Propositions

1 The intermediate types found in durum and turgidum cv. groups and the large overlap in microsatellite alleles between them implies that the groups are not separated and that gene flow between these cv. groups occurs frequently.
(This thesis)

2 An integrated approach that includes the collection of data on morphological characters, storage proteins, and microsatellites and in some cases, chromosome analysis is an effective diversity way to perform diversity assessment in tetraploid wheat.
(This thesis)

3 The variation of storage proteins in Ethiopian tetraploid wheat landraces might be relevant for the food processing technology.
(This thesis)

4 The occurrence of specific translocations in Ethiopian tetraploid wheats makes these chromosomal rearrangements valuable tools for diversity assessment work.
(This thesis and in Taketa S; Kawahari T 1996)

5 The fact that in Ethiopian tetraploid wheat the within accession component of variation is higher than the between accession component can be ascribed to the prevailing Ethiopian agricultural systems.
(This thesis)

6 The origin of wheat with purple seed colour that is unique to Ethiopian high land populations is not well known . This unique trait may favour the assumption that Ethiopian regions could have been one of the centres of origin for tetraploid wheats.
(Zeven AC 1991)

7 The fact that Ethiopia is recognised as the centre of origin for many plants and animals makes it very useful to establish a genetic institute that could study the potential of these genetic resources.

8 The gradual rise in global temperature by altered global weather conditions can be considered as a sudden event that has a negative effect on genetic resources. Therefore proper conservation strategies have to be developed to avoid loss of genetic diversity.

9 In the present world society where parts of the countries invest in the exploitation of resources for future use and other parts are still struggling against poverty, further increase of the economic difference between rich and poor countries seems unavoidable.

10 Fights between groups of people might be considered as a natural phenomenon; however, the fact that this happens at such a wide scale in East Africa might be associated with the high ethnic diversity in that part of the world.

These propositions are part of the thesis, "Multidisciplinary approach in estimating genetic diversity of Ethiopian tetraploid wheat (Triticum turgidum L.) landraces" by Tesfaye Messele, Wageningen University, 18 September 2001.

Multidisciplinary approach in estimating genetic diversity of Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces

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Tesfaye Messele

Multidisciplinary approach in estimating genetic diversity of Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces

Multidisciplinaire benadering in het schatten van de genetische diversiteit van Ethiopische tetraploïde landrassen van tarwe (*Triticum turgidum* L.)

Proefschrift
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Contents

*Abbreviations**

AFLP	Amplified Fragment Length Polymorphism
A-PAGE	Acid - polyacrylamide gels
C-bands	Constitutive heterochromatin bands
CTAB	HexadecylTrimethylammoniumbromide
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
Dst	Diversity between population
FISH	Fluorescence *in situ* Hybridisation
FITC	Fluorescein isothiocyanate
Gst	Coefficient of differentiation
HMW	High molecular weight
Hs	Diversity within populations
Ht	Total diversity
IBCR	Institute of Biodiversity Conservation and Research
ICNCP	International code for nomenclature of cultivated plants
LMW	Low molecular weight
NOR	Nucleolar organiser region
OTUs	Operational Taxonomic Units
PBs	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rDNA	Ribosomal DNA
Rnase	Ribonuclease
SDS-PAGE	Sodium dodecylsulfate - polyacrylamide gels
SSC	Standard Saline Citrate
SSR	Simple sequence repeats
UPGMA	Unweighted pair group method algorisms
UPOV	International Union for the Protection of New varieties
WMS	Wheat microsatellites

*) All abbreviations of morphological traits used in this study are discribed in Table 2-1, page 21.

1.

General introduction

1. Ethiopia: The Impact of geographic, climatic and population factors in vegetation variation

1.1. Geographic and climatic features

Ethiopia is a tropical country located in eastern Africa between 30° and 18° north latitude, and between 33° and 48° east longitude. The country has various geographic features including ragged mountains, hills, flat-topped plateaux, deep gorges, roaring rivers, incised valleys, undulating plains and sharp cliffs, whereas altitude ranges from 110 m below sea level up to 4620 m (Anonymous 1995).

Despite its tropical location, the central and most densely populated part of Ethiopia has a mild climate due to its high altitude, with a mean annual temperature of 18 °C (Anonymous 1995). Rainfall is seasonal and amounts from 500 to 2800 mm per annum. Relative humidity closely follows the rainfall pattern. The latter, along with extreme temperatures of under 10 °C and above 30 °C, together with the geographic variation provide a large diversity in ecosystems, which leads to a large variation in vegetation (Alemayehu 1991).

1.2. Population

Currently, Ethiopia has an estimated population of over 57 million, with more than 70 nationalities, each with their own culture and language. About 88% of the total population are considered as rural dwellers. Environmental factors such as altitude, mild climate, favourable conditions for agriculture such as soil fertility and economic activities influence the settlement pattern of the population. The Ethiopian highlands (above 1800 m), which cover 37% of the total area, are inhabited by about 80 - 88% of the country's population and represent about 95% of the regularly cultivated land. About 75% of the live-

stock are present in these regions (Bekele and Tesema 1997; Anonymous 1986). Dense population of these highlands of the country resulted in over-grazing and severe degradation of the vegetation. The lowlands, being affected by insufficient rainfall and high temperature are sparsely populated. This unequal distribution of human population is one of the important factors that affect productivity of agricultural lands and the conservation and management of biological resources (Anonymous 1995).

2. Ethiopia: a centre of origin and diversity

Cereals, pulses, oil crops, root and tuber crops and other less prominent but potentially important crop species are cultivated for many centuries in Ethiopia. For several economically important cereals such as wheat (*Triticum* spp), barley (*Hordeum* spp) and sorghum (*Sorghum bicolor*), the country is a centre of diversity (Vavilov 1929) and also a centre of origin for crops like anchote (*Coccinia abyssinica*), chat (*Catha edulis*), coffee (*Coffea arabica*), enset (*Ensete ventricosum*), gesho (*Rhamnus prinoides*), gomenzer (*Brassica carinata*), noog (*Guizotia abyssinica*), Oromo potato (*Coleus edulis*) and teff (*Eragrostis teff*). The degree of diversity and distribution within the country including the diverse cultural use of some crops such as tetraploid wheats, barley, cowpea, finger millet, okra, sorghum suggest that these crops are either of Ethiopian origin or have been introduced in the country since ancient times.

Wheat is one of the five major cereal crops grown in Ethiopia. It is grown over a wide range of environments different in soil fertility, incidence of weeds, disease, pests (Hailu *et al.* 1991; Workneh 1989) and water logged conditions. The most suitable areas for its production are between 1900 - 2700 m (Hailu 1991). Estimations during 1987 - 1989 revealed that the area under wheat cultivation in Ethiopia ranged from 700,000 to 750,000 ha, of which about 60 % is covered by durum wheat and 40 % by bread wheat (Hailu 1991; Seme 1991). Durum wheat is usually grown by farmers (Hailu *et al.* 1991) as a mixture of different morphotypes (Abebe and Giorgis 1991). Compared to other cereal species, tetraploid wheat shows an enormous genetic variability, which makes Ethiopia an outstanding centre of diversity (Vavilov 1929).

2.1. Taxonomy

Taxonomically wheat belongs to the genus *Triticum* of the tribe Triticeae within the subfamily Pooideae of the Gramineae family. Seven species of the genus *Triticum* are found in Ethiopia (Tesfaye and Jamal 1982). These include *T. dicoccum*, *T. durum*, *T. turgidum*, *T. polonicum*, *T. aestivum*, *T. compactum* (Vavilov 1929), while the seventh species, *T. pyrimidale* was split from *T. durum* (Abebe and Giorgis 1991). The genus comprises a series of diploid, tetraploid and hexaploid species with x=7. Tetraploid wheat, *T. turgidum* L., is assigned the genome formula AABB, where A and B stand for the putative parental species *T. monococcum* (or *T. urartu*) and *Aegilops speltoides*, respectively. Within tetraploid wheat two

groups, *T. turgidum* and *T. durum* Desf., have been distinguished, which have previously been classified as species, or as intraspecific entities, even of each other (*T. durum* subsp. *turgidum* (L.) V. Dorof, *T. turgidum* subsp. *turgidum* convar. durum (Desf.) MacKey). In view of this extremely unclear taxonomic situation and because these entities are cultivated groups rather than wild taxa, in the present work they will be classified as cultivar-groups within the species *Triticum turgidum*. Following the rules of the International Code for Nomenclature of Cultivated Plants (ICNCP, Trehane *et al.* 1995) their nomenclature thus becomes *Triticum turgidum* Turgidum Group and *Triticum turgidum* Durum Group. Informally, they will be indicated as turgidum or durum cv groups.

2.2. Genetic diversity in Ethiopia Wheats

The genetic variation present, especially in Ethiopian tetraploid wheat is very high. This was already recognised by Vavilov (1929), who observed in his study on Ethiopian wheats a large phenotypic variation within and between populations. The many endemic characters such as violet grained, beardless and half-bearded hard durum types and similar forms of turgidum wheats impressed Vavilov. A study conducted in tetraploid wheat landraces by Ghiorgis and Dibaba (1994) based on the morphological characters of 3300 accessions collected from different regions of Ethiopia showed a wide variation of botanical forms. The highest variability was detected on characters such as spike form and density, colour and size of awn, glume colour, absence or presence of hairs and pigmentation in glumes, kernel colour, size and vitreousness. Tesfaye *et al.* (1991), Getachew *et al.* (1993), Tesfaye *et al.* (1993), Jain *et al.* (1975) and Luciano *et al.* (1996) also confirmed the existence of considerable morphological variability and the uniqueness of certain genetic traits in Ethiopian tetraploid wheats (Melaku 1991; Luciano *et al.* 1992). The results obtained from isozyme studies (Seifu 1996) and chromosome portrait analyses (Getachewl *et al.* 1994) of Ethiopian tetraploid wheats also showed high variation. In addition, the presence of specific translocations confirmed the uniqueness of Ethiopean tetraploid wheats (Kawahara and Taketa 2000).

Variation of Ethiopian wheat landraces in respect to tolerance to various stress conditions including diseases, pests and variation in the content of valuable protein, lysine and other basic amino acids has also been reported (as cited by Abebe *et al.* 1989). The evaluation of resistance and/or susceptibility to two important Ethiopian wheat diseases *i.e.*, rusts and leaf blotches (*Puccinia recondita*, *P. graminis tritici* and *P. striiformis*) in 502 accessions showed that 78.1% of the studied wheat landraces were resistant to all of the three rust types (Hailu 1986).

2.3. The uses of landraces

Landraces are defined as a mixture of morphotypes evolved through human and natural selection (Harlan 1975). Through centuries of cultivation the landraces have developed a broad spectrum of resistance to various biotic and abiotic threats, which makes them very useful as starting material for breeding of modern cultivars with stress resistance and increased yield. Maxted *et al.* (1997) considered six

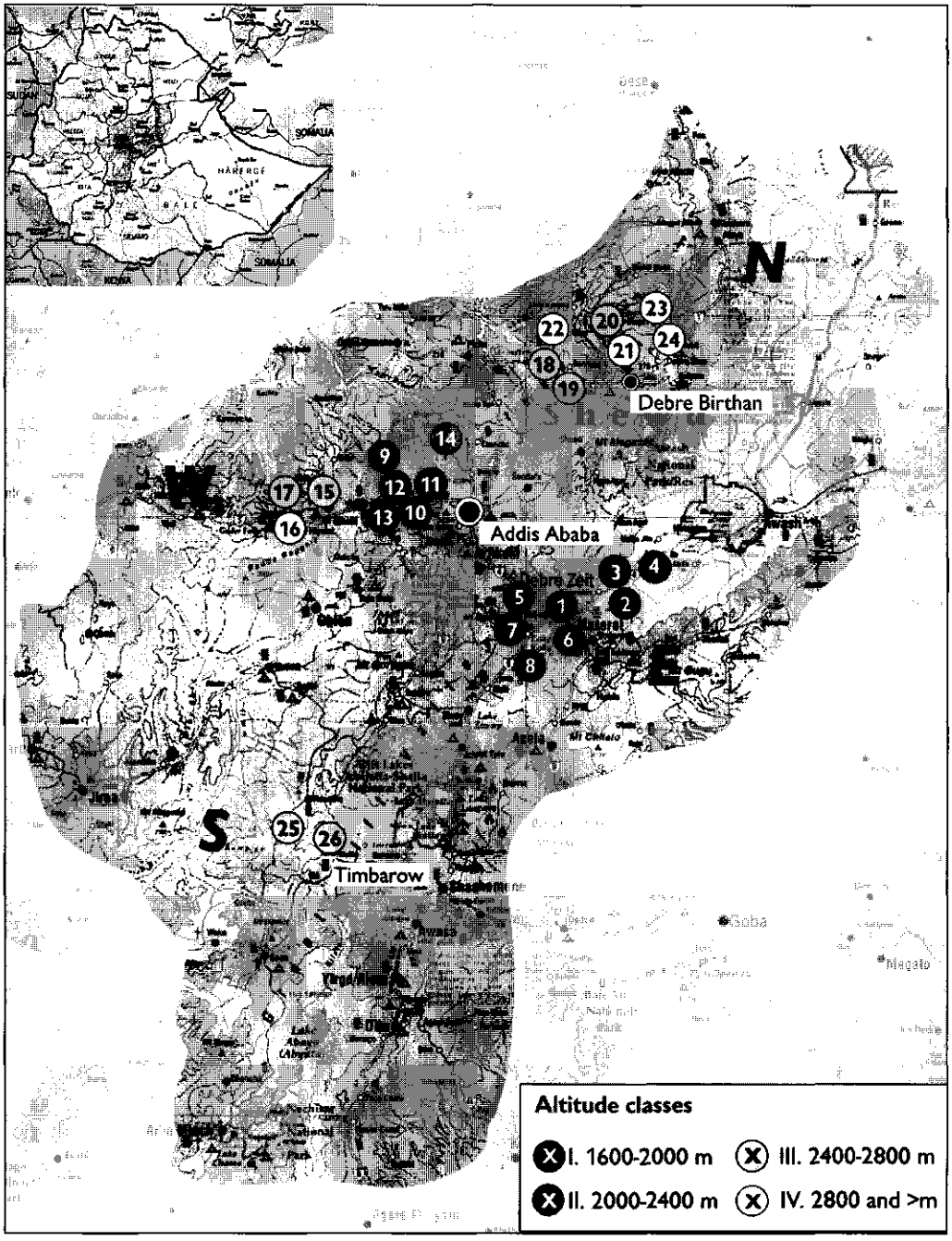

Fig. 1-1. Region of Shewa in the central part of Ethiopia (inset) where the tetraploid wheat accessions were collected. The numbers in the map refer to the collection sites (see table 1-1), the grey hues of the circles denote the altitude class.

Table 1-1. Overview of the accession, with their regions, localities and altitudes of collection. The numbers in column 1 refer to the map positions in Fig. 1-1.

Number	Accession	Region of collection	Locality	Subregion	Altitude
1	5314	Yerer & Kereyu	ADA	East	1771
2	6038	Yerer & Kereyu	ADA	East	1870
3	5180	Yerer & Kereyu	ADA	East	2300
4	5989	Yerer & Kereyu	ADA	East	2250
5	5375	Yerer & Kereyu	AKAKI	East	1675
6	5917	Yerer & Kereyu	LUME	East	2000
7	5921	Yerer & Kereyu	MOJO & WEDERA	East	2400
8	5454	Gebat & Mecha	AMBOZ	West	1772
9	7158	Gebat & Mecha	AMBOZ	West	2235
10	5941	Gebat & Mecha	DENDI	West	2350
11	6000	Menagesha	ALEMGENA	West	2120
12	6158	Menagesha	ALEMGENA	West	2150
13	5441	Menagesha	BEREH	West	2300
14	5588	Menagesha	BEREH	West	2400
15	7190	Menagesha	BEREH	West	2500
16	5473	Menagesha	SULULTA	West	2900
17	55	Tegulet & Bulga	BASOWERANA	North	2400
18	5976	Tegulet & Bulga	MORET & JIRU	North	2650
19	7118	Tegulet & Bulga	MORET & JIRU	North	2720
20	5977	Tegulet & Bulga	ANGOLELA	North	2780
21	5725	Tegulet & Bulga	KIMBIBIT	North	2810
22	6028	Tegulet & Bulga	KIMBIBIT	North	2835
23	5909	Tegulet & Bulga	BASOWERANA	North	3000
24	5908	Tegulet & Bulga	BASOWERANA	North	3020
25	5904	Kembata & Hadia	TIMBARU	South	2440
26	6022	Kembata & Hadia	TIMBARU	South	2590
27	"Boohi"	Ethiopia			
28	"Arendato"	Ethiopia			
29	"Golden Ball"	South Africa			
30	"Capeiti"	Italy			

uses of landraces for use in plant breeding, resistance to disease, adaptation to environment and changing agricultural practices, yield increase, improvement of growth pattern and growth rate of plants. So far only 10% of the total plants in the world were evaluated for their value for agriculture and medicine (Prance 1997), still more plants need to be evaluated for future utilisation.

