a total mixed ration) and buffer solution. Incubations were performed in the Daisy II Incubator (ANKOM Technology, Macedon, NY). After 48 hours of incubation, bags were washed with neutral detergent solution in an ANKOM-200 Fiber Analyzer to remove rumen fluid particles and non-cell wall materials and the difference in weight was determined. IVTD and NDF values were used to calculate the neutral detergent fiber digestibility (NDFD) following the equa-

tion: NDFD = 100*{[NDF – (100 – IVTD)]/NDF}. NDFD is the proportion of NDF digested during the 48 hour rumen fluid incubation. Each sample collected in the field was analyzed using two technical (laboratory) replications. A standard stover check was used as a reference on the NDF, ADF, ADL and IVTD assays.

Equations used to predict constituent concentrations of the biomass samples were developed using modified partial least squares (MPLS) method and selected based on high coefficient of determination (R^2) and low standard error of cross-validation (SECV) (Table 1). Prediction equations shown in Table 1 follow the D-G-S1-S2 form, where D corresponds to the derivative number, G to the gap between points over which the derivative was calculated, and S1 and S2 are the number of data points used to smooth the data (Shenk et al, 2001). The results shown in Table 1 indicate that all the predictions have substantially large R^2 and low SECV.

Statistical analysis

Analyses of variance (ANOVA) were performed using the anova function of the R software (R Development Core Team, 2012). The linear model specified with the lm R function included the fixed effect of genotype (landrace or CML) and environment (year or year-location). The genotype by environment interaction was used as the error term in the statistical model. The following model was used: $Y_{ij} = \mu + E_i + G_j + e_{ij}$, where Y_{ij} denotes the value of the jth genotype in the ith environment; μ is the grand mean; E_i is the effect of the ith environment; G_j is the effect of the jth genotype, and e_{ij} is the random experimental error. Spearman's rank correlations between environments were performed using the cor.test function of R to determine if environments could be combined for further analysis. Cluster analysis of the phenotypic data based on principal components analysis (PCA) was performed with the prcomp function of R software. The pairwise.t.test function of R software, with Bonferroni correction for multiple testing was used to compare the mean of each constituent between environments. Boxplots and histograms were also drawn using the R software.

Results and Discussion

Cell wall composition and digestibility in Mexican highland, tropical and subtropical landraces

Cell wall composition and digestibility were evaluated using measurements of NDF, ADF, ADL, IVTD and NDFD in a set of 150 Mexican landraces (hereafter refer to as landraces) and a set of 187 elite inbred lines (hereafter refer to as CMLs). All three groups of landraces (highland, tropical and subtropical) showed a substantial range of variability for all constituents evaluated (Figures 1 and 2). The range of NDF variation found in the landrace internodes (46.8 to 72.9%, overall mean 64.3%) was larger than that observed in a previous study of diverse inbred lines derived from temperate ancient landraces (38 to 54.7%, overall

mean 45.0%) in whole plant samples (Barrière et al, 2010).

Highland landraces

For the highland landraces (Supplementary Table 1) we found a weak genotype effect for NDF (p<0.10), not significant effect for ADF (p=0.11) and NDFD (p=0.16), but significant genotype effect for ADL and IVTD (p<0.05). For NDF the variability ranged from 46.8 (landrace Zaca 187, race Tablilla de Ocho) to 72% (Chih 139, race Palomero), with an overall mean of 63.8%. IVTD varied from 66.4 (Pueb 552, race Cacahuacintle) to 81.6% (Zaca 187) whereas NDFD varied from 50.2 (Jali 141, race Complejo Serrano de Jalisco) to 61.8% (Vera 648, race Mushito). Even though the extreme genotypes showed very different values of NDF, IVTD and NDFD, most of the variation in the highland landraces was accounted for by differences between environments (years 2010 and 2011) and not by differences among genotypes. The low mean square genotype observed in the highland landraces was due to the presence of few genotypes with extreme values within this group.

Tropical and subtropical landraces

In the case of tropical landraces, we observed no significant effect of genotype (p>0.10) and a large environmental effect (p<0.001) for all constituents evaluated (Supplementary Table 3). This was partially expected given that in 2011 extreme weather conditions devastated a large number of plots and only 18 of the 60 landraces evaluated were shared between the 2010 and 2011. The rank correlations were not significant (p>0.10) for all components, except NDF (Supplementary Table 4). Given the low number of landraces shared between the two environments, the comparison between genotypes was not performed in this group.

In contrast to highland landraces, tropical landraces had smaller values of IVTD and NDFD (Figure 2). This reflects the fact that tropical germplasm is expected to have lower digestibility due to the acceleration of the lignification process produced by higher temperatures during the growing season (Van Soest, 1994). According to Buxton and Casler (1993), plants grown at high temperatures have higher concentration of cell wall constituents and lower digestibility than plants grown at low temperatures when evaluated at the same physiological stage. Importantly, the inferior biomass quality generally observed in tropical germplasm might be a direct reflection of the growing conditions (i.e. the environment) and may not be an indicative of the absence of favorable alleles that could be potentially used in breeding. Therefore, tropical germplasm should still be considered a potential source of genetic variation that can be utilizing to improve not only tropical but also temperate germplasm.

A significant genotype effect was observed for all constituents (NDF, ADF, and IVTD, p<0.01; ADL and NDFD, p≤0.05) for the subtropical landraces

(Supplementary Table 5), indicating the existence of genetic variation that can be exploited in breeding programs. Interestingly, landrace Guer 230 (race Pepitilla) showed the lowest percents of NDF and ADL (53.8 and 7.3%, respectively) and the largest values of IVTD and NDFD (76.2 and 55.6%, respectively); whereas landrace Jali 87 (race Tabloncillo) had the largest NDF and ADL percents (72.5 and 10.7%, respectively) and the lowest IVTD (60.6%). These results highlight the fact that cell wall carbohydrate concentration and digestibility are highly correlated as discussed later. The subtropical landraces showed convertibility values (IVTD and NDFD) that were intermediate between the tropical and the highland landraces, but more similar to tropical landraces than to highland landraces (Figure 2). There was significant effect (p<0.05) of the environment for all constituents, except for ADF. The rank correlations were significant for all constituents evaluated and ranged from 0.29 to 0.47 (Supplementary Table 6). As observed for the highland landraces, IVTD appears to be quite stable across environments (r = 0.46, p<0.001).

