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Original Research

Classifying Ethiopian Tetraploid Wheat (*Triticum turgidum* L.) Landraces by Combined Analysis of Molecular & Phenotypic Data

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Abstract

The aim of the study was to investigate the extent of the genetic diversity among gene bank accessions of Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces using microsatellite markers, qualitative and quantitative data. Thirty five accessions of Ethiopian tetraploid wheat (*T. turgidum* L.) landraces were grown in the greenhouse at IFA Tulln, Austria during spring 2009 for DNA extraction. The same accessions were already grown in spring 2008 at BOKU Vienna, Austraia for their phenotypical characterisation. DNA was extracted from each approximately one month old plant according to Promega (1998/99) protocol. A total of 10 µl reaction mixture per sample was used for DNA amplification by PCR. The amplified mixture was loaded to PAGE (12%) containing TE buffer (1×) in CBS electrophoresis chambers and run in an electric field for 2 hrs. The fragments were visualized by scanning with Typhoon Trio scanner. Six and ten quantitative and qualitative morphological traits data respectively were used for combined analysis. Genetic variation was significant within and between wheat species and within and between altitudes of collection site. Genetic distances ranged from 0.21 to 0.73 for all accessions while it ranged from 0.44 within *Triticum polonicum* to 0.56 between *T. polonicum* and *T. turgidum*. Genetic distance between regions of collection ranged from 0.51 to 0.54 while for altitudes it ranged from 0.47 (≤2200 m) to 0.56 (≤2500 m). Cluster analysis showed that *T. polonicum* accessions were grouped together whereas *T. durum* and *T. turgidum* formed mixed clusters indicating *T. polonicum* as genetically more distinct from the other two species. We suggest combined analysis of molecular and morphological data for a better classification of accessions.

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INTRODUCTION

Microsatellites are tandemly repeated short DNA sequences that are favoured as molecular-genetic markers due to their high polymorphism index (Mun *et al*., 2006). Tandem repeat in DNA is a sequence of two or more contiguous, approximate copies of a pattern of nucleotides and tandem repeats occur in the genomes of both eukaryotic and prokaryotic organisms (Sokol *et al*., 2006). Microsatellite markers are the best DNA markers so far used for genetic diversity studies and fingerprinting of crop varieties. Microsatellites motifs are conserved in species and their unique behaviour abundance, co-dominance, robustness and easiness for PCR screening make them the best DNA markers for the evaluation of crop genetic diversity. Furthermore microsatellite markers have many advantages for tracing pedigrees because they

represent single loci and avoid the problems associated with multiple banding patterns obtained with other marker systems (Powell *et al*., 1996). However, developing microsatellite markers for a plant species requires prior knowledge of its genomic sequences, lack of which makes this technology very expensive and time consuming (Yu *et al*., 2009).

Microsatellite markers have been applied for genetic diversity studies in many crop plants including wheat (Powell *et al*., 1996; Gupta & Varshney, 2000; Li *et al*., 2002; Röder *et al*., 2002; Alamerew *et al*., 2004; Khlestkina *et al*., 2004; Hailu *et al*., 2005; Teklu *et al*., 2006a; Teklu *et al*., 2007), rice (Zeng *et al*., 2004), pearl millet (Kapila *et al*., 2008), underutilized crop species (Yu *et al*., 2009). Microsatellite markers were also applied for checking the identity of commercial crop varieties and it was proved that the markers correctly identified between different varieties, e.g. varieties of olive for oil production (Pasqualone *et al*., 2007). Diversity studies based on phenotypic traits in Ethiopian wheat species are ample. However, studies based on molecular markers are few (Alamerew *et al*., 2004; Hailu *et al*., 2005; Teklu *et al*., 2006a; Teklu *et al*., 2007).

Combined analysis of data from continuous, ordinal and non-ordinal variables were applied for germplam classification e.g. by Franco *et al*. (1997a), Franco *et al*. (1998) and Tsivelikas *et al*. (2009). According to Franco *et al*. (2001) classifying genotypes into clusters based on DNA fingerprinting and/or agronomic attributes for studying genetic and phenotypic diversity is a common practice. A minimum number of molecular markers combined with morpho-agronomic characters can result in well classified genotypes. Using this strategy Franco *et al*. (2001) found compact and well-differentiated groups of genotypes for maize, wheat and tomato. In the present study data from microsatellite markers, non-ordinal and continuous traits were combined. Studies based on solely phenotypic traits variations may not be sufficient to characterize genebank accessions. Hence, the objective of this study was to investigate the extent of the genetic diversity among genebank accessions of Ethiopian tetraploid wheat using microsatellite markers, qualitative and quantitative data.