2.4. Genetic erosion

The loss of biodiversity is irreversible and irreparable, and includes the erosion of the gene pool within a species to its total extinction (Frankel and Bennett 1970). Recurrent drought, displacement of native varieties by higher yielding either native or exotic cultivars, and changes in crop pattern and land use, largely affect genetic diversity by increasing the speed of genetic erosion. Genetic diversity in the Ethiopian landraces was only little affected until the seventies, but the threat of genetic erosion increased tremendously since then (Anonymous 1995). The preference of farmers for crops with high economic return pose the greatest threat to genetic diversity of crops in Ethiopia. Economically important crops like durum wheats replace less profitable crops like teff.

2.5 Institute of Biodiversity Conservation and Research (IBCR)

Genetic erosion requires a strategy for long term preservation of the genetic resources of Ethiopia. As one of the strategies to meet the challenge, the Ethiopian gene bank currently known as Institute of Biodiversity Conservation and Research (IBCR) was established in 1976. Since its establishment this institute has undertaken a series of expeditions to collect and conserve the country's dwindling genetic resources, including wheat. Until now, the institute has built up a collection of over 54,000 accessions covering 101 different crop types of which 12,000 are wheat samples (accessions) collected from various agro-ecological zones. In addition, materials were obtained through either donations or repatriations from various countries (Mehari 1994).

3. Methods for assessing biodiversity

The conservation efforts need to be efficient, based on the knowledge of the genetic structure of the crop and its eco-geographic distribution. Collection and conservation without proper identification is not adequate. This necessarily involves the measurement of genetic variation. Scientific studies of the landraces are becoming exceedingly important and constitute a pre-requisite towards the exploitation of the species in question. Previous studies were focussed on recording morphological information. Such activities include characterisation of the landraces based on morpho-agronomic traits. So far, more than 60% of all accessions, collected or received, have been characterised based on such agro-morphological characteristics (Abebe *et al.* 1989). However, agro-morphological characters alone can not be regarded as ideal tools for landrace characterisation (Ayad *et al.* 1995). The reason for this is that a single description technique discloses a minor part of the whole genome. Moreover, the extent of variation observed in different landraces can differ greatly based on the type of the technique applied. Ayad *et al.* (1995) recommended that for a successful study of the extent of genetic diversity within and between landraces a combination of different methods, preferably a group of techniques, which are based on neutral genetic characters and affecting different parts of a genome, should be employed.

Over the last two decades identification and estimation of the genetic diversity of genetic resources largely involved a combination of morphological methods, along with biochemical techniques like analysis of storage proteins or molecular techniques. Some of the methods frequently used for identification and estimating genetic diversity are described below.

3.1. Morphological methods

Morphological methods are based on measuring visually or rating naturally occurring morphological traits or group of traits. According to Liu (1998) morphological traits often have a one-to-one correspondence with the genes controlling the traits so this makes them reliable genetic markers. Newbury and Ford (1993), and Hardon et *al.* (1994) suggest that, although morphological traits are often influenced by environmental conditions, the method is still useful and easy to apply for classification, estimating diversity and registration of cultivars. So far, morphological methods have been used in estimating diversity within and between populations of tetraploid wheats (Tesfaye et *al.* 1991; Getachew et *al.* 1993; Jain et *al.* 1975; Vavilov 1951).

Morphological traits can be divided into discontinuous characteristics, in which traits are independent from environmental factors, and continuous ones, which usually vary with differing environments. Both types are heritable, but the discontinuous traits are favourable in providing more reliable characterization and identification of genotypes. Since most morphological analyses can make use of only a limited number of discontinuous traits, additional continuous traits have to be included.

Morphological methods are important in classification and identification between groups of closely related plants. For example the durum group and the turgidum group are classified on the basis of morphological traits. Turgidum morphotypes are characterised by short awed lemmas, compressed spikes, usually narrow spike face, strongly curved or geneculate beak shape, while for durum long awed lemmas, lax to compressed spikes, strait to moderately curved glume beak are typical.

3.2. Chromosome marker methods

3.2.1. C-banding

The C-banding technique is based on differential resistance to alkaline denaturation and SSC re-annealing of heterochromatic regions, and gives complex banding patterns in the chromosomes of most cereal species with relatively large genomes (rye, wheat, barley, oat and maize). Their darkly stained Giemsa bands are generally confined to telomeres (rye), pericentromeres (A- and D-genome wheat species) or interstitial regions (barley, B genome of wheat). The reliable and highly reproducible patterns thus can distinguish rye and wheat genome chromosomes in triticale (rye x wheat hybrid). However, the degree of polymorphism is in most cases not sufficient to detect chromosome band polymorphism at the level of subspecies and cultivars. Other banding techniques like N-banding, H-banding and DAPI-fluorescence emerged, but their application for cereal chromosomes is still limited.

3.2.2. *Fluorescence* in situ *Hybridisation*

New cytogenetic techniques, however, which have been developed in the last two decades, proved to be reliable markers for taxonomic research. The more recent fluorescence *in situ* hybridisation techniques of total genomic DNA and repetitive DNA families further improved the possibilities of chromosome and genome identification, even at the level of subspecies and cultivars.

Far more versatile are the DNA : DNA *in situ* hybridisation protocols for locating specific DNA sequences on the chromosomes and nuclei. Especially the introduction of different probes detected and visualised with their own fluorochrome (multicolour fluorescence *in situ* hybridisation, FISH) has now become the most powerful strategy for discriminating parental chromosomes and chromosome regions.

3.3. Molecular methods

Molecular markers based on DNA restriction and PCR technologies have become available for assessing genetic diversity (Russell et *al.* 1997). RFLPs, RAPDs and other marker technologies displaying DNA polymorphisms revealed minor differences in DNA sequences, thus allowing the direct comparison of related genomes and circumventing any environmental effect on gene expression. The most important aspect of such markers based on DNA polymorphism are likely the nearly unlimited numbers of such markers present in a genome and the relative easiness of analysing them on a large scale. However, the low sensitivity of RFLP compared to PCR based methods and costs of the RFLP technology, together with the insufficiently reproducibility of the RAPDs are major drawbacks and make them less suitable. More appropriate are the AFLP and SSR (microsatellite) technologies.

3.3.1. *AFLP*

AFLPs, which enable the analysis of large numbers of randomly distributed DNA sequences in a single PCR reaction, makes it possible to survey the entire chromosomal complement more efficient than ever before. The technique provides in general 30 - 100 genomic bands per lane on high-resolution polyacrylamide gels (Vos et *al.* 1995) and was found in a comparison of genetic variation among barley cultivars to give the highest diversity index when AFLPs were compared to RFLPs and RAPDs (Russell et *al.* 1997). In another study in barley it was found that this marker technology was highly effective in distinguishing genotypes from geographically distinct areas (Pakniyat et *al.* 1996).

3.3.2. *Microsatellites*

Microsatellites or SSRs (Simple Sequence Repeats) are based on repeat length variations of simple sequences, generally between 2 - 8 base pairs. They occur in large numbers in the genome and are distributed on all chromosomes. Their presence has been well documented for many species including cereals (Plaschke et *al.* 1995; Röder et *al.* 1995). Their robustness and large allelic variation make microsatellites ideal markers for population and evolutionary genetic studies (Bruford and Wayne 1993). SSRs are identified by PCR using synthetic oligonucleotides from the surrounding monomorphic

DNA as primers. Primer matching these unique flanking sequences constitutes a single locus, which is often multiallelic by variation of copy numbers in the tandem repeats.

3.3.3. Storage proteins

Storage proteins are based on the identification and analysis of glutenin and gliadin storage proteins of wheat. Plant materials such as seeds, can be analysed to establish the identify of the genotype. The storage proteins are less influenced by environmental conditions than the morphological characters and have the advantage that they can be analysed in a relatively quick and easy way. The disadvantage can be their insufficient differences between genotypes (Hardon et *al.* 1994).

4. Outline of the thesis and general objectives

1. To assess the magnitude of genetic diversity within and between selected accessions of Ethiopian tetraploid wheat landraces.
2. To identify the pattern of genetic diversity within and between geographical areas of collection
3. To compare the outcome of the analyses of
   - Morphological traits
   - Karyotypes including Giemsa C-banding and FISH of tandem repeats
   - Molecular markers (AFLP and microsatellites)
   - Seed storage proteins
4. To make an overall evaluation of the different methods for the assessment of biodiversity of the Ethiopean tetraploid wheat accessions.

2.

Morphological diversity in Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces

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Abstract

To estimate the level of diversity within and between accessions twenty-six accessions and two cultivars from the Ethiopian National Gene Bank, together with two other cultivars from the Netherlands Gene Centre were examined. Each accession was represented by fifteen OTUs (Operational Taxonomic Units), which were reared in the green house in triplicate, each replicate containing five OTUs. The diversity for the 450 OTUs thus representing all 30 accessions was established using 14 morphological traits, which were classified into 3-7 continuous or discontinuous classes. The within and between accession variation was calculated with the Shannon-Weaver diversity index. In addition, UPGMA dendrograms were constructed to distinguish redundancy and to observe level of similarity between the 30 accessions. We observed duplicate OTUs within accessions in five cases, and between accession in 21 cases involving 45 OTUs. Out of the 30 accessions used for this analysis 12 of them showed high Shannon-Weaver diversity values. Diversity compared between durum and turgidum cv. groups based on trait variation showed that in 11 traits durum had higher Shannon-Weaver diversity values than turgidum, while for turgidum relatively higher values were found for the traits Coleoptial AnthoCyanin pigment, CAC (0.79), Days to maturity, MTD (0.59) and Glume Beak Shape, GBKS (0.70).

Among the four altitude classes, class IV (above 2800 m) demonstrated a relatively high Shannon-Weaver value (0.41). High diversity was also observed for the subregion South (0.46). The OTUs dendrogram showed high similarity between OTUs from the same accessions compared to those from different accessions. The dendrogram constructed showed that similarities between the 26 accessions is in the range of 80 to 90%. The relationship between the durum and turgidum cv. groups was established by calculating and analysing principal components (PCA), in which the first three PCs accounted for 49.8% of the total variation. The two cv. groups plotted against PC2 and PC3 had the best separation of durum and turgidum compared to PC1 and PC2, and PC1 and PC3. Five traits, *i.e.*, awn length, awn colour, lower glume shoulder width, glume beak shape and lower glume shoulder shape, were considered major factors for separation of the durum and turgidum cv. groups.

This study showed that the morphological traits are helpful in identification of the closely related cv. group durum and turgidum and for estimating diversity between accessions and to indicate geographical trait distributions.

Keywords

Triticum durum, *Triticum turgidum*, accessions, Ethiopian tetraploid wheat, biodiversity, morphological markers

Introduction

The Ethiopian National Gene Bank, currently known as Institute for Biodiversity Conservation and Research (IBCR), has collected over 54,000 accessions of 101 plant types. In this institute the 12,000 wheat landraces belong to the major groups of its collection. It is unlikely that in this self-fertilising species all accessions are different, although much variation is present (Jain *et al.* 1975; Bekele 1984; Dominici *et al.* 1988; Tesfaye *et al.* 1991). Since Ethiopian farmers used to grow mixtures of various different botanical forms the occurrence of redundancy within an accession is likely relatively high (Tesfaye *et al.* 1991). Thus for sustainable collection and preservation, diversity studies by using efficient and accurate marker tools become imperative. For this purpose, morphological characters have been successfully used (Getachew *et al.* 1994; Afshan and Ahsan 1991; Fekadu 1995; Ghiorghis and Hailu 1988; Jain *et al.* 1975; Spagnoletti and Qualset 1987, 1990) and are still in use as markers for estimating diversity among groups of wheat plants. Newbury and Ford-Lloyd (1997) mentioned that, although some of the morphological traits are influenced by environmental conditions, the use of these characters has played a central role as a tool in the characterisation, evaluation and analysis of variation in landraces.

The morphological characters that are frequently used to characterise wheat traits are ear form, glume colour, spike density, awn length and colour, degree of glume hairiness, seed characters such as seed colour, size, virtuousness, and flowering time and maturity (Tesfaye 1991b; Tesfaye *et al.* 1993; Porcedu *et al.* 1994; Getachew *et al.* 1993; Davis and Heywood 1963). These descriptors enabled estimations of diversity and classification of closely related types such as durum and turgidum cultivar groups. The turgidum group features less bearded and lax to compressed spikes, a narrow ear width, short or absent awned lemmas, flat spikes, overlapping to single glume rows, usually curved glume beaks and single seeded glumes (Grubben and Soetjipto 1996). The durum cv. group usually displays longer awned lemmas and has straight to moderately curved glume beaks.

The aim of this study was to estimate the diversity of the 450 OTUs representing 26 landraces and four modern cultivars using fourteen morphological traits. Our study focused on the identification of two closely related cv. groups, durum and turgidum. We also detected the extent of diversity between these two cv. groups and between geographical areas like between and within four altitude classes and between and within four subregions.

Materials and Methods

Twenty-six tetraploid wheat (*Triticum turgidum* L.) and four cultivars (two Ethiopian, "Boohai" (Et1), "Arendato" (Et2) and two non-Ethiopian cultivars: South African "Golden Ball" (Er1) and Italian "Capeiti" (Er2) received from CEN (Centre for Genetic Resources, the Netherlands) were studied (see Table 1-1 in Chapter 1). The twenty-six wheat accessions are from the collection of the Institute for Biodiversity Conservation and Research (IBCR). The IBCR collections are from farmers fields, threshing areas, farmers stores etc. For this study landraces collected from the central region of Shewa, which was subdivided into four subregions, were used (see Fig. 1-1 in Chapter 1). Accessions representing each

region and altitude were randomly selected. Each altitude class is comprised of accessions collected within the range of 400 m *i.e.*, 1600-2000, 2000-2400, 2400-2800 and above 2800 m. Each accession represented by the selfed progeny of fifteen randomly selected seeds, hereafter called OTUs (Operational Taxonomic Unit), was grown in a green house of Wageningen University starting from April 1999. Each line was replicated three times in a randomised complete block design (total 450 OTUs).

The morphological study was conducted using fourteen morphological traits, out of which twelve were ear characters and two were flowering and maturity time. (See Table 2-1). This classification was based on the descriptor list produced by the International Union for the Protection of New Varieties of Plants, UPOV (Anonymous 1994).

Data analysis

To estimate the variations within and between accessions, and within and between altitude classes and within and between sub-regions we calculated Shannon-Weaver diversity index (H) as:

$$H = -\sum_{i=1}^{n} p_i \ln p_i$$

Where n is the number of phenotypic classes for a particular trait and p_i is the proportion of the total number of entries (n) in the i^{th} class. Ht was calculated for each trait and for all accessions, by dividing H by the logarithm of the number of trait classes.

Hs is the within component of variation and calculated as

$$Hs = Ht - Dst$$

Dst is the between accession component of variation and calculated

$$Dst = Ht - Hs$$

Gst compares the within and the between components

$$Gst = \frac{Dst}{Ht}$$

The phenetic relationships between the OTUs and between the accessions were analysed by constructing UPGMA dendrograms. The relation between durum and turgidum was visualised on the basis of the 14 morphological traits and a Principal Component Analysis (PCA). For PCA we used data from all 450 OTUs, while for the UPGMA dendrograms we used only the 150 OTUs that we selected for the other markers (AFLP, microsatellites and storage proteins). The significant level for trait distribution by altitude class and subregions were computed with the standard χ^2-test.