Genotype by environment interaction in landraces

For all the constituents evaluated, we found highly significant effect of the environment (i.e. year effect: 2010 vs. 2011) for the highland landraces. The study of the environment and genotype by environment interaction is of interest to breeders for the development of cultivars that have stable performance across environments (e.g. locations, years, location-year combinations). The environment effect is the result of a combination of interacting components including abiotic (e.g. temperature, light, photoperiod, water and nutrients availability) and biotic factors (e.g. insects, diseases, beneficial microorganisms) that influence plant growth and development and hence, cell wall composition and digestibility. Jung and Buxton (1994) found significant environmental effect for several cell wall composition constituents and in vitro degradability of cell wall polysaccharides in 45 temperate maize inbred lines. A study carried out by Kruse et al (2008) also showed the existence of a moderate effect of the environment on NDF, ADF and ADL. They concluded that the variation in cell wall composition observed was more influenced by the environment than by the genotypic effect. Barrière et al (2010) also found a significant genotype by year interaction for NDF, cellulose and hemicellulose in a study utilizing diverse maize inbred lines derived from landraces.

The landraces evaluated in this study are adapted to specific mega-environments (highland, tropical and subtropical) and therefore they had to be evaluated in locations representing their adaptation requirements. To assess the impact of the environment on cell wall constituents two different years (2010 and 2011) were sampled within each location (BA, AF, and TL) for each group of landraces (highland, tropical and subtropical), respectively. The highland land-

races grown at BA experienced a frost (less than -4 °C) early in the early reproductive stage of the plants. It is worth to notice that the remarkable stressful condition suffered by these plants at BA in 2011 resulted in a significant increase (p<0.001) in NDF values (55.1 vs. 76.5% in 2010 and 2011, respectively) and a significant (p<0.001) reduction in IVTD values (73.4 vs. 71.0% in 2010 and 2011, respectively). This resulted in significantly (p<0.001) larger NDFD values in 2011 (51.9 vs. 62.2%, in 2010 and 2011, respectively), mostly due to the larger NDF values observed in 2011. It has been shown that low temperature stress can result in cell wall thickening, a physiological response to increase plant hardiness (Chalker-Scott and Fuchigami, 1989). An increase in phenolic compounds has been observed as a response to cold stress and acclimation.

The importance of the genotype by environment interaction can be assessed statistically by the Spearman's rank correlations between environments. For highland landraces, these correlations were generally low and not significant, except for ADL and IVTD (Supplementary Table 2). The significant Spearman's correlation (r = 0.35, p<0.10) and the minor differences between environments observed for IVTD, suggest that this constituents is relatively more stable across environments. According to Van Soest (1994) temperature is the main environmental variable that affects forage quality. The highland landraces are generally grown in high altitudes ranging from 2000 to 2700 meters above sea level and are exposed to low temperature during development (mean temperature of 14 °C). These two aspects contribute to a delayed lignification process (Van Soest, 1994). The highland landraces evaluated in this study showed a similar distribution of variability for cell wall composition constituents compared to tropical and subtropical landraces (Figure 1). However, their distribution of IVTD and NDFD is shifted towards higher values compared to the subtropical and tropical landraces. Additionally, highland adapted germplasm tend to accumulate anthocyanin in leaf midribs and stalk pith, which is associated with low lignin contents and high soluble polyphenolic matter (Barrière and Argillier, 1993; Estrada-Flores et al, 2003). Indeed, Van Soest (1994) reported that brown midrib maize mutants, that have higher digestibility values, were originally discovered in a collection of highland Mexican landraces. Thus, highland landraces can be a potential germplasm source in breeding for enhanced biomass quality in maize.

Genetic variation within and between landraces

It has been recognized that the level of variation within a landrace can be larger than the variation among landraces (Reif et al, 2003; Reif et al 2004). In this study, ten plants for each landrace in each environment were collected and pooled to form a single sample. Although this approach does not allow the assessment of variation within a landrace, the overall

mean of a landrace can be used to identify promising landraces to use in future studies. This initial characterization of 150 Mexican landraces will enable selection of a reduced number of landraces for more detailed studies, including individual-to-individual variation within a landrace. This plant-to-plant variation can be exploited in breeding programs through the identification of favorable genotypes and alleles (within a landrace) that can be used in the development of improved open-pollinated varieties and/or inbred lines to be used in hybrid development.
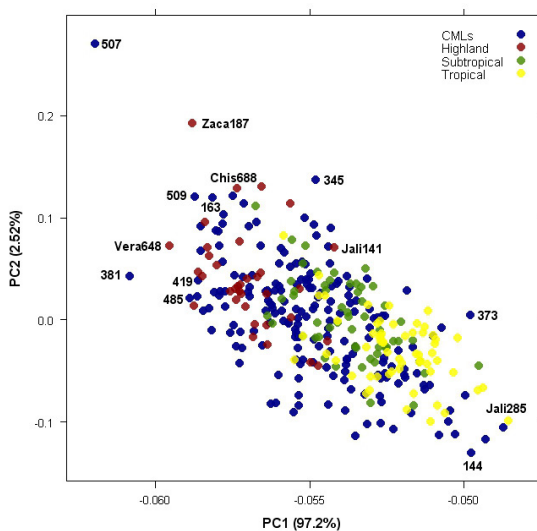
Cell wall composition and digestibility in CML