MATERIALS AND METHODS

Plant Material

Thirty-five accessions of Ethiopian tetraploid wheat (*T. turgidum* L.) landraces (Table 1) were grown in the greenhouse at IFA Tulln, Austria during spring 2009. Ten seeds per accession were planted in order to have enough plants per accession for DNA extraction. The same accessions were already grown in spring 2008 at BOKU Vienna, Austria for their phenotypical characterisation

DNA Extraction

DNA was extracted according to Promega (1998; 1999) protocol. DNA was extracted from each approximately one month old plant. Ten to fifteen centimetres long young leaves were taken and chopped in 2-ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and left open to dry for four days in plastic bags containing silica gel. The dried leaves were grounded and leaf tissues were lysed by adding 600 µl of nucleic lysing solution to each of the tubes. The tubes were vortexed for 1-3 minutes to wet the cell uniformly and incubated in hot water at 65°C for 15 min. Ribonucleic acids (RNAs) were dissolved by adding 3 µl (4 mg ml^{-1}) RNase solution. Mixing was done by inverting the tubes 2-5 times. The mixture was incubated at 37°C for 15 min and then cooled at room temperature. 200 µl protein precipitation solution was added to each sample and vortexed vigorously for 20 Sec and then centrifuged for 3 min at 16000Xg. The precipitated proteins formed a tight pellet. The supernatant was carefully removed and transferred to another new 1.5 µl micro centrifuge tube containing 600 µl room tempered isopropanol. The solution was gently mixed for each sample by inversion until a thread like mass of DNA strand was visible. Then the mixture was centrifuged at 16000Xg for 2 min at room temperature. The supernatant was carefully decanted for each sample. 600 µl of room tempered ethanol (70%) was added and the tubes were gently inverted several times to wash the DNA and then centrifuged at 16000× g for 2 min at room temperature. The ethanol was carefully decanted and the tube containing the sample was inverted on clean absorbent paper and the pellet was air dried for 15-20 min. 100 µl TE buffer solution was added to re-hydrate the DNA and incubated at 65°C for 1 hr. For subsequent use of DNA in PCR, it was diluted by 1:50 (v/v) DNA/dH_2O.

Table 1. Accession codes and regions and/or altitudes of collection sites of Ethiopian tetraploid wheat landraces.

Accession	Region	Altitude of collection site
T. durum		
5325	Kefa	2667
5613	Shewa	2400
5768	Shewa	2300
5888	Shewa	2920
5982	Shewa	2930
6078	Arsi	2740
6137	Shewa	2670
6915	Gojam	2030
7073	Arsi	2480
7472	Welo	2920
8317	Gamu gofa	2680
T. polonicum		
6102	Shewa	2430
209774	–	–
214370	Shewa	1975
226469-1	–	–
6325-1	–	–
T. turgidum		
5326	Kefa	–
5585	Shewa	2650
5880	–	–
6125	Shewa	2720
6370	–	–
7028	Arsi	2880
7135	Shewa	2820
8085	–	–
8314	Gamugofa	–
204708	Eritrea	2400
226637	–	–
241959	Gojam	2125
241988	Welo	2845
241994	Tigray	2965
241996	Tigray	2445
241999	Shewa	3030
241982-1	Gonder	3080
241990-1	Welo	2445
241997-1	Tigray	2445

Polymerase Chain Reaction (PCR)

A total of 10 µl reaction mixture per sample was used for DNA amplification by PCR. The 10 µl PCR mixture contained 0.025 µl forward primers (10 µM), 0.25 µl (10 µM) reverse primers, 0.225 µl of fluorescent M-13 labelled tail of 10 µM (HEX or FAM), 5 µl GoTaq®Green master mix (Promega Corporation, Madison, USA) (a, b), and 1.2 µl dH_2O. GoTaq®Green master mix (a, b) contains dNTPs (dATP, DGTP, dCTP and dTTP), $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. GoTaq® Green master mix (a, b) (Flanagan *et al*., 2005) is a premixed ready to use solution containing a non-recombinant modified form of TaqDNA polymerase that lacks 5´→3´exonuclease activity. It also contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. PCR program SSR M13 was used for amplification. The following temperatures and times were used for PCR amplification of genomic DNA: (1) 95°C for 2 min (to heat the lid); (2) 95°C for 45 s to denature the double stranded DNA; (3) 68°C for 45 s to anneal the primers to the single stranded DNA; (4) 72°C for 1 min for TaqDNA polymerase to extend the primers. Steps 2 to 4 were repeated for 7 times; (5) 95°C for 45 s to denature the DNA; (6) 54°C for 45 s to anneal the primers to the single stranded DNA; (6) 72°C for 1 min for TaqDNA polymerase to extend the primer ends and steps 5 to 6 were repeated 30 times; (7) further extension of primers was done at 72°C for 5 min by TaqDNA polymerase; (8) finally the reaction was stopped and cooled at 8°C.