Result

Duplicates

We observed only five cases of identical OTU pairs (duplicates) within five different accessions. For 45 OTU duplicate pairs (20 cases) and triplicates (1 case) were found in 24 different accessions representing a total of 21 morphological different OTUs.

Shannon-Weaver accession and trait variation

The total average diversity value (Ht) over all traits and accessions was 0.80. The mean diversity value for accessions ranged from 0.02 to 0.58. Twelve out of 26 accessions showed significantly high Shannon-Weaver diversity values ($P<0.05$). The highest mean diversity value was observed in accession 6022 and 5921, followed by the accessions 5441 and 6028. Two accessions (5314 and 7118) showed a very low Shannon-Weaver diversity value (0.02).

Regarding trait variation Shannon-Weaver diversity values ranged from 0.22 to 0.53. Of the 14 traits the highest Shannon-Weaver diversity value was observed for the trait KRL (0.53), followed by LGSS (0.43). The Shannon-Weaver diversity value for the trait CAC showed the lowest variation (0.22) compared to the other thirteen traits used in this study.

Percentage trait occurrence by trait classes

To determine the pattern of each trait class occurrence, the percentage trait class distribution was calculated by pooling each trait over all the 26 accessions and displayed in table 2-1. For some traits the percentage occurrence of classes is highly pronounced, for example in the case of flowering date and kernel colour where a class with medium flowering date (FLD, 71.0%) and kernel colour (71.8%) showed a higher percentage occurrence as compared to other classes of these traits.

Table 2-1. Descriptor used for estimating morphological diversity in Ethiopian tetraploid wheat accessions, trait diversity by number of classes and percentage occurrence of trait classes

Trait	Observed classes	Occurrences (%)
Coleoptial anthocyanin pigment (CAC) *Presence/absence and extent of coloration at seedlings stage*	1. Absent/very weak 2. Weak 3. Medium 4. Strong/very strong	41.8 23.9 10.0 24.4
Days to flowering (FLD) *Number of days from sowing to ear emergence*	1. Early (35-45 days) 2. Medium (46 -56 days) 3. Late (> 56 days)	16.4 71.0 12.6
Days to maturity (MTD) *Days from sowing to complete physiological maturity (yellowish uppermost internode) occurred.*	1. Early (65-70 days) 2. Medium (71-75 days) 3. Late (> 76 days)	41.3 56.2 2.6

Ear density (ERD) *The number of spiklets at 10 cm internode of the rachis in central part of the spike*	1. Very lax 2. Lax 3. Medium 4. Dense 5. Very dense	36.9 26.7 16.4 19.2 0.8
Ear length (ERL) *Length in cm from base to top of the spike*	1. Short (< 4 mm) 2. Medium (4-6 mm) 3. Long (6-8 mm) 4. Very long (8-10 mm)	1.3 33.1 58.7 6.9
Ear color (ERC) *Color at maturity*	1. Yellow to white 2. White to yellow 3. Brown to red 4. Red to brown 5. Dark brown or black 6. Yellow and dark	62.8 4.1 10.0 21.3 0.5 1.3
Awn length (AWL) *Length in cm from base to top of awn*	1. Short (< 3 mm) 2.Medium (3-6 mm) 3. Long (6-9 mm) 4. Very long (> 9 mm)	15.9 18.2 59.2 6.7
Awn color (AWC) *Color at maturity*	1. White to yellow 2. Red to brown 3. Purple to black 4. Yellow to white	55.1 12.6 1.3 31.0
Lower glume shoulder width (LGSW) *Width in profile*	1. Absent 2. Very narrow 3. Narrow 4. Medium 5. Broad 6. Very broad	26.4 3.9 25.4 29.2 6.4 8.7

Lower glume shoulder shape (LGSS) *Shape in profile*	1. Sloping 2. Slightly sloping 3. Strait 4. Elevated 5. Strongly elevated 6. Elevated and sloppy 7. Strongly elevated with second point present	17.7 2.3 43.9 5.4 11.3 4.6 14.9
Glume beak shape (GBKS) *Shape in profile*	1. Strait 2. Slightly curved 3. Moderately curved 4. Strongly curved 5. Geniculated 6. Strongly geniculated	40.0 1.8 25.9 8.5 8.7 15.1
Spikelets per spike (SPLsp) *Number of spikelets per spike*	1. 1 - 10 2. 11 - 20 3. 21- 30 4. 31 - 40	2.6 44.9 49.2 3.3
Kernel length (KRL) *Length in millimeters* *(Longitudinally measured)*	1. Very short (< 5 mm) 2. Short 5 - 7 mm 3. Medium 6 - 8 mm 4. Long (> 8 mm)	5.9 19.5 44.9 29.7
Kernel colour (KRC) *Colour of mature seed*	1. White to gray 2. Red to brown 3. Light to dark purple	71.8 14.4 13.6

*) The descriptor list used for this study was adopted from the manual used by the international union for the protection of new varieties of plants (UPOV).

Variation within durum and turgidum types

Table 2-2 presents Shannon-Weaver diversity values calculated to compare durum and turgidum variation by using fourteen morphological traits within each cv. groups. In all traits, except CAC, MTD and GBKS, the total diversity in durum is higher compared to turgidum.

Principal component analysis

The principal component analysis (Fig. 2-2) was carried out to distinguish the extent of variation or similarity and divergence between the 450 OTUs. This analysis did not effectively reveal the relationships. Instead it showed good separation between the durum and turgidum cv. groups. The first three principal components accounted for 21.74, 15.92 and 12.15, respectively, explaining 49.8% of the total variation (Table 2-3). Here we see that the five characters that showed relatively high factor loading on these PCs, *i.e.*, AWL, AWC, LGSW, LGSS and GBKS can be considered the main characters separating the durum and turgidum groups. This separation is obvious in the scatter plot of PC2 and PC3 (Fig. 2-2). The PC also showed the clustering of few durums to the turgidum cv. group and turgidum to durum cv. groups, indicating that they are intermediate types. The four cultivars were dispersed in different directions within the PC.

Table 2-2. Durum and turgidum total (Ht) Shannon-Weaver diversity index values for fourteen morphological traits.

trait	Durum	Turgidum
CAC	0.75	0.79
FLW	0.80	0.74
MTD	0.55	0.59
ERD	0.74	0.66
ERL	0.77	0.74
ERC	0.69	0.52
AWL	0.80	0.71
AWC	0.75	0.64
LGSW	0.88	0.79
LGSS	0.88	0.40
GBKS	0.69	0.70
SPLsp	0.88	0.81
KRL	0.88	0.78
KRC	0.76	0.39
mean	0.77	0.68

Variation by geographic areas

a. Variation by altitude classes

The 26 accessions were subdivided into four altitude classes. Shannon-Weaver diversity index values per altitude class were calculated and compared (Fig. 2-1a). The mean diversity indexes for Shannon-Weaver per altitude class were 0.32, 0.35, 0.32 and 0.41, showing, except altitude class IV (>2800 m), no significant difference between altitude classes.

b. Trait variation by altitude classes

Average Shannon-Weaver trait variations by altitude classes were calculated to compare the pattern of diversity within the four altitude classes. For altitude class I five traits (ERD, AWL, LGSW, LGSS and KRL), for altitude class II two traits (LGSS and KRL), for altitude class III four traits (LGSW, GBKS, KRL and KRC) and for altitude class IV six traits (ERD, ERC, AWL, LGSW, GKKS and KRL) out of the fourteen traits had significantly high ($P<0.05$) Shannon-Weaver diversity index values.

Table 2-3. Morphological traits showing highest factor loading for the first three principal components

Traits	Principal components		
	1	2	3
	Factor loading		
CAC	-0.28	-0.26	0.04
FLW	0.31	0.19	0.24
MTD	0.32	0.08	0.26
ERD	0.37	0.18	0.19
ERL	-0.34	0.17	0.00
ERC	-0.24	0.24	0.11
AWL	0.06	0.45	-0.38
AWC	-0.14	0.48	0.10
LGSW	0.28	-0.02	-0.40
LGSS	0.36	-0.13	-0.35
GBKS	0.09	-0.29	0.57
SPLsp	0.19	0.35	0.17
KRL	-0.10	0.33	0.17
KRC	-0.36	0.05	-0.03
Percentage per PC	21.74	15.92	12.15

Percentage total variation for the first three principal components amounted 49.81%.

The within altitude class percentage trait distribution

The distribution of OTUs among trait classes was significantly associated with their distribution over altitude and subregion groups (Table 2-4).

c. Variation by subregion

Fig. 2-1b presents the Shannon-weaver diversity values calculated for accession and pooled for the four subregions (East, West, North and South). The values were calculated to determine the level of variation per and among subregions. The total Shannon-Weaver means diversity calculated per subregion

were: East (0.34), North (0.31), South (0.46) and West (0.37), showing no significant difference in variation between subregions.

d. Trait variation by subregions

For subregion East ten (FLD, MTD, ERD, ERL, AWL, AWC, LGSW, LGSS, SPLsp and KRL), for subregion North ten (FLD, ERD, ERL, ERC, AWL, AWC, LGSW, LGSS, GBKS and KRL), for subregion South ten (MTD, ERD, ERL, AWC, GBKS, LGSW, LGSS, KRC, KRL, SPLsP) and for subregion West nine traits (ERL, ERC, AWL, LGSW, LGSS, GBKS, SPLsp, KRL and KRC) out of the fourteen traits observed have significantly higher ($P<0.05$) Shannon-Weaver diversity values.

The within subregion mean percentage trait class distribution

The within subregion mean percentage trait distribution by subregions that were calculated to determine the distribution of trait classes by subregions showed in all traits the distribution is significantly different and some traits like FLD for class medium in subregions West and South, and trait KRC for class white to gray in subregion West and North even displayed highly significant values. The 93% trait occur-

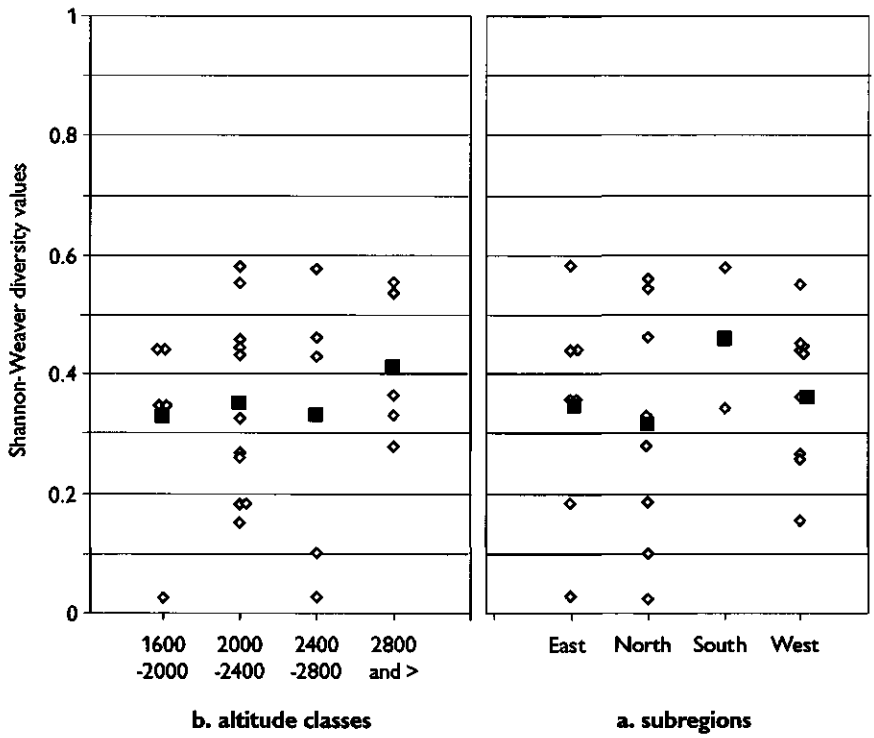

Fig. 2-1. Distribution of Shannon-Weaver indexes for accessions grouped in the four different regions and altitude classes. Average values are represented by solid squares.

rence in trait ERC for class yellow to white shows the tendency of fixation of this class by subregion in this case for subregion South (Table 2-5).

Dendrograms

Dendrograms constructed to depict the relation among the 150 OTUs and among the 30 accessions are presented in the Figs. 2-3 and 2-4. The dendrogram for the 150 OTUs was subdivided into two main groups (I and II) in which group II is composed of a subgroup (IIa) containing three cultivars and three landrace OTUs (Fig. 2-3). Clustering by cv. group is less pronounced, except in few cases where durum and turgidum OTUs clustered together. Three of the control cultivars (Er1, Er2 and Et1) showed close similarity among themselves, while the group of Et2 cultivar clustered in subgroup II.b.2.2. There is no apparent clustering according to region or altitude classes.

The dendrograms for 30 accessions (Fig. 2-4) revealed two main groups. The three cultivars Et1, Er1 and Er2 showed close clustering in the same group. No clustering for altitude classes or subregions was observed.

Discussion

The morphological method helped to distinguish between the closely related durum and turgidum groups (Table 2-2 and Fig 2-2) and also enabled to observe duplicate OTUs within accessions. Although OTUs from the same acessions could be distinguished most of the times, the morphological analysis did not revealed a strong differentiation between regions and altitude classes. That flowering and maturity time (FLW, MTD) showed low diversity values can be explained by the fact that growing of the Ethiopian tetraploid wheats is depended on a short period of seasonal rain water

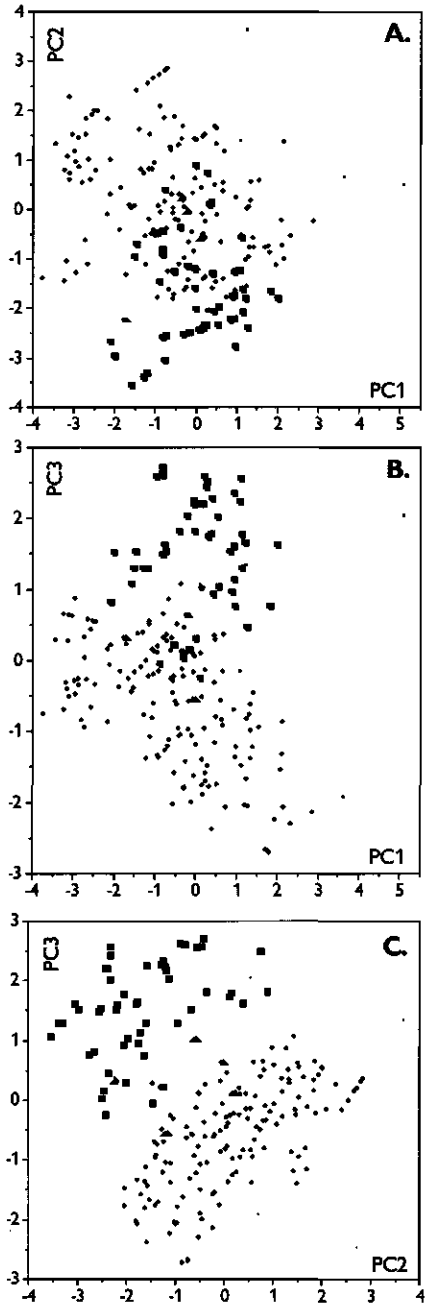

Fig. 2-2. Scatterplot of the 450 OTUs plotted against PC1 by PC2 (A); PC1 by PC3 (B), PC2 by PC3 (C), displays the pattern of durum (dots) and turgidum (squares) relation. The triangles and small spheres are the cultivars.