CMLs are inbred lines derived from different sources of germplasm including tropical lowland, mid-altitude tropical, sub-tropical, and tropical highland lines (Ortiz et al, 2010). CMLs are lines developed from the CIMMYT germplasm based on large general combining ability (GCA) for grain yield and a significant number of important traits such as drought tolerance, nitrogen use efficiency, acid soil tolerance, disease, insect and parasitic weed resistance. CMLs are frequently used as parental lines of hybrids adapted to different maize mega-environments. The CMLs are of main interest given the wide range of adaptation they represent. Currently, there is an increasing interest in broaden the genetic base of maize breeding programs worldwide, not only for enhancing grain yield but also for improvement of silage and stover composition. For instance, maize breeding programs in Europe are broadening the genetic diversity of early-maturing germplasm by introgressing medium-to-late maturity germplasm (Barrière et al 2009). Genetic characterization of the CMLs have highlighted the large amount of allelic diversity of these fixed

lines (Warburton et al 2002; Xia et al, 2004; Xia et al, 2005; Warburton et al, 2008). However, CMLs have not been extensively evaluated for cell wall composition and biomass digestibility. The ANOVA show that there was a significant genotypic effect (p<0.001) for all the constituents evaluated (Supplementary Table 7). These results highlight the fact that there is genetic variation among CMLs that can be exploited and that the environment places a major role in cell wall composition as mentioned before.

Large range of variability was found among the CMLs for all traits evaluated (Figures 1 and 2). For NDF the variability ranged from 42 (CML 507) to 78% (CML 405), with an overall mean of 65.5%, whereas IVTD varied from 57.3 (CML 144) to 87.4 (CML 507). The considerable large levels of IVTD observed in CML 507 are directly related to the substantial low levels of structural carbohydrate content (i.e. NDF). Interestingly, CML 405 is a late maturing line, whereas CML 507 is an early to inter-medium maturity line. In general terms, a late maturity genotype that remains vegetative for a longer period of time will tend to be less lignified than an early-maturity genotype (Van Soest, 1994) at any given point in time. A negative correlation between digestibility and earliness has been found in forage species such as orchardgrass (Lentz and Buxton, 1991). Nevertheless, other studies have found contrasting relationships, depending on the maturity stage at which the biomass was harvested and other management practices (see Buxton and Casler, 1993, for a discussion). Lundvall et al (1994) found an apparently smaller range of variation for in vitro digestibility (from 46.5 to 72.7%) evaluated in stems of 45 maize temperate inbred lines. A 2.2-fold difference between the ADL genotype means was found (from 5.2 to 11.6% ADL, CML 507 and CML 264, respectively). The range of variation observed for CMLs was somewhat larger than the variation for cell wall content found in 45 temperate maize inbred lines –from 52.9 to 77.3% (Jung and Buxton, 1994). The relatively low level of NDF (an indirect way to measure total cell wall concentration) observed in the CML 507 (42.3%) was substantially lower to the values observed in the study of Jung and Buxton (1994). This suggests the existence of genetic variation in non-temperate germplasm that can be exploited in the improvement of forage quality.

There was a significant environmental effect for all the evaluated constituents (p<0.001). The CMLs were grown in two contrasting locations AF (tropical) in 2010 and TL (subtropical) in 2010 and 2011, so it was not surprising to find strong environmental influence in structural carbohydrates and convertibility measurements. Spearman's rank correlations were all significant (p<0.001) and ranged from 0.29 to 0.52 for different combination of environments and constituent (Supplementary Table 8).

The Spearman's correlation between environments (TL2010 vs. TL2011, TL2010 vs. AF2010 and

Figure 3 - Principal component analysis for cell wall and digestibility constituents evaluated in CMLs and landraces. Principal component 1 (PC1) is plotted against principal component 2 (PC2) for 337 genotypes.
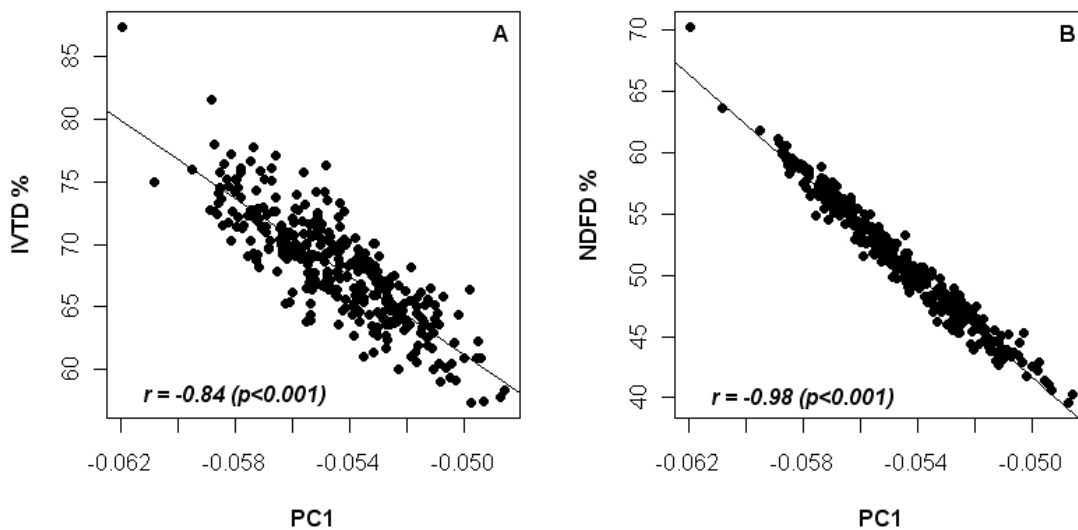
Figure 4 - Pearson's correlation between principal component 1 (PC1) with NDFD (A) and IVTD (B) for landraces and CMLs. A set of 337 the genotypes were evaluated, of which 150 are landraces and 187 are CMLs. Principal components were determined for all constituents evaluated in this study.

AF2010 vs. TL2011) was larger for the two digestibility measurements, IVTD and NDFD, compared to carbohydrate concentration measurements. These results suggest that, as previously mentioned, convertibility measurements such as IVTD and NDFD might be more stable across environments than carbohydrate concentration measurements such as NDF and ADF Improved digestibility has been considered one of the major goals in breeding for enhanced forage quality (Buxton and Casler, 1993). According to these authors, the existence of genetic variation for digestibility is sufficient to warrant the development of genotypes with improved forage quality. The reduced genotype by environment interaction effect on the biomass digestibility of CMLs and the importance of digestibility for increasing forage quality highlights the importance of IVTD and NDFD for the development of maize cultivars with superior characteristics for ruminant feeding, fiber and biofuel production with a potential wide range of adaptation.