Polyacrylamide Gel Electrophoresis (PAGE) and Scanning

The amplified mixture was loaded to PAGE (12%) containing TE buffer (1×) in CBS electrophoresis chambers (C.B.S. Scientific Co., Del Mar, USA) and run in an electric field for 2 hrs. The fragments were visualized by scanning with Typhoon Trio scanner (GE Healthcare Europe GmbH, Regional Office Austria, Vienna).

Microsatellite Loci

Microsatellite loci were selected based on available information. Out of 30 micro satellite loci only 11 of them gave polymorphic bands that can be scored as either 0 or 1. However, the microsatellite markers *Xgwm181* and *Xgwm340* are located on the same chromosome arm, i.e. 3BL, very near to each

Table 2. Repeat number, chromosomal location and genetic diversity for SSR markers.

Primers	Chromosome	Repeat	Annealing temperature	Number of alleles	Genetic diversity
Xgwm294	2A	(GA)9TA(GA)15	55°C	4	0.62
Xgwm495	4B	(GA)20	60°C	5	0.57
Xgwm340	3B	(GA)26	60°C	5	0.41
Xgwm160	4A	(GA)21	60°C	2	0.39
Xgwm135	1A	(GA)20	60°C	6	0.53
Xgwm397	4A	(CT)21	55°C	4	0.61
Xgwm626	6B	(CT)5(GT)13	50°C	5	0.37
Xgwm595	5A	(GA)39imp	60°C	5	0.44
Xgwm400	7B	(CA)21	60°C	4	0.60
Xgwm344	7B	(GT)24	55°C	2	0.09
Total				**42**	**4.63**
Mean				**4.2**	**0.46**

imp, imperfect repeat (Source: Röder *et al.,* 1998)

other (Röder *et al.,* 1998). Hence, only fragments from *Xgwm340* were considered for the analysis. Chinese Spring wheat was used as size standard marker. The microsatellite primers are presented in Table 2.

Molecular and Phenotypic Data

Data from the 10 microsatellite markers were recorded in a binary way (0 or 1). Zero means no allele for the locus while 1 means there is an allele. In total 42 alleles were present. Quantitative data of six morphological traits, i.e. days to heading, spike density, awn length, thousand kernel weight, yellow pigment content and protein content which were used for the combined analysis. Furthermore ten qualitative traits included beak shape, beak length, glume colour, awn color, glume hairiness, seed color, seed size, seed shape, vitreousness and seed plumpness were used.

Statistical Analysis

Gene diversity among accessions for microsatellite markers was calculated according to Nei (1973):

$$Gene\ diversity = 1 - \sum P_{ij}^2$$

where P_{ij} is the frequency of the j^{th} allele for the i^{th} locus summed across all alleles of the locus. The gene diversity coefficient is also referred to as the allelic polymorphic information content according to Anderson *et al*. (1993). Data from SSR marker, qualitative and quantitative traits were combined and analysed modified after Franco *et al*. (1997a). Regions with only a few number of accessions were pooled together and four groups were formed, i.e. Northern (Eritrea, Tigray, Welo, Gonder, Gojam), Central (Shewa) and Southern (Arsi, Kefa, Gamu Gofa) Ethiopia. Accessions with no available information of their original collection site were pooled together in one group. Similarly, altitudes of collection sites were classified as ≤2200 m, ≤2500 m, ≤2800 m, >2800 m and genotypes with no available information. Genetic distances between accessions, within and between species, within and between regions, and within and between altitudes were computed using Gower's distance (Gower, 1971). Using the dissimilarity distances between accessions a GLM analysis of variance was run for species, regions and altitudes to check significances between these effects and in order to obtain means and standard errors. Hierarchical cluster analysis was performed for all genotypes using the dissimilarity matrix of Gower's distance and the Ward fusion method. All analyses were carried out using SAS Vers. 9.1 software (SAS Institute, Cary, USA).