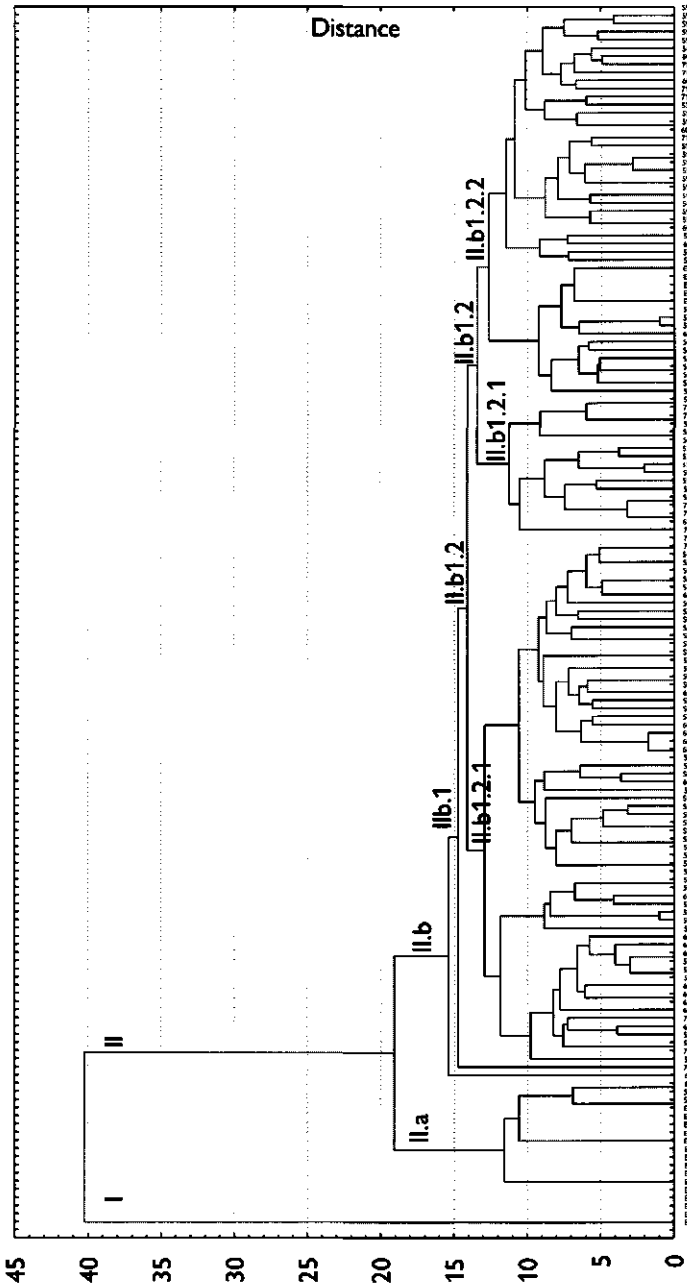

Fig. 2-3. UPGMA dendogram of the 150 OTUs constructed on the basis of 14 morphological traits. The letters D, T and X stand for durum, turgidum and intermediate type, respectively.

Table 2-4. Percentage trait class (see table 2-1) occurrence by altitude classes

trait & classes	Altitude classes (%)				trait & classes	Altitude classes			
	1	2	3	4		1	2	3	4
CAC					LGSW				
1	55	28	35	65	1	16	33	43	7
2	15	33	31	7	2	8	0	12	0
3	9	6	6	23	3	21	30	16	28
4	21	33	28	5	4	31	22	12	61
FLD					5	9	10	0	3
1	47	8	8	12	6	15	5	17	1
2	47	76	87	69	LGSS				
3	7	16	5	19	1	24	15	8	28
MTD					2	8	2	55	35
1	37	36	39	59	3	23	53	13	3
2	63	62	61	32	4	0	5	0	25
3	0	2	0	9	5	9	9	4	0
ERD					6	7	6	0	4
1	24	45	36	32	7	29	10	20	5
2	31	20	21	43	GBKS				
3	12	18	19	15	1	56	42	29	31
4	33	16	20	11	2	0	4	0	0
5	0	0	4	0	3	28	33	16	17
ERL					4	5	3	9	23
1	0	3	0	0	5	4	5	20	9
2	44	37	33	13	6	7	12	25	20
3	56	52	53	83	SPLsp				
4	0	8	13	4	1	13	0	0	0
ERC					2	41	52	49	28
1	47	68	73	41	3	45	48	47	59
2	47	7	0	5	4	0	0	4	13
3	7	10	12	9	KRL				
4	0	12	12	44	1	3	5	8	9
5	0	0	3	0	2	13	19	27	20
6	0	3	0	0	3	51	52	48	21
AWL					4	33	25	17	49
1	11	17	35	0	KRC				
2	19	10	9	45	1	79	68	60	85
3	68	60	53	55	2	5	18	20	11
4	3	13	3	0	3	16	14	20	4
AWC									
1	40	65	72	31					
2	5	12	15	19					
3	0	3	0	0					
4	55	19	13	51					

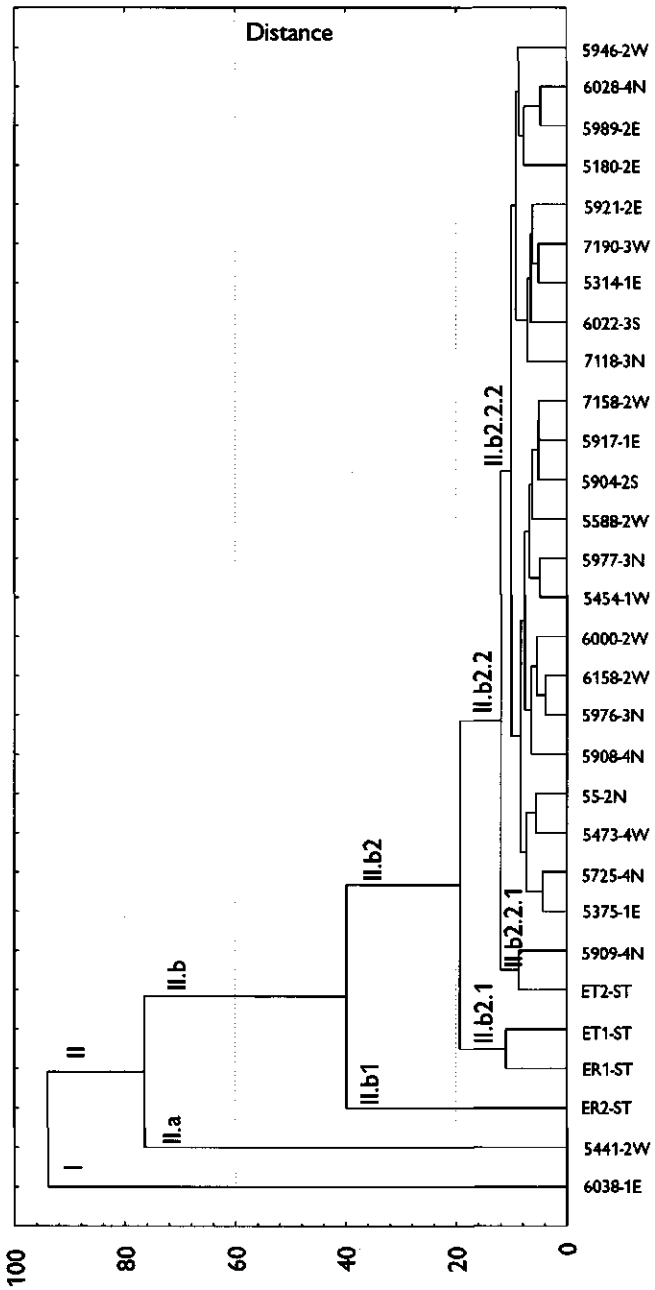

Fig. 2-4. UPGMA dendogram of the 30 accessions constructed on the basis of 14 morphological traits. The letters W,E, S, and N refer to the subregions West, East, South and North, respectively.

Table 2-5. Percentage trait class (see table 2-1) occurrence by subregion classes

trait & classes	Subregion classes (%)				trait & classes	Subregion classes (%)			
	E	W	N	S		E	W	N	S
CAC					LGSW				
1	32	36	63	17	1	30	26	24	23
2	43	16	18	17	2	2	3	2	23
3	5	13	14	0	3	28	24	32	0
4	20	36	5	67	4	19	36	32	27
FLD					5	9	7	2	17
1	23	15	11	23	6	12	5	9	10
2	64	78	68	77	LGSS				
3	13	7	21	0	1	14	23	19	0
MTD					2	4	4	0	0
1	40	42	35	67	3	50	34	55	23
2	60	56	59	33	4	4	4	4	23
3	0	2	6	0	5	8	19	8	0
ERD					6	5	4	3	17
1	28	54	31	17	7	16	13	11	37
2	19	21	33	53	GBKS				
3	19	11	17	30	1	57	44	29	7
4	34	14	17	0	2	0	5	16	0
ERL					3	26	25	14	70
1	0	0	4	0	4	4	7	18	10
2	40	41	25	7	5	3	7	0	0
3	55	50	68	73	6	10	13	23	13
4	5	9	3	20	SPLsp				
ERC					1	10	0	0	0
1	56	67	57	93	2	35	54	38	63
2	0	11	1	0	3	55	46	51	37
3	10	16	5	0	4	0	0	11	0
4	33	6	32	7	KRL				
5	0	0	2	0	1	6	8	5	0
6	0	0	4	0	2	19	16	23	23
AWL					3	45	60	31	33
1	11	16	23	0	4	30	16	41	43
2	16	17	20	23	KRC				
3	62	57	57	70	1	69	73	83	33
4	10	10	0	7	2	5	21	8	43
AWC					3	27	7	9	23
1	38	65	53	77					
2	10	13	13	17					
3	0	4	0	0					
4	52	18	33	7					

(Vavilov 1957). The second reason could be a combination of natural and human selection pressure prevailed to provide an escape mechanism, which might contribute to sustaining medium and earlier types (Engels 1993) and reducing the late flowering and maturing types.

Our observation that ERC showed low variation compared to the study conducted in Ethiopia under field conditions by Tesfaye (1991a) indicate that the difference in the environment may have hindered the detection of variation in ERC. In general pigmentation increases under specific stress conditions, such as low temperatures and high light intensity, which may be true especially in regions at high altitude. Pigmentation in wheat ear colour as a physiological change due to the change in environment has been reported by Nastasi (1964) cited by Hailu (1991). Based on Nastasi's (1964) report wheat grown at altitudes ranging 2200-2700 m has deeper pigmentation of the spikes, while wheat grown at altitude ranging 1800-2000 m have yellow-kernels and a complete absence of pigmentation of the glumes and awns. The relative small sample size used for this study may also have led to low variation in ERC by chance. For subregion South ERC shows a tendency of clinal variation by subregion and the trait seems to be fixed based on subregion (Table 2-5). Zeven (1991) suggested that purple seed colour was absent at lower altitudes below 2400 m, however, a small proportion (7%) purple seeds was observed in this study which were collected from altitude ranging from 1660 to 2000 m.

The presence of intermediate types between durum and turgidum could be a reflection of their common ancestry and implies that these tetraploids cv groups have not yet undergone reproductive isolation and because genetic exchange between types is possible, species distinction is not justified. The lack of taxonomic distinction to discriminate these intermediate types or most probably con-specific types (which are not yet diverged to species level) may be caused by this.

The reason for the existence of high tetraploid wheat variation in Ethiopia may be attributed to an exposure of the early migrant wheats to the large diversity of agro-ecological niches of new environments (Ethiopia) coupled with the prevalent diverse Ethiopian culture and agricultural system that may have resulted in the development of new genotypes and, as a result of a series of mutation and subsequent natural cross-fertilisation, the new genotypes may widen in variation. In addition, the common practice of growing mixtures, probably to limit the risk that a specific genotype cannot provide a crop failure in particular years or conditions, might contribute to more maintenance of variation.

The reason for high diversity in the high altitudes could be that more than 80% of the Ethiopian farming population are concentrated in altitudes greater than 2000 m and frequent harvest and diverse agricultural practice including different cultural needs of the farmers may have imposed adaptation for different traits and evolution to be continued. The low diversity detected in the east and north sub-regions may be associated either to substitutions by improved varieties or by replacement with other local crops such as oats.

3.

Chromosomal polymorphisms in Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces

Tesfaye Messele, Jannie Wennekes and Hans de Jong

Abstract

This chapter presents a pilot study on chromosomal polymorphisms in six Ethiopian tetraploid wheat accessions and one European durum wheat cultivar using C-banding and Fluorescence *in situ* Hybridisation (FISH) with repetitive DNA sequences as probes. Comparison of their C-band patterns in the karyotypes of metaphase complements revealed 12 polymorphic heterochromatin sites. An additional weak band on chromosome arm 6BL of the European cultivar was absent in the Ethiopian accessions, whereas one of the interstitial bands on chromosome arm 6BL of all Ethiopian accessions was absent in the European cultivar. The FISH study with the 45S rDNA, 5S rDNA and the pSc119.2 tandem repeat probed to metaphase complements of the Ethiopian accessions demonstrated polymorphisms for 45S rDNA on chromosome 6B and for pSc199.2 on chromosome arm 6BL. Although many more chromosomal polymorphisms can be demonstrated with both heterochromatin banding techniques and multicolour FISH using a great variety of tandem repeats, these cytogenetic methods are considered far too laborious if compared with biodiversity studies based on polymorphisms of morphological, molecular and storage protein markers. Chromosomal polymorphism and other cytogenetic methods still remain important for those cases where large scale chromosomal rearrangements between accessions are considered.

Keywords

Triticum durum, *Triticum turgidum*, accessions, Ethiopian tetraploid wheat, biodiversity, C-band polymorphism, FISH, repetitive sequence polymorphism

Introduction

Common wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L.), and most other Triticeae species have highly differentiated chromosomes and well studied karyotypes. Upon Giemsa C-banding or N-banding these chromosomes depict clear heterochromatin bands, which are most helpful in the identification of individual chromosomes in the complement and allow the distinction of chromosomes from different genomes (e.g., Gill and Kimber 1974; Gill *et al.* 1991; Gerlach 1977). The chromosomes from the A-genome, considered to be originated from *T. urartu, have small bands in the centromere regions and few diagnostic bands at distal and interstitial positions. The B-genome chromosomes, which are most similar to the wild species Aegilops speltoides*, have far more differentiated chromosomes with conspicuous heterochromatin blocks in the proximal regions of all chromosomes and several other minor bands in the short and long arms. The D-genome chromosomes, with *T. tauschii* as putative parent display relatively few bands at the centromeres and at distal and interstitial regions. The Ethiopian durum and turgidum landrace wheats studied in this thesis, contain the A-genome and B-genome chromosomes, but lack the D-genome chromosomes, which are typical for the hexaploid bread wheats.

A second group of techniques that allow identification of individual chromosomes is the Fluorescence *in situ* Hybridisations (FISH), using repetitive DNA sequences as probes (e.g., Cuadrado and Jouve 1994). FISH techniques not only allow simultaneous detection of various repeat families in a multiprobe - multicolour detection (Mukai *et al.* 1993), they also can be combined with genome painting using total

genomic DNA as probes, or with the detection of single copy DNA sequences (Dong and Quick, 1995). Well known examples of repeats for wheat are the ubiquitous 45S and 5S rDNAs, and the pSc119 tandem repeats.

Heterochromatin blocks, as shown by chromosome banding, and large blocks of tandem repeats, as demonstrated by FISH protocols, can reveal a large variation of position and size between individual plants. These chromosomal polymorphisms, which generally have no or negligible effects on viability and fertility, can easily be demonstrated between accessions, landraces and ecotypes. However, only very few reports appeared on polymorphic variations in remote accessions and wild relatives of wheat (Friebe *et al.* 1992; Friebe and Gill 1994; Friebe *et al.* 1996). Badeva *et al.* (1994) and Taketa and Kawahara (1996) used C-banding and F1 analysis to study translocations in Ethiopian accessions.

In this pilot study we explore both C-banding and FISH of tandem DNA repeats for establishing chromosomal polymorphisms in the Ethiopian accessions. Due to the complexity of the experimental procedures, we selected only six of the available 26 accessions, plus one of the European cultivars as external control. Selection was based on differences in altitude and region, and dissimilarity of molecular markers (see Material and Methods and Chapters 2, 4 and 5).

Material and Methods

We selected six representative accessions from the Ethiopian collection, both durum and turgidum and known to originate from different altitude and found genetically different based on microsatellite analysis as shown in Chapter 4. The Italian cultivar "Capeiti" (Er2, Chapter 1) was used as a unrelated control. Actively growing root tips were collected in the morning from young plant in dry, aerated pot soil and pre-treated in ice water for 24 hours. The material was fixed in fresly prepared acetic acid - ethanol (1:3) and stored at -20 °C until further use. For preparing chromosome slides we first rinsed the root tips in milli-Q water. Mild hydrolysis in 0.2 N HCl for 1 hour at room temperature was done for C-band preparations. For FISH we rinsed the root tips slides twice in 50 mM citrate buffer, pH 4.5 (described below), before they were incubated the tips in a mixture of 0.1% cellulase Y23, 0.1% pectolyase and 0.1% cytohelicase, citrate buffer, pH 4.5, at 37 °C. After 100 minutes of enzymatic digestion the softened root tips were transferred to a clean slide and squashed in acetic acid 45%. The slides were frozen on dry carbon ice, the cover slip removed with a razor blade and finally left to dry.