The distribution of phenotypic variability for cell wall composition and digestibility observed in the CMLs was similar to the variability observed in the landraces. Interestingly, there was a wider range of variation for all the evaluated constituents in the CMLs compared to the three groups of landraces. The CMLs do not represent a specific adaptation group. They were originated from distinct germplasm groups including tropical lowland, mid-altitude tropical, sub-tropical, and tropical highland lines. These results reaffirm the potential of the CMLs already shown by the genetic studies (Warburton et al, 2008) and it demonstrates that despite CIMMYT breeding programs focus mainly on yield potential, a large level of variation for cell wall composition and digestibility

has been maintained in the development of the CML. These large levels of genetic variation can be further exploited in the development of dual-purpose (grain and biomass) maize inbred lines and hybrids.

Principal component analysis of CMLs and landraces

It was of interest to conduct a multivariate analysis (principal component analysis, PCA) to determine the existence of clusters of landraces (highland, tropical and subtropical) and CMLs adaptation groups (Africa mid-altitude subtropical, Asia lowland, highland, lowland, South America, South America late tropical, subtropical and transition). Figure 3 shows the PCA of the CMLs and all landrace groups (highland, tropical and subtropical). As previously indicated, the CMLs did not form a distinct cluster that can be separated from the landraces in terms of cell wall composition and digestibility but rather they were widely spread along the PC1 and PC2 axes (Figure 3). This again supports the finding that breeding for yield GCA and good agronomic performance has not reduced the diversity for cell wall composition and digestibility constituents in the CMLs. The first principal component (PC1) explained more than 97% of the phenotypic variance (Figure 3). Interestingly, PC1 had a very large (negative) correlation (Pearson's correlation) with IVTD and NDFD (Figure 4), indicating that the digestibility values were sufficient to group the individuals in this study, and that larger values of PC1 were associated with lower digestibility. PC2 explains less than 3% variance, but it has a strong correlation with IVTD ($r = 0.95$, $p<0.0001$). It is important to notice that the clustering of these groups was merely based on biomass quality constituents. It is expected that the use of agronomical traits in the classification of these
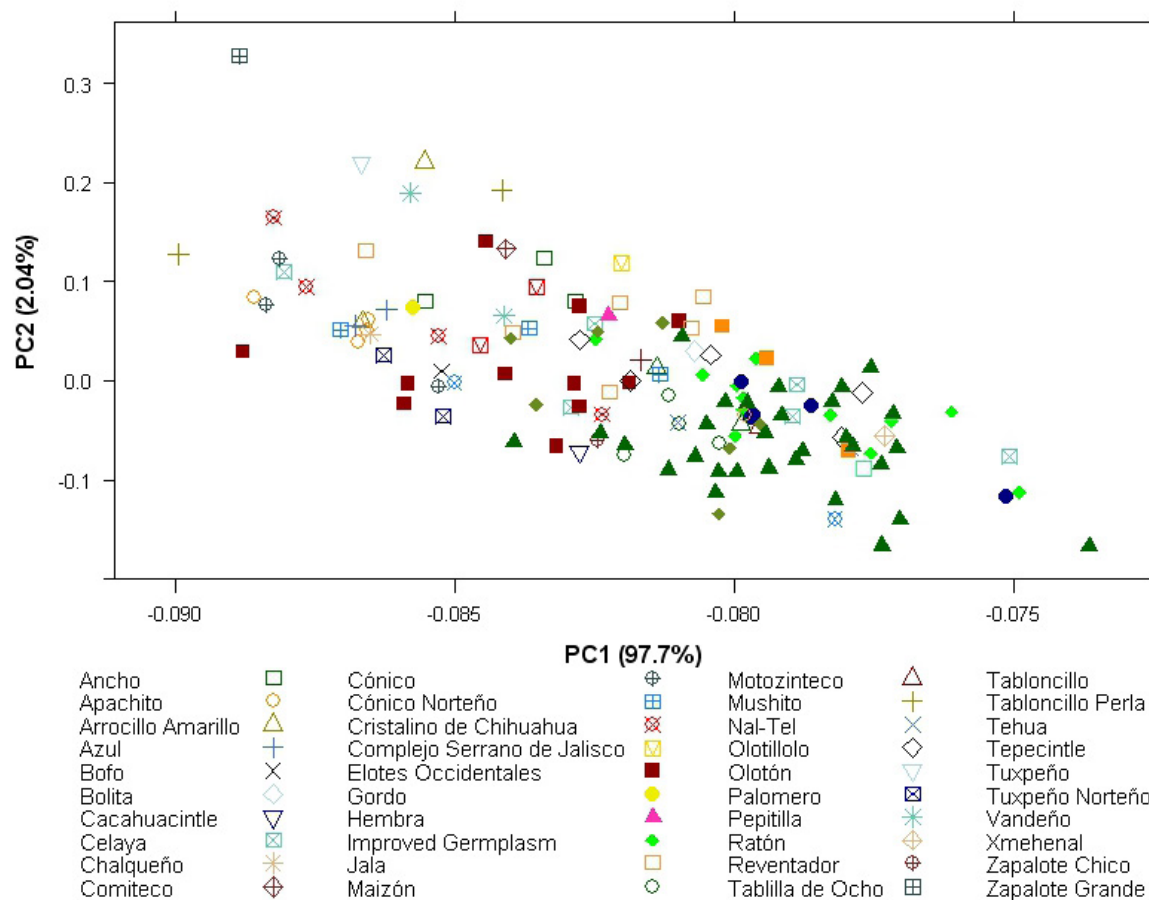
Figure 5 - Principal component analysis of 150 Mexican landraces. The 40 different races evaluated are shown with different plotting symbols and colors.

germplasm groups would provide additional insights.