RESULTS

The used microsatellite markers revealed a total of 42 alleles. The number of alleles per locus ranged from two for *Xgwm160* and *Xgwm344* to six for *Xgwm135.* Genetic diversity ranged from 0.09 (*Xgwm344*) to 0.62 (*Xgwm294*) (Table 2). Based on combined data Gower's dissimilarity ranged from 0.21 between *ID 5585* and *ID 241997-1* (*T. turgidum*) to 0.73 between *ID 241982-2* and *ID 209774* (*T. turgidum* and *T. polonicum*, respectively).

Analysis of variance of the Gower dissimilarity matrix showed that the difference within and between species and altitudes were significant (P<0.0001), whereas the differences within and between regions were not significant (P>0.05) (Table 3). Mean dissimilarities within and between species, regions and altitudes are presented in Tables 4, 5 and 6, respectively. At species level the dissimilarity ranged from 0.44 (within *T. polonicum*) to 0.56 (between *T. polonicum* and *T. turgidum*). On the other hand, within species variability was higher for *T. durum* and *T. turgidum* genotypes. Within region dissimilarity ranged from 0.51 for Central Ethiopia to 0.53 for accessions of unknown origin while between regions dissimilarity ranged from 0.51 between Central and Southern Ethiopia to 0.54 between accessions of unknown origin and Northern and/or Southern Ethiopia. Generally, accessions of unknown origin had higher within and between regions dissimilarities. The most probable reason is that these accessions have been collected in different regions of Ethiopia.

Table 3. ANOVA for species, region & altitude.

Source of variation	DF	Mean Square	Pr>F
Species	5	0.045	<0.0001
Region	9	0.008	0.2590
Altitude	14	0.017	<0.0004

For altitude, within altitude dissimilarity ranged from 0.47 (≤2200 m) to 0.56 (≤2500 m) while between altitudes dissimilarity ranged from 0.49 between ≤2200 m and accessions of unknown altitude and between ≤2800 m and >2800 m to 0.55 between ≤2200 m and ≤2500 m. Clustering of genotypes using Gower's dissimilarity matrix grouped the 35 genotypes into 6 subgroups (Figure 1). The most remarkable result of the dendrogram is that almost all *T. polonicum* accessions are grouped together, indicating the indigenous evolution of this tetraploid wheat species. *T. durum* and *T. turgidum* accessions were randomly mixed together throughout all clusters.

DISCUSSION

In the present study of combined analysis of molecular marker and quantitative and qualitative phenotypic data variation within and between tetraploid species of Ethiopian origin was evident. Due to the larger number of *T. durum* and *T. turgidum* genotypes variation within these two species were higher than within *T. polonicum*. Genetic dissimilarity within *T. polonicum* was lower than within the other two species. The lower variation within *T. polonicum* genotypes is most probably due to the fewer number of investigated genotypes and the narrower, more indigenous evolution of this species. Therefore, dissimilarity between *T. polonicum* and the other two species is significantly higher than within dissimilarity. The higher variation within T. durum and T. turgidum and the random mixing of these species in the clusters following cluster analysis of Gower's dissimilarity matrix is not astonishing

Table 4. Genetic dissimilarity within and between wheat species.

Species	Mean	Standard Error
T. durum	0.51	0.010
T. polonicum	0.44	0.024
T. turgidum	0.52	0.006
T. durum vs T. polonicum	0.53	0.010
T. durum vs T. turgidum	0.52	0.005
T. polonicum vs T. turgidum	0.56	0.008

Table 5. Genetic dissimilarity within and between regions.

Region	Mean	Standard Error
Northern Ethiopia	0.52	0.012
Central Ethiopia	0.51	0.011
Southern Ethiopia	0.52	0.017
Unknown origin	0.53	0.017
Northern vs Central	0.52	0.008
Northern vs Southern	0.53	0.009
Northern vs unknown origin	0.54	0.009
Central vs Southern	0.51	0.009
Central vs unknown origin	0.53	0.009
Southern vs unknown origin	0.54	0.011

Table 6. Genetic dissimilarity within and between altitudinal classes.