C-banding

For C-banding we followed the BSG-protocol of Gill *et al.* (1991), with minor modifications. Briefly, preparations were denatured in saturated barium hydroxide (6%) at 37 °C for 10 min. After a thorough rinse in running tap water, the material was incubated in 2xSSC (0.3 N NaCl + 0.03 N Na-citrate, pH 7.0) at 60 °C for 1 hour, rinsed in 50 mM phosphate buffer (pH 7.2) and stained in 5% Giemsa in the same phosphate buffer. The slides were then briefly destained in buffer and distilled water and air-dried. The slides, mounted in Entellan-Neu (Merck), were observed in a bright field microscope.

Fig. 3-1. C-banded metaphase chromosomes of six Ethiopean tetraploid wheat accessions and a European durum wheat cultivar. Chromosomes were ordered according to the nomenclature for wheat chromosomes in Gill et *al.* 1991. Differences in chromosome size are due to contraction differences between metaphase sets. Asterisks show examples of C-band polymorphisms between the accessions; arrows show typical polymorphisms occurring only in the accessions and not in the cultivar.

Table 3-1. Accessions used for assessing chromosomal polymorphism in tetraploid Ethiopian wheat. Distinction of microsatellite groups was based on their position in the UPGMA dendrogram (see Fig. 4-2)

Accession	OTU	cv. group	Altitude / Region
5909	221, 222, 226	durum	3000 / N
6028	18, 21	turgidum	2835 / N
5946	2	durum	2350 / W
5921	69, 70	turgidum	2400 / E
5314	270	durum	1771 / E
5375	246	turgidum	1675 / E
Er2	"Capeiti"	durum	-

Fluorescence in situ Hybridisation

The following DNA sequences were used as probes: 1) The 45S rDNA repeat pTa71 (Gerlach and Bedbrook 1979) was labelled with biotin according to the random primed labelling method (Manufacturer's instruction); 2) The tandem repeat from rye, pSc119.2 (Bedbrook *et al.* 1980), was labelled with digoxigenin according to the random primed labelling method; 3) the 5S rDNA repeat, pTa794/8 (Gerlach and Dyer 1980) was PCR labelled with biotin-16-dUTP. The chromosome squash preparations were pre-treated in 100 µg/mL RNase A at 37 °C for 1 hour, followed by a 20 minutes 5 µg/mL pepsin and 10 minutes 1% formalin in PBS. The preparations were then dehydrated through an ethanol series and finally air-dried.

For the hybridisation we first boiled the probes (concentrations between 10-20 ng were empirically determined) in a hybridisation mix containing the hybridisation buffer HB50 (50% formamide) and 10% dextran sulphate. Preparation with 20 µL hybridisation mix were then heated on a plate at 80 °C for 2 - 2.5 minutes and left overnight at 37 °C for hybridization. Stringency washes were carried out in 10 min 2xSSC, 10 min. 20% formamide in 0.1xSSC and 0.1xSSC, all at 42 °C. Probes, labelled with biotin, were detected and amplified with streptavidin-Cy3, biotinylated-anti-streptavidin and streptavidin-Cy3, respectively. The digoxigenin was detected with sheep-anti-digoxigenin-FITC, and rabbit-anti-sheep-FITC. Finally, the preparations were mounted in Vectashield (Vector Laboratories, Inc.) containing 5 µg/mL DAPI (4',6 diamidino-2-phenylindole).

Image capturing and karyotype analysis

Well-spread metaphase plates of both C-band and FISH preparations were selected and photographed with a Zeiss Axioskop II FISH microscope on 400 ISO colour negative film. The film was scanned at 1000 dpi for digital processing. Another part of the cells was captured on a Zeiss Axioskop II FISH

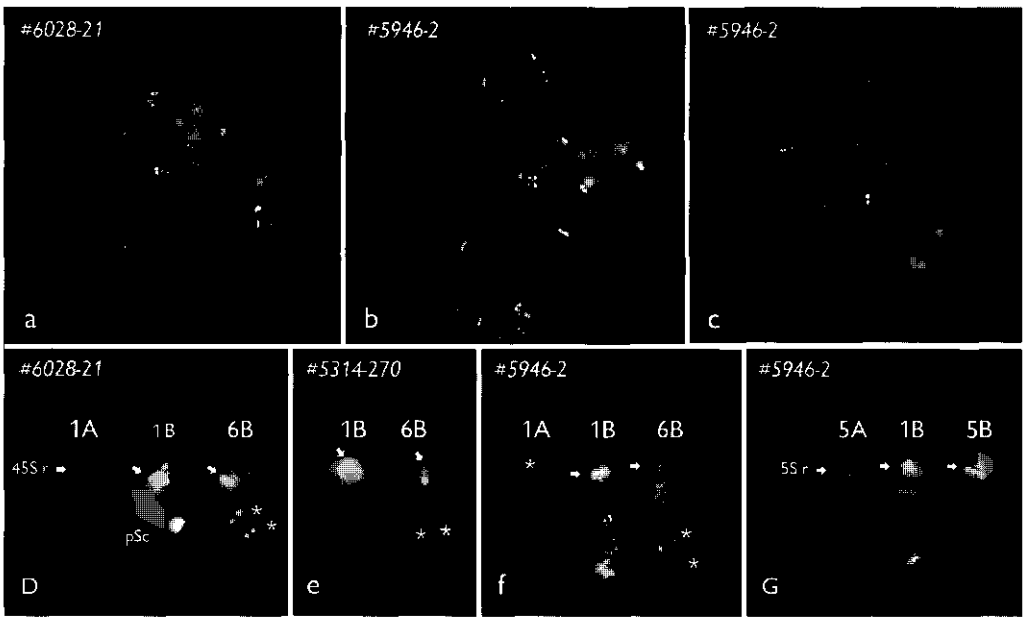

Fig. 3-2. FISH images of the tandem repeats 45S rDNA (pTa71), 5S rDNA (pTa794/8) and pSc119.2 probed and hybridised on metaphase chromosomes of Ethiopean accessions. a) Accession 6028-21, hybridised with 45S rDNA (red fluorescence) and pSc119.2 (green fluorescence). b) Accession 5946-2, hybridised as a. c) Accession 5946-2 hybridised with 5S rDNA (red) and pSc199.2 (green). d) The chromosomes 1A, 1B and 6B from the metaphase in Fig. 3-2a. e) The chromosomes 1B and 6B from a metaphase of OTU 5314-270. f) The chromosomes 1A, 1B and 6B from the metaphase in Fig. 3-2b. g) The chromosomes 5A, 1B and 5B from the metaphase plate in Fig. 3-2c. The arrows in the figures d-e show red fluorescence signals of the 45S rDNA probe (d-f) and of the 5S rDNA probe (g). There are no clear cases of polymorphism for 45S rDNA in chromosome 1A and pSc199.2 the chromosomes 6B.

microscope equipped with a 1400x1000 Photometrics CCD camera and Genius® Software from Applied Images Inc. Images of metaphase plates and individual chromosomes were further processed with Corel Draw and Adobe Photoshop image processing software programmes.

Results

Our chromosome identification was based on the nomenclature for wheat chromosomes in Gill *et al.* (1991). Chromosomes from at least 5 metaphase plates per accession were scored and chromosomes were identified on the basis of their length and centromere position, and diagnostic C-bands. Fig. 3-1 displays a set of representative chromosome sets for each of the six Ethiopian wheat accessions and the Italian cultivar Capeiti (Er2). Where chromosomes were overlapping in a metaphase complement or where not clear for some other reason, a chromosome from a different complement was used for the karyotype. Some variation between cells from the same accession were observed, but these were considered as local differences in Giemsa staining intensity. Only 12 cases of C-band pattern variation could

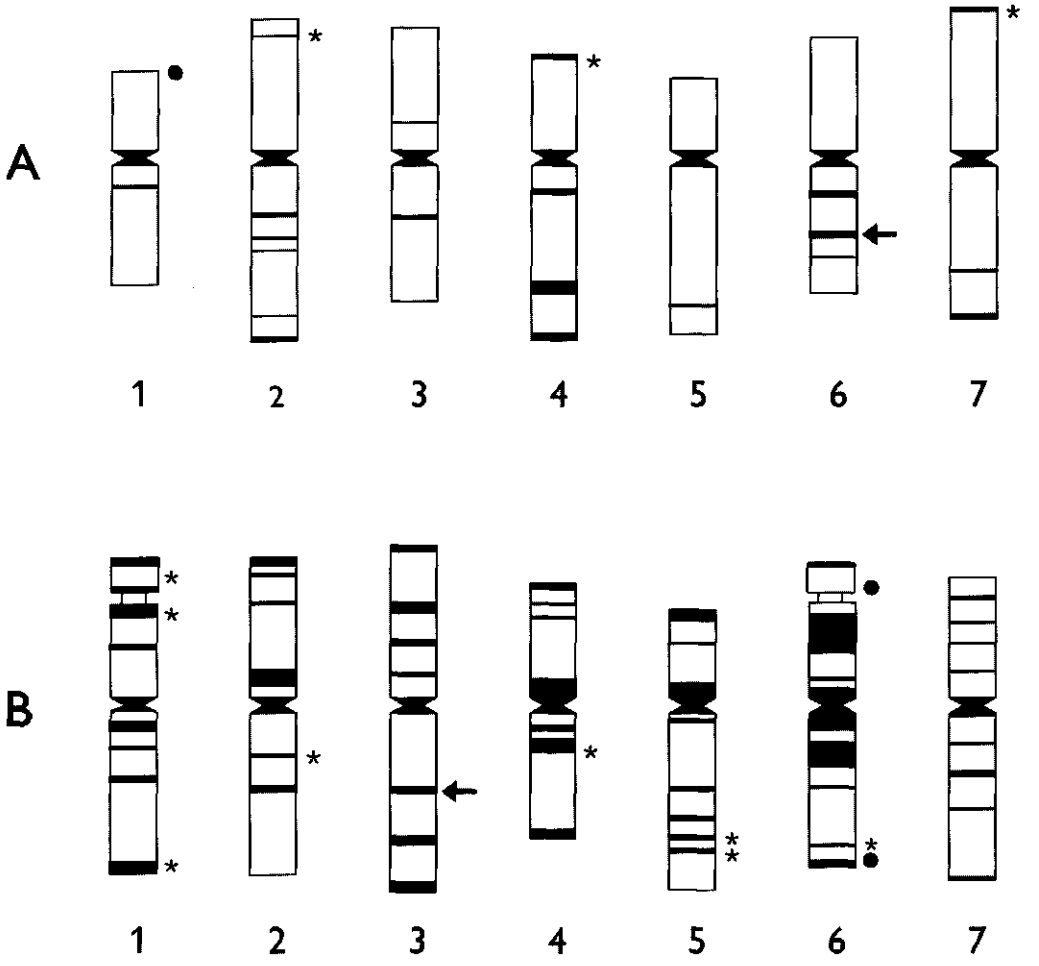

Fig. 3-3. Generalised ideogram of the Ethiopian tetraploid wheats diplaying chromosomal polymorphisms. Asterisks denotes observed cases of C-band polymorphisms and solid spheres show repetitive DNA polymorphisms. The arrows on chromosomes 6A and 3B point at bands that were found in most of the Ethiopian accessions and not in the European cultivar (Er2).

be detected between the accessions. Most striking were the (sub-)distal C-band in chromosome arm 2AS, which was found only in the accessions 5909, 6028 and 5946. The two faint interstitial bands on chromosome 6A were never observed together in the Ethiopian accessions, in contrast to Er2, which shows both bands faintly. The subtelomeric band on chromosome A7 was observed in accession 5921 only. The B-genome chromosomes showed more cases of polymorphisms. We observed variation in C-band sizes and NOR size on the chromosome 1B, and C-band polymorphisms on the chromosomes

1B (distal), 2B (interstitial), 3B (interstitial), 5B (interstitial) and 6B (interstitial). Only in the case of chromosome arm 6BL an interstitial C-band was observed that occurred in all Ethiopian accessions and was absent in the European cultivar.

Examples of FISH signals with the 45S rDNA repeat (pTa71); the 5S rDNA repeat (pTa794/8) and the tandem repeat (pSc119.2) are shown in Fig. 3-2. We observed polymorphisms for the 45S rDNA repeat on the chromosomes 6B. The 5S rDNA repeat was found on the chromosomes 5A,1B and 5B. The pSc199.2 tandem repeats could only be studied on the chromosomes showing 45S rDNA and 5S rDNA signals; other chromosomes with only pSc199.2 could not be identified in this study. So far we could demonstrate only polymorphism for this tandem repeat on the chromosomes 6B (distal long arm). An ideogram for all A- and B-genome chromosomes summarizing the observed C-band and tandem repeat polymorphisms is given in Fig. 3-3.

Discussion

Heterochromatin as demonstrated by C-banding gives the highest number of bands in tetraploid wheat species yet known. Based on the study of Gill *et al.* (1991) and others later on, A-genome and B-genome chromosomes display numerous bands at their telomeres, centromeres and interstitial positions. In total about 150 bands can be recognised of which 70% occur on the B-genome chromosomes. However, most bands are rather small and can only be detected in a limited number of preparations obtained under optimal technical conditions. Polymorphisms are based on either significant size differences between bands or on the presence - absence of such bands in the chromosome sets of different plants. Our study revealed twelve cases of polymorphisms between the chromosome sets of the six Ethiopian accessions, most of them on the B-genome chromosomes. An additional weak band on chromosome arm 6BL of the European cultivar was absent in the Ethiopian accessions, whereas one of the interstitial bands on chromosome arm 6BL of all Ethiopian accessions was absent in the European cultivar. Many more cases of polymorphisms may exist, but far more extensive C-band analyses of this material will be required to demonstrate such differences in heterochromatin sites unambiguously.

In the first few FISH experiments we were able to demonstrate tandem repeat polymorphisms for the 45S rDNA and the pSc199.2 repeats, but extensive studies can likely reveal many more examples of such chromosome variations. Using various repeat simultaneously in a multicolour FISH can potentially demonstrate many more differences between homologous chromosomes from related accessions. Not only is the detection of tandem repeats more reliable than chromosome banding patterns, lengths differences in tandem repeat lengths can also better be standardised. In addition, by combining the three tandem repeats used in this pilot study (45S rDNA, 5S rDNA and pSc199.2) with other already identified repeat family classes in wheat species a complex multicolour chromosomal bar code can be obtained (Schmidt and Heslop-Harrison 1998).

The use of polymorphisms at the chromosomal level for biodiversity studies is still limited compared to the large variation we obtained with morphological, molecular and storage protein traits. In addition,

C-band analysis and FISH experiments are relatively laborious, time consuming and less reproducible as the molecular marker technologies. Yet, cytogenetic studies remain unreplaceable where the detection of large translocations, inversions and other chromosomal rearrangements are considered. Using C-banding of metaphase complements and meiotic analyses of F1 hybrids, Belay and Merker (1998) evidenced the existence of a 5BS.6BS translocation in three tetraploid Ethiopian landraces. The outcome of our C-band analysis could not confirm the existence of such a rearrangement in the Ethiopian accessions. A second translocation, as recently shown in a C-band study of Kawahara and Taketa (2000) of fifteen Ethiopian tetraploid wheats, clearly demonstrated a unique 2A.4B translocation in Ethiopian landraces, supporting indications for a monophyletic origin of Ethiopian wheats. In our material it is likely that the accessions 5909, 6028 and 5921 do contain this translocation, whereas the accessions 5921, 5314 and 5375 do not. Our observation is in contrast to the results of Kawahara and Taketa (2000), who suggest that this translocation occurs in all tetraploid Ethiopian wheats, a chromosomal rearrangement that was used to support their argument for the monophylogenetic origin of Ethiopian wheats. Meiotic studies of F1 hybrids of these accessions will be needed to get more certainty about the general occurrence of this translocation in the Ethiopian wheats.

4.