As depicted in Figure 3, the inbred CML 507 (Africa mid-altitude subtropical group) that showed the lowest values of NDF, ADF and ADL (42.3, 26.5 and 5.2%, respectively) and the largest values of IVTD and NDFD (87.4 and 70.3%, respectively) in the entire set of genotypes evaluated in this study was far apart from the other genotypes. Additionally, inbreds CML 163 (lowland group), CML 345 (lowland group), CML 501 (lowland group) and CML 509 (Africa mid-altitude subtropical) showed low values of NDF, ADF and ADL and large values of IVTD. On the other hand, CML 381 had the second largest NDFD percent of all the lines evaluated, following CML 507. However, this line has relatively large values of NDF (68.4%) and high values of IVTD (75.03%). This suggests that structural carbohydrates of this genotype were easily digested by rumen microorganism, which explains the large cell wall digestibility (NDFD) observed (63.6%). CML 485 and CML 419 were also of interest given their large values of NDFD, NDF and intermediate-to-high IVTD values (~73%). All of these lines are potential germplasm sources for breeding programs focusing on the

improvement of biomass quality. Structural carbohydrate measurements are still relevant because their concentration in hybrids is not strongly associated with that of their parental inbreds (Dolstra et al, 1993; Argillier et al, 2000).

Even though, most of the CMLs with outstanding cell wall composition and digestibility belong to the Africa mid-altitude subtropical and the lowland groups, we did not find any pattern in the principal component analysis of the CMLs (Supplementary Figure 1). The highland landrace Zaca 187 (race Tablilla de Ocho) showed low NDF, ADF and ADL (46.8, 29.5 and 6.1%, respectively) and large IVTD and NDFD (81.6 and 60.8%, respectively). After CML 507, this landrace showed the most interesting cell wall composition and digestibility in the study (low structural carbohydrates and large convertibility values). The landrace Vera 648 (race Mushito) showed relatively large IVTD (75.9%) and very large NDFD (61.8%). Another interesting highland landrace is Chis 688 (race Olotón), that showed low NDF, large IVTD and intermediate-to-high NDFD. The tropical landraces studied were clustered with high values of

PC1 and low values of PC2 (Figure 5). This group is characterized by having higher values of NDF, ADF and ADL and lower values of IVTD and NDFD, as previously observed (Figure 1). This is expected given the tendency of these types of tropical germplasm to tolerate to higher temperatures, which accelerate the lignification processes (Van Soest, 1994; Estrada-Flores et al, 2006). Additionally, lignin has higher energy content than cellulose or hemicelluloses and constitutes a considerable metabolic sink (Novaes et al, 2010). Therefore, the greater solar radiation and higher temperatures of commonly found in tropical environments is likely to favor lignin biosynthesis.

Most of the variation in the landraces PCA was explained by PC1 which again was strongly correlated with NDFD and IVTD (Figure 5, Supplementary Figure 2). The tropical landraces Tuxpeño and Tuxpeño del Norte as well as the improved germplasm evaluated in this study tended to form a cluster that corresponded to lower values of NDFD and IVTD (larger values of PC1 and lower values of PC2, Figure 5). Most of the Improved Germplasm landraces have a large background of Tuxpeño and that helps explains their relatively similar clustering. If breeding for dual purpose maize in the subtropics is the goal, races like Tuxpeño are less desirable given its trend to smaller cell wall digestibility values. However, this landrace is largely recognized and used in breeding programs focused on the improvement of grain yield due to its outstanding performance and grain productivity (Wen et al, 2012; Pena-Ramos et al, 2003). Given that Tuxpeño race has a large potential to improve biomass yield, the information provided in this study can be used for the development of specific crosses to exploit both high biomass yielding landraces and high forage quality landraces to develop cultivars to be used as livestock feed.

It is clear from this study that there is substantial variation to be used in stover quality improvement especially for the subtropical regions. Although some studies have demonstrated the existence of substantial amounts of variation for stover composition in current temperate elite breeding programs (Lewis et al, 2010; Lorenz et al, 2009), the information on diversity found here might provide new sources of variation that could complement the currently available germplasm. Moreover, the results discussed up to this point create the opportunity for further stud-

ies focusing on the identification of molecular markers associated with low lignin accumulation such as COMT enzyme mutations (Barrière et al, 2003; Guillet-Claude et al, 2004), and also for the development of transgenic plants with altered lignin biosynthesis, which has been shown to be effective in increasing the digestibility of maize biomass (Piquemal et al, 2002; He et al, 2003; Pichon et al, 2006).

Correlation among constituents

Previous studies using maize lines derived from landraces have found a strong relationship between cell wall digestibility, measured as *in vitro* dry matter digestibility (IVDMD) and ADL/NDF (R2 = 0.77) (Barrière et al, 2010). Very similar results were found in this study, with linear correlations IVTD-NDF and IVTD-ADL across all germplasm groups of -0.68 and -0.61, respectively (Table 2). The structural carbohydrate concentration measurements, NDF and ADF were strongly correlated with each other, suggesting that NDF can be sufficient for preliminary characterizations of diversity of cell wall content. NDF was highly (negatively) correlated with IVTD but no rank correlation was observed between NDF and NDFD. Similar Pearson's correlations nearly equal to zero were shown between NDF content and cell wall digestibility based on long term in vivo experiments with maize hybrids (Barrière et al, 2004). These results are also consistent with those reported by Casler (2001) that showed that a genetic reduction of the concentration of structural carbohydrates (measured via NDF) resulted in increased in vitro dry matter digestibility. However, the negative correlation between NDF and IVTD reflects a dilution effect, in which the reduction of NDF represents an increase in the proportion of cell content (highly digestible soluble components) which, in turn, results in higher dry-matter digestibility (Casler, 2001). For this reason, Dolstra and Medema (1990), Argillier et al (1995), and Casler (2001) indicate that NDF digestibility is a more useful trait than IVTD as selection criteria to improve cell wall digestibility per se. Thus, selection based on NDFD is likely to affect the structure and/or composition of the cell wall but not its concentration.