Altitude (m)	Mean	Standard Error
≤2200	0.47	0.045
≤2500	0.56	0.015
≤2800	0.49	0.020
>2800	0.51	0.013
Unknown altitude	0.53	0.013
≤2200 vs ≤2500	0.55	0.016
≤2200 vs ≤2800	0.52	0.018
≤2200 vs >2800	0.51	0.015
≤2200 vs unknown altitude	0.49	0.015
≤2500 vs ≤2800	0.53	0.011
≤2500 vs >2800	0.53	0.009
≤2500 vs unknown altitude	0.55	0.009
≤2800 vs >2800	0.49	0.011
≤2800 vs unknown altitude	0.53	0.011
>2800 vs unknown altitude	0.53	0.009

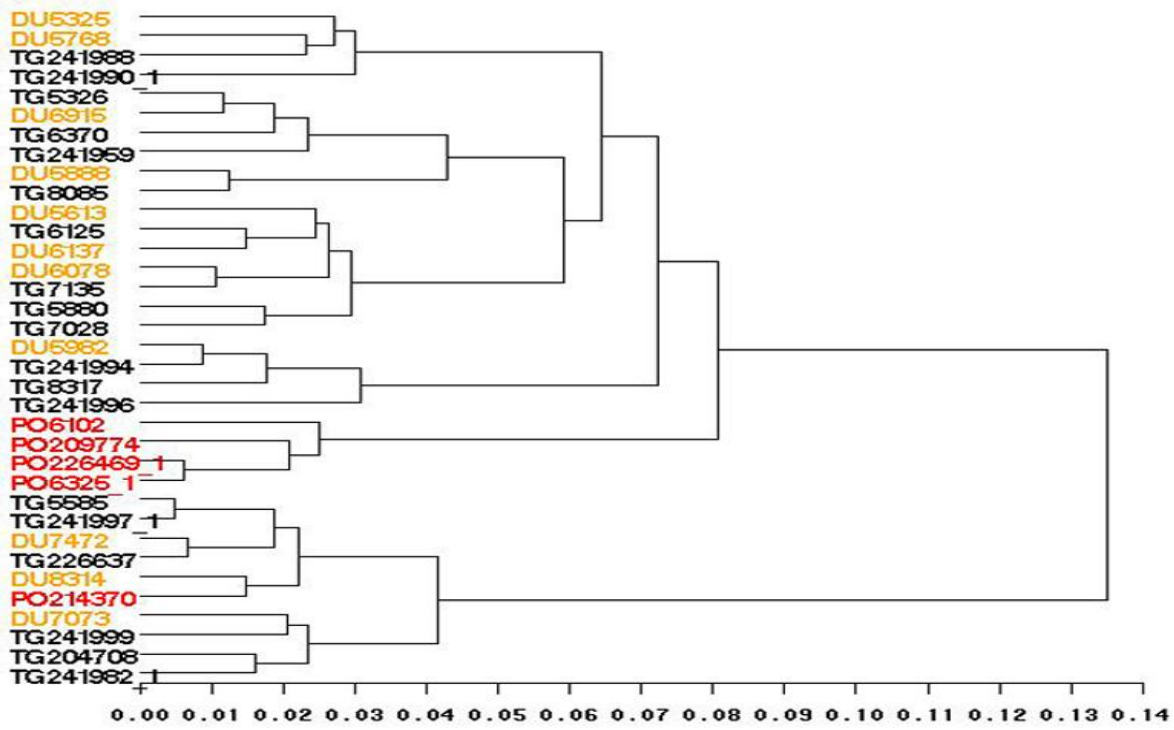

Figure 1. Cluster analysis for 35 genotypes of tetraploid wheats using Gower's distance dissimilarity matrix.

considering the different developments in wheat taxonomy. Dorofeev *et al*. (1979) clearly differentiated between *T. durum* and *T. turgidum* at species level, whereas MacKey (1988) classified durum wheat as convariety of subspecies *turgidum* of species *turgidum*, i.e. *T. turgidum* subsp. *turgidum* convar. *durum*, van Slageren (1994) followed this idea at the subspecies level, i.e. *T. turgidum* subsp. *durum*, and Kimber & Sears (1987) classified all tetraploid wheats with a BA genome as *T. turgidum* (for a *Triticum* comparative classification table see http://www.k-state.edu./wgrc/Taxonomy/comptri.html).