The use of Microsatellites to measure genetic diversity in Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces

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Abstract

Ethiopian cultivated tetraploid (*Triticum turgidum* L.) wheat is known for its considerable genetic variation. In a project to describe this variation, a representative selection of 26 accessions and two cultivars from Ethiopia, along with two European cultivars were used in a study on microsatellite polymorphisms. We used twelve wheat microsatellite (WMS) located on different A- and B-genome chromosome arms. A total of 96 alleles were scored, with an average of 7.9 alleles per locus, ranging from sixteen for WMS577 to only three for WMS160. Genetic diversity estimations, based on Nei's diversity index, revealed relatively high values for eight out of the 26 accessions, while two other accessions had a low diversity. Gene diversity among accessions (Dst values) ranged from 0.02 - 0.42 with a mean of 0.26. The relative coefficient of gene differentiation for accessions (Gst) showed that the within-accessions component accounted for 45% of the total diversity, while 55% of the total was due to the interaccessional component. Accessions were grouped into four geographical subregions and four classes of altitude. We observed for both subregions and altitude classes that genetic diversity was much higher for the within than for the between class component. The UPGMA dendrogram that was constructed on the basis of microsatellite allele similarities for all accessions demonstrated that most of the accessions could be loosely grouped according to altitude and, to a lesser extent, to region of collection. Our study demonstrated the power of microsatellite markers in establishing genetic diversity of Ethiopian tetraploid wheat landraces.

keywords

Triticum durum, *Triticum turgidum*, accessions, Ethiopian tetraploid wheat, genetic diversity, microsatellites

Introduction

Twenty-three percent (12,000 accessions) of the total IBCR (Institute of Biodiversity Conservation and Research) collection is wheat landraces (Mehari 1994). Although part of them surely represents different genotypes it is tempting to believe that the actual number of genotypes is far less than the total gene bank collection. To allow a sustained and efficient (*ex-situ* and *in-situ*) conservation of these materials a thorough knowledge of genetic diversity and relationships between the accessions is required.

Until now the characterisation of wheat landraces preserved at IBCR is based on morphology. These characters are prone to environmental conditions and/or are controlled by multiple genes (Newbury and Ford-Lloyd1997). Recently also isozyme marker technology was included for the characterisation of the landraces (Tsegaye *et al.* 1996), but the number of polymorphisms in isozyme markers is limited and their expression has been shown to be affected by environmental conditions as well.

Paetku and Strobeck (1994) suggested that the use of robust marker tools is required for a more reliable assessment of genetic diversity in wheat. Barrett and Kidwell (1998a) confirmed that molecular markers have great value in the design of conservation programmes. Rapid progress in molecular biology in recent years has resulted in the development of a number of techniques for detecting variation at the DNA level. Such DNA marker systems are based on either hybridisation with single copy probes of

DNA digested with specific restriction fragments revealing restriction fragment length polymorphism (RFLP) or on the amplification of genome segments between arbitrary or specific oligonucleotide priming sets using PCR (polymerase chain reaction) amplification (Karp et al. 1996). A specific example of the latter group are microsatellites that are based on single locus PCR analysis of DNA fragments, using primers complementary to the flanking regions of tandem repeats of 2-6 base pairs. It appeared that those repeats frequently show length polymorphism at the species and subspecies level.

Microsatellites often display various co-dominant alleles per locus (Plaschke et *al.* 1995; Edwards et *al.* 1991, 1992) and they have been used successfully for genotype identification (Allen et *al.* 1995; Scribner et *al.* 1994; Chakraborty 1984) and for estimating genetic and geographic diversity (Paetku and Strobeck 1994; Bruford and Wayne 1993; Gottelli et *al.* 1994; Roy et *al.* 1994; Bowcock et *al.* 1994; Forbes et *al.* 1995).

This study aims at the evaluation of the results of an analysis of microsatellite markers for establishing genetic diversity in a representative sample of 30 Ethiopean tetraploid wheat accessions. Alleles were scored and subsequently diversity was quantified using Nei's (1973) diversity index. The unweighted pair group method algorithm (UPGMA) was used for cluster analysis. The results were compared for differences in altitude and subregional distribution.

Material and Methods

Per accession five tandemly selected plants were used (detailed descriptions on materials used are found in chapter 1 and 2) and planted in a greenhouse. Each plant was described as an OTU (Operational Taxonomic Unit). Ten to eleven days old leaves representing each OTU were collected and stored in 15mL screw capped glass tubes. The leaves were either directly further processed or stored in liquid nitrogen until later use.

DNA extraction and amplification

DNA was extracted according to the CTAB protocol published by Van der Beek et *al.* (1992), with minor modifications. Two grams of frozen leaf tissue were homogenised in 3 mL 2% CTAB-buffer. The homogenate was incubated at 65 °C for 90 min, while gently mixing at a 10 minutes interval. After two chloroform extractions, the DNA was precipitated and dissolved in 0.5 mL TE buffer. The microsatellites were studied as sequence-tagged microsatellite markers by PCR amplification (Bredemeijer et *al.* 1998). Amplification reaction was performed as described in Röder et *al.* (1998), Plaschke et *al.* (1995) and Bredemeijer et *al.* (1998). For each PCR reaction a volume of 20 µL containing 5 µL wheat genomic DNA, 2.5µL fluorescently labelled forward primer (Pharmacia) and 2.5mL unlabeled reverse primer (Isogen, The Netherlands), 2.5 mL each dNTP, 2.5 µL MgCl2, 2.5 µL 10x PCR buffer, 1 µL 0.5 U/µL ampliTaq gold (Perkin Elmer) and 1.5 µL distilled water was used.

The forward primer (P1) was labelled at the 5' end with Cy5 (Pharmacia). Amplification were done using Hybaid Omni Gene thermal cycler. Cycling condition for amplification were 94 °C for one min,

annealing temperature (60 °C or 55 °C) for one min, at 72 °C one min, after 45 cycles leave five min at 72 °C, finally the amplified samples were stored at 4 - 25 °C.

A 6 % acrylamide sequencing gel was used to separate the PCR products on automated laser fluorescence (ALF express) sequencer (Pharmacia). Per gel we combined 6 µL PCR products with 6 µL mix of loading buffer and size standard. Then 2 to 4 µL of the mix was loaded to each gel lane in the presence of 1x TBE gel running buffer. Prior to loading, each DNA sample was denatured by heating the mix at 90 °C for 4 min followed by quenching on ice. DNA fragment sizes were analysed with the computer program Fragment Manager (Pharmacia) by comparing with size standards and finally translated in to a binary (presence 1 and absence 0) data matrix.

Previous studies have assigned the microsatellites used in this study to the chromosome 1A, 2A (S), 3A, 4A, 6A, 7A, 1B (S), 2B, 3B, 4B, 6B and 7B (Röder *et al.* 1998).

Data analysis

Nei's (1973) diversity index (H) was computed as follows

$$H = 1 - \sum_{i}^{a} p_i^2$$

in which variable pi is the frequency of variant *i* and *a* the number of variants. Ht is the Nei index based on all landrace OTUs.

Dst and the proportion of between group difference (Gst) were computed as

$Dst = Ht - Hs$ and

$Gst = \frac{Ht - Hs}{Ht}$ which gives the relative contribution of the between group variation.

When a Gst value equals 1, groups are fixed for different alleles.

For the construction of the UPGMA (Unweighted Pair Group Method Algorithm) dendrograms for the relations among and between OTUs and accessions we used the SAS statistical software (SAS institute, Cary, NC, version 6, 1989).

Results

The 150 OTUs representing the 26 accessions and four cultivars showed for the twelve markers a total of 96 polymorphic bands. The number of alleles per locus varied from 3 to 17. The highest number (17) of alleles was detected for WMS577 and the lowest (3) for WMS160.

The number of different alleles per locus amounted one to four for the accessions and one or two, incidentally three (WMS577) for the cultivars. Cases of no polymorphism (one allele within accessions) greatly differed between loci and between accessions and cultivars. For the loci WMS631, 18, 619, 493, 513 and 680 fifty percent or more of the accessions were not polymorphic. The accessions 5441, 5588, 5725, 6038, 5180, and 5976 had nine or more polymorphic loci. Cultivars were less polymorphic (Ht

0.32) than the accessions (0.54, see tables 4-1 and 4-2) and four markers were identical for all four clutivars. In the cultivars, the B-genome was more differentiated (Gst 0.21) than the A-genome (Gst 0.13, Table 4-2). Heterozygosity was observed for locus WMS577 in five OTUs (5588-184, 6038-210 - 213, 5180-277 - 285), for locus WMS155 in one OTU (7190-80), for locus WMS18 in one OTU (7190-80), for locus WMS631 in the cultivar Er1 and for the locus WMS513 in the cultivar ER2, in one OTU each. Rare alleles were found in seven OTUs for locus WMS18, in five OTUs for locus WMS513, in four OTUs for locus WMS357 and in one OTU for locus WMS577.

Table 4-1. Nei's diversity index for individual primers based on all accessions

Microsatellite marker	chromosome position*	Ht	Hs	Dst	Gst
WMS357	1A	0.64	0.37	0.27	0.42
WMS95	2A	0.78	0.36	0.42	0.54
WMS155	3A	0.65	0.28	0.36	0.57
WMS160	4A	0.62	0.21	0.41	0.66
WMS169	6A	0.66	0.30	0.36	0.55
WMS631	7A	0.08	0.06	0.02	0.25
WMS18	1B	0.59	0.26	0.33	0.56
WMS619	2B	0.25	0.14	0.11	0.44
WMS493	3B	0.29	0.19	0.10	0.34
WMS513	4B	0.46	0.38	0.08	0.18
WMS680	6B	0.57	0.31	0.26	0.46
WMS577	7B	0.84	0.45	0.39	0.46
Total mean		0.54	0.28	0.26	0.45

*) According to Röder et *al.* (1995)

Table 4-1 shows Nei's diversity index values, which were calculated to estimate diversity for total accession (Ht), mean within accessions (Hs), mean among accessions (Dst) and coefficient of gene differentiation (Gst). The average total genetic diversity per marker (Ht) was 0.54, ranging from 0.08 to 0.84. Accessions like 5314 and 6028 had relatively high diversity values for all loci, except for the loci WMS619

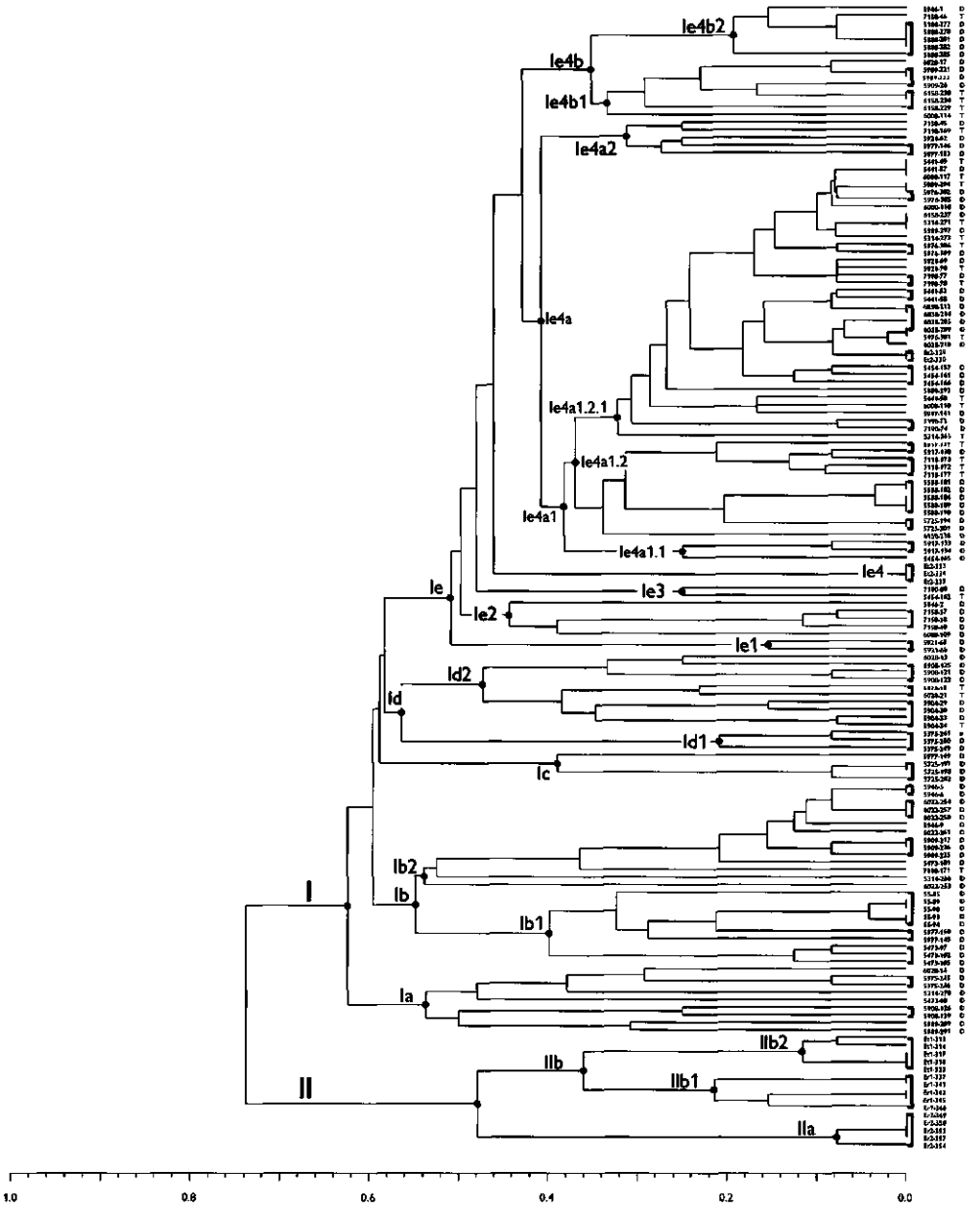

Fig. 4.1. UPGMA dendrogram based on the microsatellite data of the total 150 OTUs. D and T denotes durum and turgidum cv groups, respectively; Et1, Et2, Er1 and Er2 are the cultivars. Brackets show the identical microsatellites. of related OTUs.

and 631. Average diversity for all accessions and loci was 0.28. The differentiation within accessions accounts for only slightly more than 50% of the variation (Gst 0.45), which is due to the high within accession variation. The landraces are as much as differentiated from each other (Dst 0.26, Table 4-1) than the cultivars (0.24, Table 4-2). The highest differentiation coefficient (Gst 0.66) was observed for WMS160.

Table 4-2. Nei's diversity index for cultivars, the number of different alleles per cultivar and per microsatellite marker.

Primers	Ht	Hs	Dst	Gst	Number of different alleles			
					Et1	Et2	Er1	Er2
WMS357	0.32	0.08	0.24	0.25	1	1	1	2
WMS95	0	0	0	0	1	1	1	1
WMS155	0.32	0.08	0.24	0.25	2	1	1	1
WMS160	0	0	0	0	1	1	1	1
WMS169	0.32	0.08	0.24	0.25	2	1	1	1
WMS631	0	0	0	0	1	1	1	1
Mean	0.16	0.04	0.12	0.13				
WMS18	0	0	0	0	1	1	1	1
WMS619	0.32	0.08	0.24	0.25	1	1	1	1
WMS493	0.96	0.24	0.72	0.25	2	2	2	1
WMS513	0.32	0.08	0.24	0.25	1	1	1	2
WMS680	0.32	0.08	0.24	0.25	1	1	1	1
WMS577	0.94	0.24	0.7	0.26	1	3	1	2
Mean	0.48	0.12	0.36	0.21				
Total mean	0.32	0.08	0.24	0.17				

To estimate the diversity within the durum and the turgidum types from all accessions together, we found that 60 of the alleles (63%) were common in durum and turgidum. Thirty-five alleles (37 %) were specific for durum and only one allele was specific for turgidum. The Nei's mean total diversity values (Ht) for the durum and turgidum cv. groups were 0.52 and 0.40, respectively (tables 4-3). A comparison of the two tetraploid wheat cultivar groups indicates that variation is higher in the durum group than in

the turgidum group (table 4-3). The Gst value between the two groups (0.15) indicate that there is differentiation between groups.

Table 4-3. Comparisons of durum and turgidum cv groups, Nei's diversity index values and number of different alleles.