Conclusions

Substantial phenotypic variation was observed for all compositional traits evaluated for both CMLs and landraces. The range of variation for NDF was from

Table 2 - Pearson's and Spearman's correlations between constituents evaluated in 187 CMLs and 150 landraces. Pearson's (above diagonal) and Spearman's correlations (below diagonal) between constituents were estimated (n=335). *** p<0.001, ** p<0.01, * p<0.05, ns not significant (p>0.10). NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; IVTD: *in vitro* true digestibility; NDFD: NDF digestibility.

	NDF	ADF	ADL	IVTD	NDFD
NDF		0.96***	0.80***	-0.68***	-0.12*
ADF	0.96***		0.75***	-0.70***	-0.18***
ADL	0.77***	0.72***		-0.61***	-0.19***
IVTD	-0.61***	-0.65***	-0.56***		0.80***
NDFD	-0.04ns	-0.12*	-0.14ns	0.79***	

47 and 73% for the three groups of landraces and from 42 to 78% for CMLs. The distribution of variation for IVTD and NDFD formed three distinct groups, in which highland landraces have larger mean than subtropical landraces and these, in turn, had a larger mean than tropical landraces. The analysis of all 40 races included in this study, showed that the tropical race Tuxpeño, widely used in CIMMYT breeding programs, formed a cluster characterized by high levels of cell wall content and low biomass digestibility.

Of all the constituents evaluated in this study, IVTD was the most stable across environments. Moreover, IVTD and NDFD were the most useful constituents to group landraces and CMLs. These results support the large recognized importance of cell wall digestibility as the major component for the improvement of forage quality.

The large level of variation observed in the germplasm evaluated in this study provides a vast source of untapped genetic variation that can be exploited in the improvement of maize biomass composition for the dual purpose of this crop, food and animal feed. Although, there are other non-food sources for biofuel production, biomass sources with enhanced quality are essential for the sustainable development of bioenergy crops.