The present data was enough to depict variation within and between species. Using 22 SSR markers Alamerew *et al*. (2004) studied genetic diversity among Ethiopian wheat accessions (*T. aestivum*, *T. aethiopicum* and *T. durum*) and found that all *T. aestivum* accessions grouped together while *T. durum* and *T. aethiopicum* accessions were not grouped into distinct clusters. Using only molecular markers data may not group accessions into the respective species/subspecies level. Combing molecular with phenotypic data might be more promising. Another study by Hailu *et al*. (2005) using 8 ISSR marker showed that genetic distances were higher between *T: turgidum* and *T. dicoccon* but lower between *T. turgidum* and *T. durum* and clustering of genotypes did not completely group according to their region of origin or species level. Using 29 microsatellite markers, Teklu *et al*. (2006a) studied genetic diversity among Ethiopian tetraploid wheat landraces and found a lower genetic distance between *T. turgidum* and *T. durum* compared to *T. turgidum* and *T. dicoccon* or *T. durum* and *T. dicoccon*.

Although within region and between regions dissimilarities were not significant accessions of unknown origin were responsible for higher dissimilarities. The most probable reason for this observation is that these accessions were collected in different regions. From our results we conclude that accessions of the Ethiopian genebank with no available information about their collection site are the most variable group and, therefore, can be valuable sources for crop improvement programmes despite the fact that more or less no passport data about their origin is available. Based on 29 SSR marker Teklu *et al*. (2006b) found highest within region genetic diversity for Shewa (Central) and Gonder (Northern Ethiopia), however, the authors did not find significant correlations between genetic distances and geographic distances. Hailu *et al*. (2005) on the other hand found lower values for between region diversity. However, the latter authors used only a few ISSR markers data for their genetic diversity study. With regard to altitude, in the present study within altitude diversity was highest for ≤2500 m while between altitudes diversity was highest between ≤2200 m and ≤2500 m. This is in agreement with Teklu *et al*. (2006b).

Molecular tools alone may not be sufficient to group wheat species and/or genotypes efficiently. To establish good groupings according to species/subspecies level, pedigree background etc. a large number of molecular data would be needed. For instance Zhang *et al*. (2002) determined the minimum number of SSR markers to completely classify common wheat varieties into parental breeding lines and large-scale breeding varieties. The authors suggested 350 to 400 alleles to be enough to cluster varieties into their respective groups. This high number of needed markers/alleles would be too costly for screening of stored genebank accessions for e.g. the development of core sets. On the other hand, Tsivelikas *et al*. (2009) studied the genetic diversity among squash accessions using RAPD data and morpho-physiological data and they found that the best groupings were obtained when molecular data were combined with morpho-physiological data. Vollmann *et al*. (2005) used both phenotypic and RAPD markers data to analyse genetic diversity in *Camelina sativa* accessions and they found similarities between the two different clustering approaches. In our study, the combined approach better grouped together *T. polonicum* genotypes than molecular or morphological data alone would have done. The uses and preferences of different statistical tools for analysis of genetic diversity in crop plants were reviewed by Mohammadi & Prasanna (2003). The authors stated that each data set (morphological, biochemical or molecular) has its own strengths and constraints and there is no single or simple strategy to address effectively various complex issues related to choice of distance measure(s), clustering methods, determination of optimal number of clusters or analysis of individual, and combined data sets by means of various statistical tools. Crossa & Franco (2004) recommended a two stage sequential clustering strategy using all variables, continuous and categorical, to produce more homogenous groups of individuals than other clustering

strategies. Franco *et al*. (1997b) applied Normix after Ward method for classifying genebank accessions of maize and obtained a good estimation of optimum group number and formation of more compact and separated groups than using only the Ward method. Gutiérrez *et al*. (2003) compared racial classification by visual observation and numerical taxonomy for the classification of maize landraces. The authors found that numerical taxonomy using the Ward-MLM (modified location model) strategy generated more homogenous clusters than the initial racial method.

CONCLUSION

The present data was enough to depict variation within and between species. Combing molecular with phenotypic data might be more promising. Although within region and between regions dissimilarities were not significant, accessions of unknown origin were responsible for higher dissimilarities. The most probable reason for this observation is that these accessions were collected in different regions. From our results we conclude that accessions of the Ethiopian genebank with no available information about their collection sites are the most variable group and, therefore, can be valuable sources for crop improvement programmes despite the fact that more or less no passport data about their origin is available. From the results of the present study the combined use of molecular markers and phenotypic data is suggested as a promising way for the characterization of genebank accessions.

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