Microsatellite Marker	H		No. of different alleles		Total number of alleles
	durum	turgidum	durum	turgidum	
WMS357	0.66	0.51	11 (106)*	5 (22)	11
WMS95	0.78	0.63	10 (107)	3 (22)	10
WMS155	0.66	0.48	8 (114)	3 (16)	8
WMS160	0.63	0.50	3 (102)	2 (22)	3
WMS169	0.91	0.55	7 (102)	4 (22)	7
WMS631	0.08	0.10	6 (108)	2 (22)	6
WMS18	0.60	0.17	7 (107)	3 (22)	7
WMS619	0.24	0.25	6 (108)	3 (22)	6
WMS493	0.15	0.38	9 (107)	3 (22)	9
WMS513	0.23	0.17	5 (108)	3 (22)	6
WMS680	0.50	0.25	6 (107)	3 (22)	6
WMS577	0.81	0.82	17 (105)	9 (21)	17
Mean all markers	0.52	0.40			

*Number of OTUs used for the microsatellite analysis

Diversity by geographical areas

Altitude

The average Nei's diversity indexes per altitude and subregion class are shown in Table 4-4. The within-altitude component of diversity (Hs) is not very different from the total diversity (Ht), which indicates that the within altitude group variation is much larger than the between altitude group variation. The difference in H values for the different groups were relatively small (data not shown).

Subregions

The total mean within subregion variation is higher than the between subregion variation, showing that the differentiation between subregions is limited (table 4-4).

Table 4-4. Nei's diversity in relation to altitude and region.

	Hs	Gst
For altitudes	0.44	0.23
For subregions	0.43	0.27

Fig. 4-1 depicts a UPGMA dendrogram with similarity distances of the 150 OTUs. In this dendrogram thirteen accessions had two or three identical OTUs, while one accession (55) showed four identical OTUs. In other cases eight identical OTUs were present in different accessions. In the dendrogram except for few cases, the cultivar groups durum and turgidum occurred mixed throughout the dendrogram, but OTUs were often clustered in their own accession. The UPGMA dendrogram in Fig. 4-2 displays the relation between the 26 accessions and also between four cultivars, and their geographic areas. The dendrogram depicts two main groups in which group I contains all the accessions and the Et2 cultivar, whereas group II contains the remaining three cultivars.

Discussion

The analysis of twelve A- and B-genome wheat microsatellite loci in 26 Ethiopian tetraploid wheat landraces has shown high levels of variation (Ht 0.54) although their values greatly varied between the loci, ranging from 0.08 for WMS631 to 0.84 for WMS577. Polymorphism between the two Ethiopian and two European cultivars (Dst) was 0.24, which was slightly less compared to that of the landraces (0.28, tables 4-1 and 4-2).

In six of the twelve loci 8% of the alleles are unique and specific for small groups of related OTUs. These alleles, which are assumed to reflect recent mutations, can be used as a representative marker for landrace identification. The presence of heterozygosity in the landraces can be ascribed to different causes. Firstly, the mixtures of various forms are preserved in the farmers' fields (Mekbib and Giorgis 1990), which is traditional agricultural practice in Ethiopia. Secondly, traditionally produced crops grown nearby will allow occasional gene exchanges (Jana 1993). Thirdly, natural cross pollination in wheat, which is very rare at high humidity was reported to be frequent in dry regions at a frequency of 3 to 4% (cf. Rao 1974). Vavilov (1951) argued that there is a possibility of high cross-pollination and gene introgression among the Ethiopian tetraploid wheats. Ethiopia's variable environmental conditions might have favoured the conditions for natural cross-pollination, and so for frequent gene exchanges.

The difference that was observed in allele number between durum and turgidum (Table 4-3) might be due to the smaller sample size. However, also total diversity is somewhat less for turgidum. How-

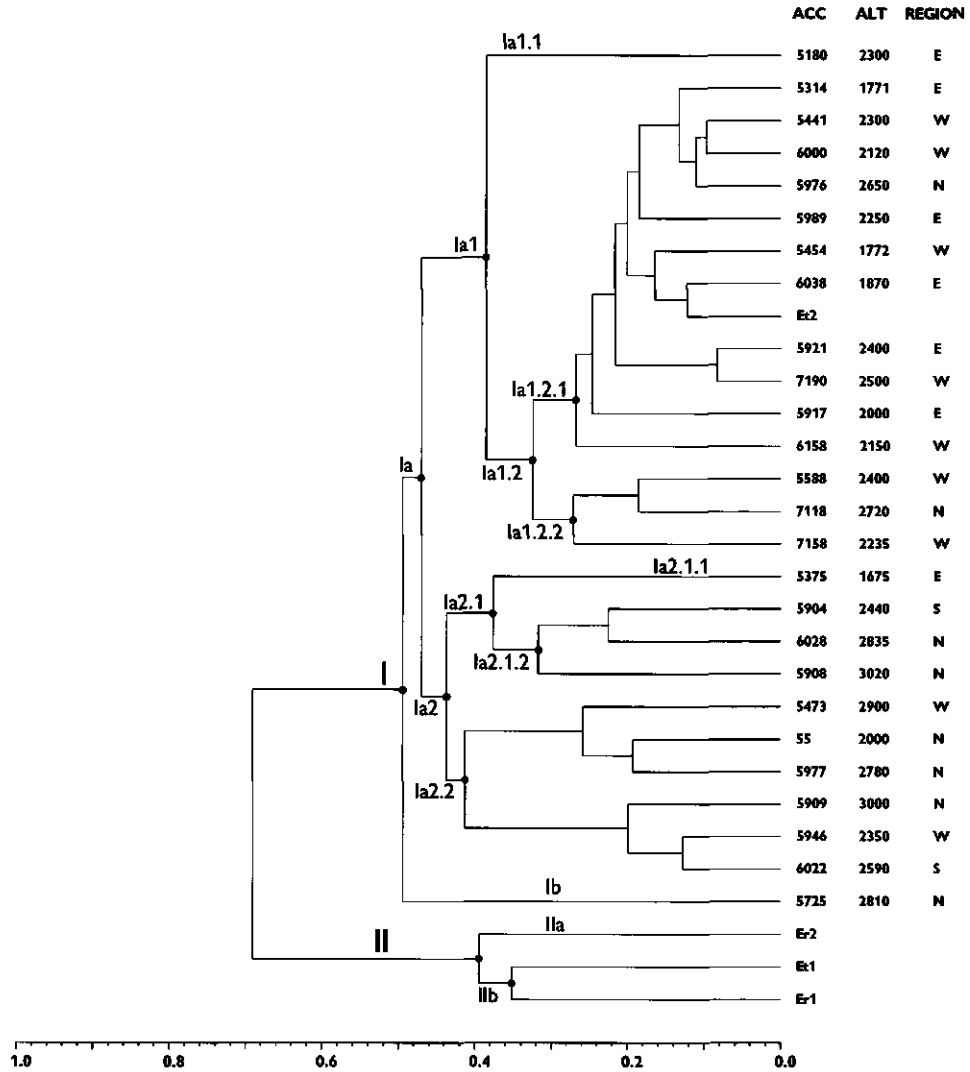

Fig. 4-2. UPGMA dendrogram constructed using 26 accessions and four cultivars. The dendrogram is labelled by altitude class and subregion (see table 1-1). Et1, Et2, Er1 and Er2 are the cultivars used in this study.

ever, the overall difference between both groups is relatively limited as shown by the large number of common alleles, the low Gst value and the UPGMA dendrograms. Probably, durum and turgidum were derived from a common ancestor, emmer wheat (*T. monococcum*, Grubben and Partohardjono 1996).

It is also possible that the durum cv groups has diverged from the turgidum by accumulation of specific alleles, especially of a number of alleles that control some morphological traits.

The dendrogram based on similarities of the 150 OTUs (Fig.4-1) showed a strong clustering of cultivars, with the exception of the Ethiopian cultivar Arendato (Et2), which displayed far higher similarity with the landraces than the other three cultivars. This indicates that the Et2 cultivar most likely, originated from Ethiopian landraces. The dendrogram revealed some differentiation of the durum and turgidum OTUs. Clustering of either similar turgidum and durum plants was mainly observed in rather related genotypes within accessions. Assignment of the OTUs to either durum or turgidum cv groups was equivocal in few cases when representative characteristics were intermediate. Such intermediates are common for Ethiopian wheat accessions and were previously described in Mekbib and Giorgis (1990) and Tesfaye *et al.* (1991). No obvious clustering was observed for altitude and subregion groups.

The farmers' seed source through rotation, exchange and market sampling may account for the maintenance of variation in accessions. Moreover, primitive populations are known to have a high plasticity due to their genetic variability (Bennett 1970) in spatially heterogeneous and often adverse environmental conditions.

The climatic conditions in the extreme altitudes i.e., less than 1700 m or greater than 2800 m may be disadvantageous for most genotypes. The isozyme work on barley landraces suggests the extreme altitude greater than 3500 m to be poor in terms of genetic diversity (Abebe and Asmud 1997 and Asfaw 1989). However, in the present research no strong differentation between altitude groups was observed.

Our study on the use of microsatellites as molecular markers in Ethiopian tetraploid wheat accessions has shown that most of these molecular markers can be used for landrace identification and reliably estimating diversity and relationships between accessions and so to improve the efficiency of germplasm collection, and proper planning the *in situ* and *ex situ* conservation programme.

5.

AFLP fingerprinting to estimate genetic diversity in Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces

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Abstract

Genetic variation of 150 OTUs representing 26 Ethiopian tetraploid wheats (*Triticum turgidum* L.) landraces and 4 cultivars were investigated at the DNA level using the AFLP method. Nine primer combinations (PCs) were used to amplify DNA segments from the genomic DNA fingerprint. A total of 84 polymorphic bands were scored, with an average of 9.3 polymorphic bands per PC, ranging from nineteen for PC E35M37 to only two for E42M36.

Genetic diversity estimation which were based on Shannon-Weaver diversity indexes, revealed relatively high values for two (#5904 and #6028) out of the 26 accessions. Diversity within accessions (Hs) ranged from 0.19 to 0.50 with a mean of 0.33. The relative coefficient of gene differentiation for accessions (Gst) showed that the within accession component accounted for 0.66 of the total, which indicates that the high accession variation is due to the within component rather than the between accessions component. Accessions were grouped into four geographical subregions and four classes of altitude. We observed for both subgroups that genetic diversity was much higher for the within- than for the between component. UPGMA dendrograms that were constructed on the basis of AFLP band similarities demonstrated that the accessions in the dendrogram do not show a clear pattern of geographical clustering.

Keywords:

Ethiopian tetraploid wheats, AFLP, diversity, landraces

Introduction

The 12,000 wheat accessions representing 23 % of the 54,000 accessions in total for the 101 crop types that were collected and preserved in the national gene bank of Ethiopia (Mehari 1994) are composed of different morphotypes. Individual accessions are often composed of various morphotypes because farmers traditionally used to grow mixtures of different types. This phenomenon together with the existing natural variation might caused to inflate the number of wheat accessions preserved in the gene bank. To limit redundancy and to represent all genotypes within the collection a diversity study using reproducible methods that are capable of discriminating genotypes on the basis of unique fingerprints is required. Such genomic bar codes for each distinct genotype thus provide an effective preservation strategy of the national wheat diversity in the gene bank.

For diversity study in cereals molecular marker technologies based on DNA fingerprinting are increasingly employed. AFLP is a molecular fingerprinting techniques that is based on PCR amplification of short restriction endonuclease - digested genomic DNA fragments onto which adapters possessing 3' selective nucleotides of one to four bases are used in a selective amplification reaction. Subsequent to selective amplification by PCR, a separation of labelled amplified products is followed by denaturing polyacrylamide gel electrophoresis (Vos *et al.* 1995).

The length polymorphisms of DNA fragments represent loci from all over the genome and can be used to measure within-species diversity. In comparison to other molecular markers AFLP has the

advantage of allowing simultaneous identification of a large number of amplification products (Qi & Lindhout 1997) as well as detecting genetic diversity between and within closely related species. AFLP markers mostly inherit dominantly and have successfully been used to study diversity in various cereals including *Triticum aestivum* and *Triticum monococcum* and *Aegilops tauschii* (Barrett and Kidwell 1998;Tracy et *al.* 1996); barley (*Hordeum vulgare*) (Pakniyat et *al.* 1996) and rice (*Oryza sativa*) (Mackill et *al.* 1996).

The aim of this work is to evaluate the use of AFLP markers as a tool to establish genetic diversity in a sample of 26 randomly selected Ethiopian tetraploid wheat landraces and four cultivars all represented by 5 individuals. Bands were scored and diversity was subsequently quantified using the Shannon-Weaver diversity index and UPGMA cluster analysis. The results were compared for groups differing in altitude and geographical distribution.

Material and Methods

Detailed description on the material used for this study can be found in Chapters 1 and 2. Ten to eleven days old leaves of each OTU were collected and stored in 15 mL screw capped glass tubes. The leaves were used either directly or stored in liquid nitrogen.

DNA extraction and amplification

Genomic DNA was isolated from fresh leaves of greenhouse grown plants using the CTAB method (Van der Beek et al 1992). The AFLP method followed essentially the protocol of Vos et al. (1995), with adaptation for the number of selective nucleotides and preamplification steps. The protocol included: 1) restriction of genomic DNA with *Eco*RI and *Mse*I, and ligation of adapter sequences to the restriction fragments in order to generate the primary template; 2) selective preamplification of this primary template with AFLP primers with various additional 3' selective nucleotides (Table 5-1); 3) selective amplification with ^{33}P-labeled EcoRI primers having three selective nucleotides and MseI primers with three 3' selective nucleotides (Table 5-1), and 4) separation of labeled amplification products on a denaturating polyacrylamide sequencing gel using an S2 sequencing gel electrophoresis apparatus (Gibco BRL, Life Technologies USA, Maryland, USA). The anodal buffer was supplemented with 1 x TBE to generate a salt gradient, which contributes to a better separation of the larger fragments. The gels were dried on Whatmann 3MM paper, exposed on X-ray films (Konica, Tokyo, Japan) at -20 °C for 3-7 days depending on signal intensity. Finger printing patterns were analysed and scored by eye.

Data analysis.

Genetic diversity per polymorphic band within accession, and the pattern of diversity within geographical regions were estimated according to the Shannon-Weaver index (see Chapter 2). H was determined per individual band for a total of 84 polymorphic bands based on the presence or absence of specific bands. In this study it was not possible to distinguish AFLP band intensity differences as an indication of heterozygosity. The presence or absence of AFLP fragments was scored on the autoradiogram and

transferred into a 1 (present) and 0 (absent) matrix over all operational taxonomic units (OTUs). The phenetic analysis was performed with the Jaccard algoritm in a UPGMA clustering analysis. Dendrograms were generated using the euclidean distance matrixes.

Table 5-1. Adapters and primers used for the detection of diversity in tetraploid Ethiopian wheat landraces and cultivars.

Adapter	Extension	Sequence
E+1	A	5'-CTCGTAGACTGCGTACCCATCTGACGCATGGTTAA-5'
M+1	A	5'-GACGATGAGTCCTGAGTACTCAGGACTCAT-5'
Primers		
E35	ACA	5'-AGACTGCGTACCAATTCAC A
E37	ACG	5'-AGACTGCGTACCAATTCAC G
E42	AGT	5'-BGACTGCGTACCAATTCAG T
M36	ACC	5'-GATGAGTCCTGAGTAAAC C
M37	ACG	5'-GATGAGTCCTGAGTAAAC G
M38	ACT	5'-GATGAGTCCTGAGTAAAC T
M40	AGC	5'-GATGAGTCCTGAGTAAAG C

Results

Number of bands

Using nine AFLP PCs the 150 OTUs representing the 26 landraces and four cultivars generated a total of 84 polymorphic bands. The number of different bands detected per PC over all accession ranged from 2 to 19. Of all the AFLP markers PC E35M37 generated the highest number of polymorphic bands (19) while PC E42M36 had the lowest (2). Out of the 84 polymorphic bands two were specific for eleven OTUs and five for the two cultivars Et1 and Er1, which are assumed to reflect rare alleles.