Acknowledgements

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A

$r = -0.88 \ (p<0.001)$

B

$r = -0.99 \ (p<0.001)$
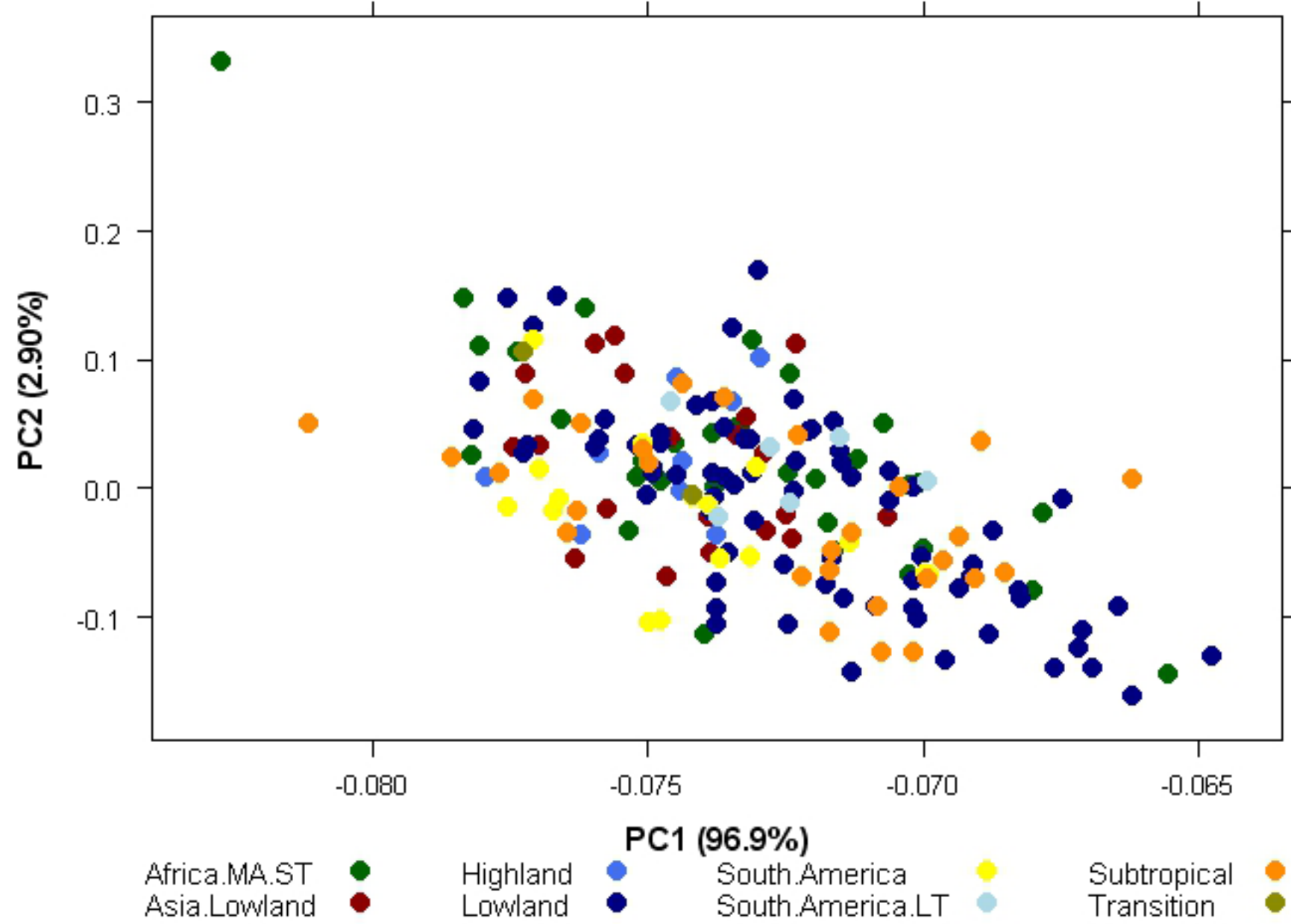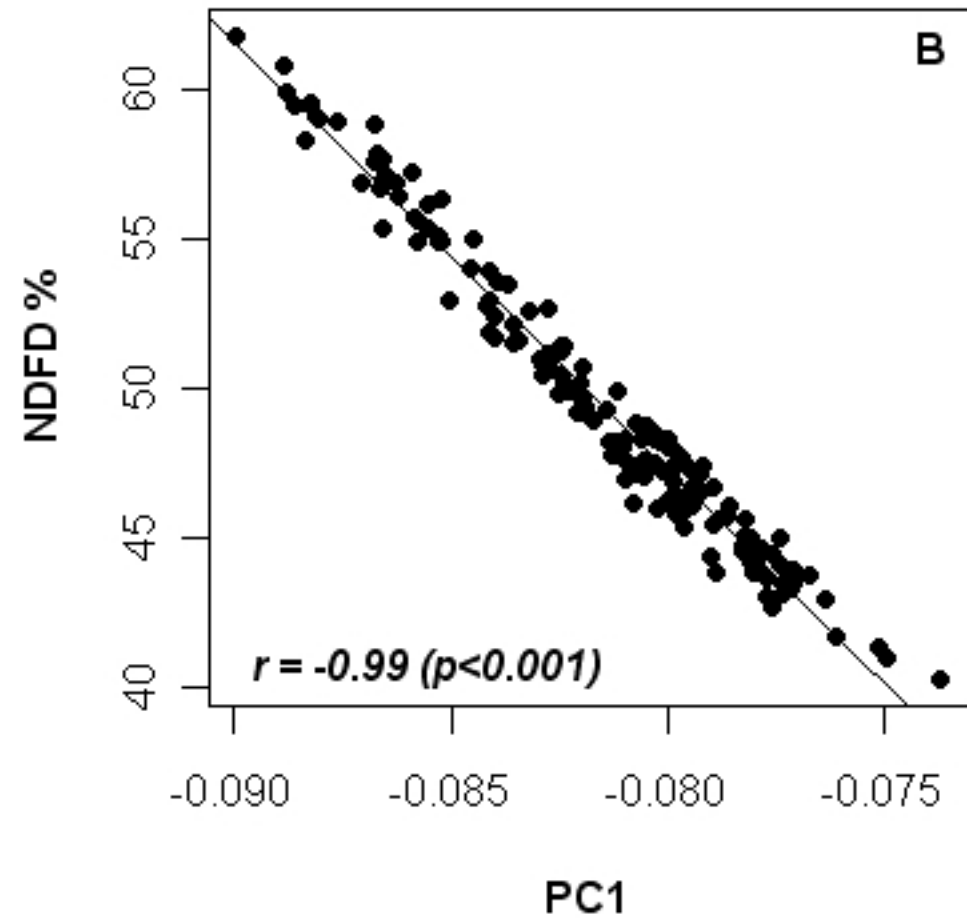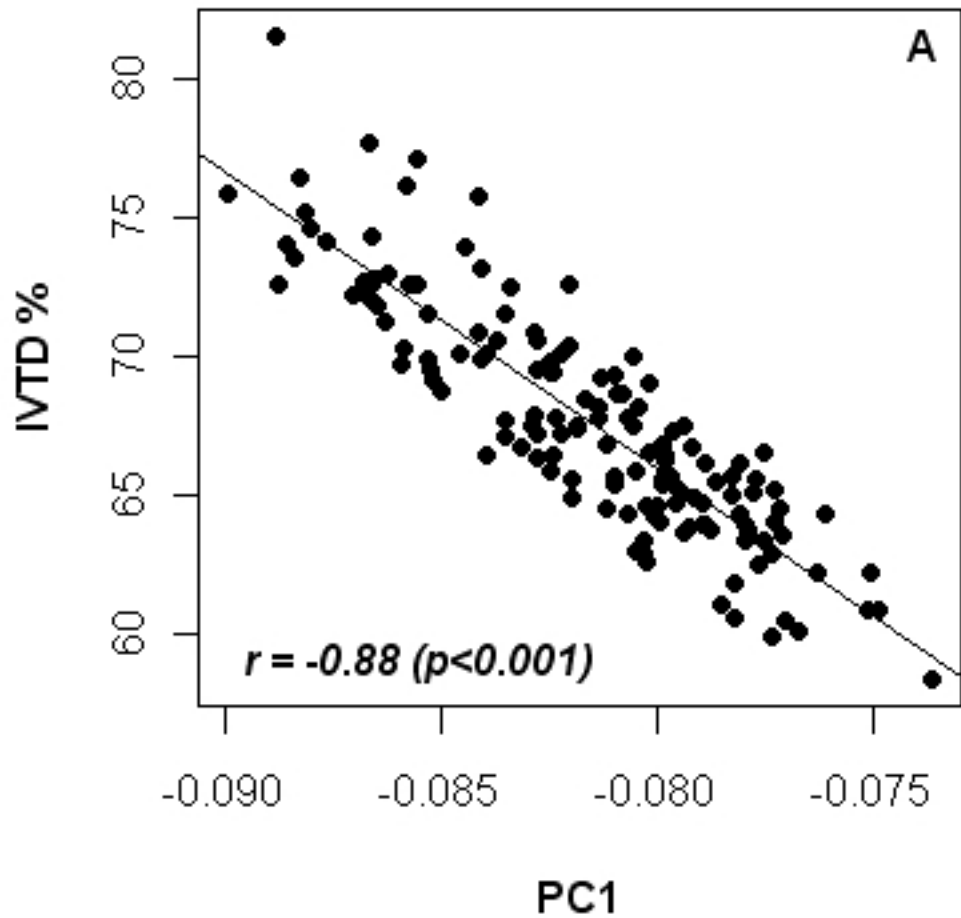
Supplementary table 1. Analysis of variance for NDF, ADF, ADL, IVTD, NDFD and EtOH yield for highland landraces grown at El Batán (temperate environment) in 2010 and 2011. The error term corresponds to the genotype by year interaction. NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; IVTD, *in vitro* true digestibility; df, degrees of freedom.

NDF Source of variation	df	Mean square	F-value	p-value
Year	1	7239.8	560.45	<0.001
Genotype	34	22.2	1.72	<0.10
Error	28	12.9	-	-
ADF Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	2014.52	184.28	<0.001
Genotype	34	17.36	1.59	0.11
Error	28	10.93	-	-
ADL Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	198.35	668.91	<0.001
Genotype	34	0.744	2.51	<0.01
Error	28	0.297	-	-
IVTD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	95.41	14.32	<0.001
Genotype	34	13.85	2.08	<0.05
Error	28	6.66	-	-
NDFD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	1665.94	154.62	<0.001
Genotype	34	15.62	1.45	0.16
Error	28	10.77	-	-

Supplementary table 2. Spearman's rank correlations to assess the level of genotype by environment interaction for each constituent in the highland landraces grown at BA in 2010 and 2011 ($n=29$). $* \ p<0.05$, † $p<0.10$, *ns* not significant ($p>0.10$).

Constituent	BA2010-2011
NDF	0.18*ns*
ADF	0.14*ns*
ADL	0.41*
IVTD	0.35†
NDFD	0.08*ns*

Supplementary table 3. Analysis of variance for NDF, ADF, ADL, IVTD, NDFD and EtOH yield for tropical landraces grown at Agua Fría (tropical environment) in 2010 and 2011. The error term corresponds to the genotype by year interaction. NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; IVTD, *in vitro* true digestibility; NDFD, NDF digestibility; df, degrees of freedom.

NDF Source of variation	df	Mean square	F-value	p-value
Year	1	1022.47	100.4	<0.001
Genotype	59	13.11	1.29	0.29
Error	17	10.18	-	-
ADF Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	230.88	38.88	<0.001
Genotype	59	8.11	1.37	0.24
Error	17	5.94		-
ADL Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	22.54	66.48	<0.001
Genotype	59	0.332	0.98	0.55
Error	17	0.339	-	-
IVTD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	77.71	10.15	<0.01
Genotype	59	8.27	1.08	0.45
Error	17	7.66	-	-
NDFD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	131.75	15.28	<0.001
Genotype	59	12.27	1.42	0.21
Error	17	8.62	-	-

Supplementary table 4 - Spearman's rank correlations to assess the level of genotype by environment interaction for each constituent in the tropical landraces (n=18). * p<0.05, *ns* not significant (p>0.10).

Constituent	AF2010-AF2011
NDF	0.48*
ADF	0.37*ns*
ADL	0.20*ns*
IVTD	0.20*ns*
NDFD	0.28*ns*

Supplementary table 5. Analysis of variance for NDF, ADF, ADL, IVTD, NDFD and EtOH yield for subtropical landraces grown at Tlatizapán (subtropical environment) in 2010 and 2011. The error term corresponds to the genotype by year interaction. NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; IVTD, *in vitro* true digestibility; NDFD, NDF digestibility; df, degrees of freedom.

NDF Source of variation	df	Mean square	F-value	p-value
Year	1	439.41	34.74	<0.001
Genotype	54	28.07	2.22	<0.005
Error	45	12.65	-	-
ADF Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	3.56	0.64	0.43
Genotype	54	15.83	2.85	<0.001
Error	45	5.11		-
ADL Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	50.88	153.39	<0.001
Genotype	54	0.58	1.74	<0.05
Error	45	0.33	-	-
IVTD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	246.24	43.66	<0.001
Genotype	54	12.66	2.41	<0.005
Error	45	5.35	-	-
NDFD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Year	1	55.96	6.26	<0.05
Genotype	54	14.32	1.60	≤0.05
Error	45	8.94	-	-

Supplementary table 6. Spearman's rank correlations to assess the level of genotype by environment interaction for each constituent in the subtropical landraces (n=46). *** $p<0.001$, ** $p<0.01$, * $p<0.05$, †$p<0.10$.

Constituent	TL2010-TL2011
NDF	0.40**
ADF	0.47***
ADL	0.27†
IVTD	0.46***
NDFD	0.29*

Supplementary table 7. Analysis of variance for NDF, ADF, ADL, IVTD, NDFD and EtOH yield for CML (elite lines) grown at three environments (combination of year-location): Agua Fría (tropical environment) 2010 and Tlatizapán (subtropical environment) in 2010 and 2011. The error term corresponds to the genotype by year interaction. NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; IVTD, *in vitro* true digestibility; NDFD, NDF digestibility; df, degrees of freedom.

NDF Source of variation	df	Mean square	F-value	p-value
Environment	2	2378	57.28	<0.001
Genotype	186	87.56	2.11	<0.001
Error	288	41.51	-	-
ADF Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Environment	2	1238.3	59.21	<0.001
Genotype	186	44.66	2.14	<0.001
Error	288	20.91	-	-
ADL Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Environment	2	123.65	99.80	<0.001
Genotype	186	2.43	1.96	<0.001
Error	288	1.239	-	-
IVTD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Environment	2	240.86	15.07	<0.001
Genotype	186	48.17	3.01	<0.001
Error	288	15.99	-	-
NDFD Source of variation	**df**	**Mean square**	**F-value**	**p-value**
Environment	2	679.18	45.76	<0.001
Genotype	186	51.45	3.47	<0.001
Error	288	14.84	-	-

Supplementary table 8. Spearman's rank correlations to assess the level of genotype by environment interaction for each constituent in the CML elite lines. For TL2010-TL2011 $n=129$, for TL2010-AF2010 $n=120$, and for TL11-AF10 $n=159$. *** $p<0.001$, ** $p<0.01$.

Constituent	TL 2010 TL2011	TL 2010 AF2010	AF2010 TL2011
NDF	0.38***	0.33***	0.29***
ADF	0.39***	0.32***	0.32***
ADL	0.36***	0.42***	0.23**
IVTD	0.41***	0.52***	0.41***
NDFD	0.49***	0.46***	0.45***