Table 5-2. Shannon-Weaver diversity per primer combination for within and between accession variation

Diversity index	E35M37	E37M36	E37M38	E37M40	E35M40	E35M36	E42M37	E42M36	E35M38	Mean
Ht	0.42	0.47	0.47	0.47	0.50	0.48	0.54	0.67	0.49	0.50
Hs	0.24	0.30	0.19	0.36	0.37	0.31	0.50	0.40	0.29	0.33
Dst	0.18	0.17	0.28	0.11	0.13	0.17	0.04	0.27	0.20	0.17
Gst	0.42	0.36	0.60	0.23	0.26	0.35	0.07	0.40	0.41	0.34

Genetic diversity

Table 5-2 shows Shannon-Weaver diversity index values calculated to estimate total (Ht) diversity, mean diversity within accession (Hs), mean among accession (Dst), and coefficient of gene differentiation (Gst).

Mean total diversity (Ht) for all accessions and loci was 0.50 and ranged from 0.42 - 0.67 per locus. We observed a far higher relative differentiation value for the within accession variation (Gst 0.66) than the between accession variation (Gst 0.34). A large difference between PCs was observed for Gst values indicating that some discriminate much more between accessions than the others. The accessions 5904 and 6028 showed relatively high diversity values.

To estimate the variation within and between durum and turgidum the Shannon-Weaver diversity values for these cultivar groups were calculated. The mean total (Ht) genetic diversity value for the durum and turgidum were 0.48 and 0.16, respectively, showing that durum is three-fold higher in total (Ht) diversity than turgidum. The Gst value (0.36) showed differentiation between both groups. This difference is not reflected by a difference in the number of polymorphic bands between the durum and turgidum cultivar groups (Table 5-3).

Table 5-3. Shannon-Weaver diversity values for durum and turgidum and numbers of polymorphic bands per primer combination and per cultivar group.

Primer combinations	Ht		Number of polymorphic bands per primer combination and cultivar group	
	Durum	Turgidum	Durum	Turgidum
E35M37	0.40	0.17	14	13
E37M36	0.45	0.21	11	10
E37M38	0.45	0.19	9	8
E37M40	0.45	0.20	7	9
E35M40	0.48	0.12	3	5
E35M36	0.47	0.12	4	4
E42M37	0.53	0.19	5	5
E42M36	0.62	0.13	2	2
E35M38	0.46	0.11	6	5
Mean	0.48	0.16	7	7

Diversity by geographical areas

The Gst values for altitude and subregion classes revealed that almost 92-96% of the variation was due to the within component (Table 5-4). The means for the four altitude class Shannon-Weaver indexes compared by t-test showed no significant difference ($P<0.05$) between the classes (data not shown).

Table 5-4. Shannon-Weaver diversity in relation to altitude and subregion.

groups	Hs value / group				Mean Hs	Gst
altitude classes I, II, III, IV	0.47	0.52	0.53	0.50	0.50	0.04
subregions E, N, S, W	0.40	0.51	0.42	0.52	0.46	0.08

Cluster analysis

In Fig. 5-1 the association among the 150 OTUs revealed by UPGMA cluster analysis are presented. The UPGMA dendrogram shows two main groups. The Ethiopean cv Boohai (Et1) and the South African (Er1) cultivar are separated from the rest of the OTUs. The Italian cultivar Capeiti (Et2) together with Arendato are within the large clusters of the landraces. The turgidum OTUs are interspersed in this cluster, but in many cases the representatives of one accession do cluster together. OTUs with identical AFLP patterns for the within accession were observed in six cases, while between accessions there were no identical OTUs observed.

The dendrogram (Fig. 5-2) for the 30 accessions was drawn to observe the between-accessions genetic distance. The similarity level of the accessions ranged from 78 to 90%. The clustering of accessions in the dendogram did not show a clear pattern of clustering according to region or altitude. Accessions from different altitude and region groups often group together.

Discussion

The AFLP technique was helpful in discriminating the 150 OTUs, representing 26 landraces and four cultivars based on nine AFLP primer combinations. The technique was sensitive and powerful in determining the extent of diversity in the landraces and cultivars, and between cv. groups durum and turgidum. The reliability of AFLP in detecting genetic variation was reported in several studies: like the study of diversity in cereals (Barrett and Kidwell 1998a), AFLP analysis for plant variety registration in wheat (John *et al.* 1998) and the analysis of genetic relatedness in barley (Roger *et al.* 1997). The experimental reproducibility, coupled with the diagnostic ability of the technique also have made AFLP a useful molecular tool for measuring diversity.

The high within accession (0.66) and the low between accession variation (0.34) can have different causes: in Ethiopian's farmers field it is common to find many types of several species of tetraploid wheat growing mixed in relatively small plots of land (Harlan 1971), which favours genetic exchange that can

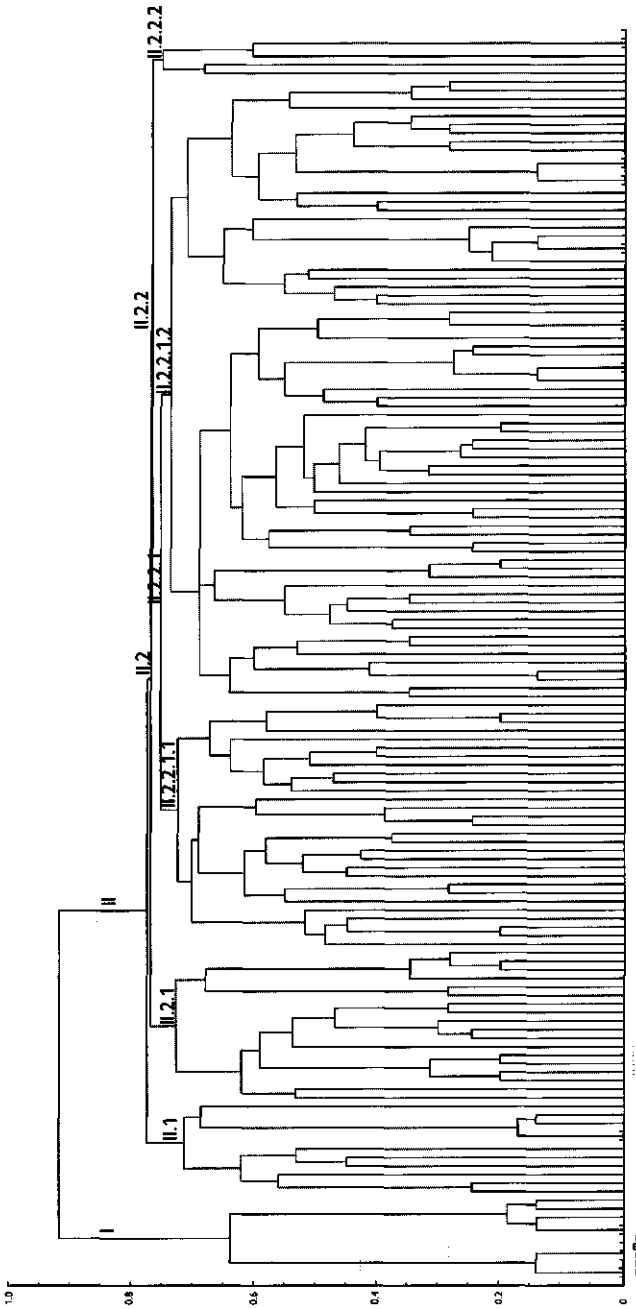

Fig. 5-1. UPGMA dendogram of the AFLP polmorphic bands for the 150 OTUs.

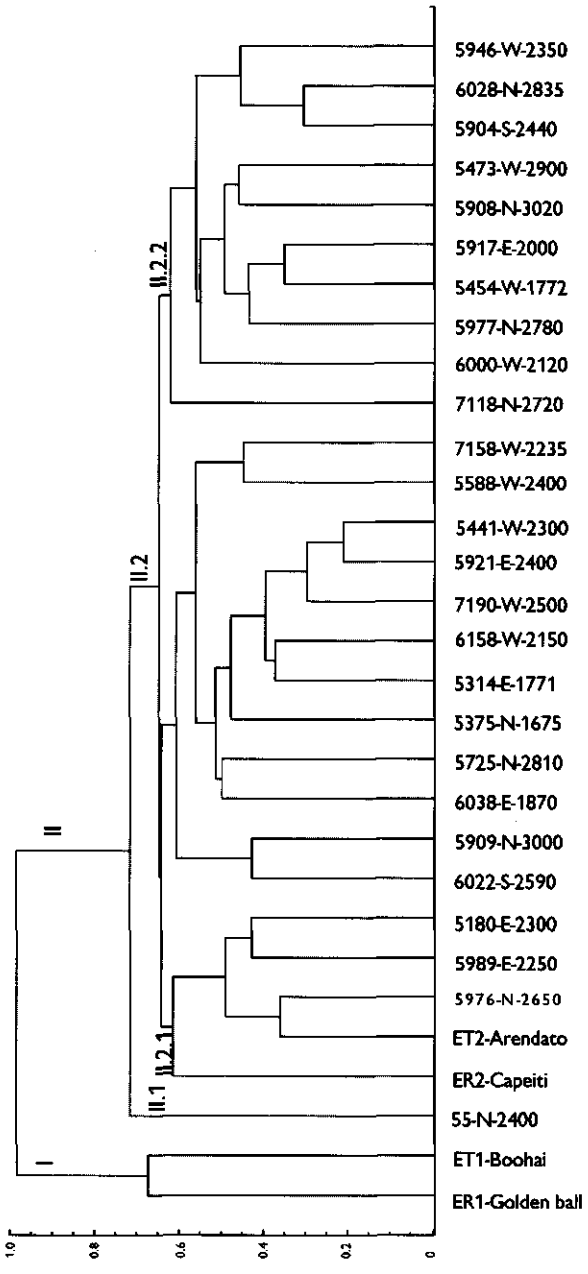

Fig. 5-2. UPGMA dendrogram of the AFLP bands grouped for the 26 accessions and 4 cultivars.

result from a low percentage of cross pollination. Cross pollination itself is not affected by mixed cultures, but this will only lead to new genotypes when it occurs between different genotypes (Moshe 1976). Vavilov (1951) observed that some of the Ethiopian tetraploid wheats open their glumes, the stamens lengthen outside the glumes and the anther hang out for some minutes enabling pollen shedding in the air and thus cross pollination.

The comparison between durum and turgidum based on genetic diversity showed that durum is more diversed than turgidum. The observation that durum and turgidum share common bands makes clear that they are very related, which may be explained by sharing common ancestors and by frequent exchange of genetic materials in the Ethiopian agricultural practice. Very little differentiation was found between altitude and subregion groups, suggesting that the genetical restraints for any particular environment cannot be observed in the present study and it also indicates that within the studied regions exchange between seeds from different subregions must be common.

6.

Analysis of gliadin and glutenin storage proteins for estimating genetic diversity in Ethiopian tetraploid wheat (*Triticum turgidum* L.) landraces

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Abstract

Ethiopian tetraploid wheat (*Tritium turgidum* L.) comprising 26 accessions from the Ethiopia National Gene Bank and six cultivars were used for this study. Five randomly selected seeds per accession were studied using gliadin and glutenin storage proteins. For these accessions and cultivars a total of 49 polymorphic bands were identified. Based on gliadin and glutenin bands we grouped the 160 OTUs (Operational Taxonomic Units) into four groups: with only γ-42 band (group I), with only γ-45 band (group II), with both bands (group III) and with neither of the bands (group IV). Using the presence and absence of γ-42 and γ-45 bands and also based on information obtained from the respondents the 160 OTUs were also classified into two use groups *i.e.*, bread making and alcohol production groups. Based on respondent information for bread making quality durum (27%) was superior to turgidum (6%).

Geographical distribution of the four classified groups showed that group II and group IV were distributed over all altitudes (1600 to 3020 m) and over all four sub-regions, while group I and III were only found within the altitude range from 2300 to 2600 m and within sub-regions north and west. The genetic diversity was estimated by calculating ShannonWeaver diversity index for each accession and for the four altitude classes and for the four subregions. The extent of variation for the within and between altitude and also within and between subregions was almost similar. We also observed that Shannon-Weaver diversity value for durum was slightly higher than that of turgidum. SW for the four altitude classes showed similar diversity values. Except subregion South the total diversity value for the three subregions are similar. Two UPGMA dendrograms to determine the level of similarity between OTUs and similarity between accessions were constructed. In the case of accessions dendrogram only two accessions that were collected from the same altitude showed 85% similarity level, while the similarity level for the 30 accessions ranged from 30 to 80%. Our study demonstrates that the gliadin and glutenin are highly informative and useful for estimating diversity in the Ethiopian tetraploid wheat landraces.

Introduction

The large number of wheat accessions (12,000) 23% of the total collection (54, 000) preserved in the national gene bank of Ethiopia, as is described in chapter 1 and 2 may contain a high incidence of redundant types. To characterise and verify these accessions efficient and reproducible markers are required. In this chapter the analysis of storage protein is presented. The storage proteins in wheat are usually classified into gliadins and glutenins, which together form the gluten fraction. The ability to use wheat flour for making bread, other baked goods and pasta is largely dependent on the property of gluten (Martin et al 1999).

Gliadins are small in size ranging from 28 to 70 kD, alcohol soluble, single chain polypeptides that can be classified into α, β, γ and ω-gliadin groups (Pogna and Mellini 1988). Glutenins are a group of heterogeneous molecules built up of different sub-units connected by disulphide bonds (Wall 1979) and range in size from 50 to 2000 kD (Payne 1987). The glutenin group is subdivided in to high molecular weight (HMW, 80-120kD group) and low molecular weight (LMW, 35-50kD group). The high molecular weight (HMW) glutenins are encoded by the *Glu-A1* and *Glu-B1* genes. Some low molecular weight (LMW) glutenins are encoded by *Glu-A3* and *Glu-B3* loci. Some are similar to gliadin in their biochemical

characteristics and amino acid sequences and the respective genes are located close to the gliadin genes. In addition to these genes (α, β, γ and ω-gliadin, HMW and LMW glutenin) encoding the major storage proteins, other loci encode minor gliadin and glutenin proteins (Pogna, Lafiandra and Masci, in press). The complexity of the genetics of storage protein is increased by the large number of alleles at various loci, which makes that varieties often differ in their electrophoretic storage protein pattern.

The presence of specific alleles has been related to the quality and technological characteristics of wheat flour. For example, Porceddu *et al.* (1998), Kosmolak *et al.* (1980), Zillman and Bushuk (1979a) and Federmann *et al.* (1994) classified closely related durum wheats into varieties with inferior and good cooking quality based on two gliadin (γ-42-gliadin = type1 and γ-45-gliadin = type2) alleles. The HMW subunit 5 + 10 seems to correlate also with good quality bread. The γ-42/γ-45 allele markers are tightly linked with the glutenin alleles at the *Glu-B3* locus (Pogna *et al.* 1990). The effect of the storage protein on wheat flour quality might imply that selection for high quality for specific use has led to fixing specific alleles.

It has been shown that seed storage proteins are highly polymorphic, which is strictly genetically determined (Benedettelli *et al.* 1990; Radic-Miehle *et al.* 1980, Khan *et al.* 1983; Damania *et al.* 1983; Autran *et al.* 1993; Wrigley and Shepherd 1973). The electrophoretic patterns can be directly related to allelic frequencies making the technique an ideal marker tool for estimating genetic diversity (Second 1982; Metakovsky *et al.* 1997). The storage proteins that include gliadin and glutenin alleles have also been used for wheat cv groups identification (Bushuk *et al.* 1978) and in estimating genetic and geographical diversity in wheat (Benedettelli et al. 1990).

In most parts of the world durum wheats are used for pasta production. However, in Ethiopia durum wheats are used for many more purposes. These include, leaven bread, injera (sort of pancake), cereals (breakfast food), porridge, Kitta (sort of pizza), pastries, whole seed boiled or roasted foods, and home-brewed non-alcoholic and alcoholic drinks. In addition, the crop-residues are used for fodder and construction. The tetraploid wheats in Ethiopia might have resulted in selection for specific protein compositions.

The aim of this work is to establish the genetic diversity in 26 Ethiopian tetraploid wheat accessions and six cultivars. Shannon-Weaver diversity index values over all accessions and for the 49 storage protein polymorphic bands were calculated and the results were used for establishing diversity for accessions, altitude classes and sub-regions. The UPGMA dendrogram was constructed to relate the 160 OTUs representing the 26 accessions and four cultivars and to determine the level of similarity between the accessions and cultivars.

Material and Methods

The 30 accessions used for this study and the geographical areas from where the accessions were collected are described in chapter 1 and 2. In this chapter in addition to the 26 accessions and four cultivars,

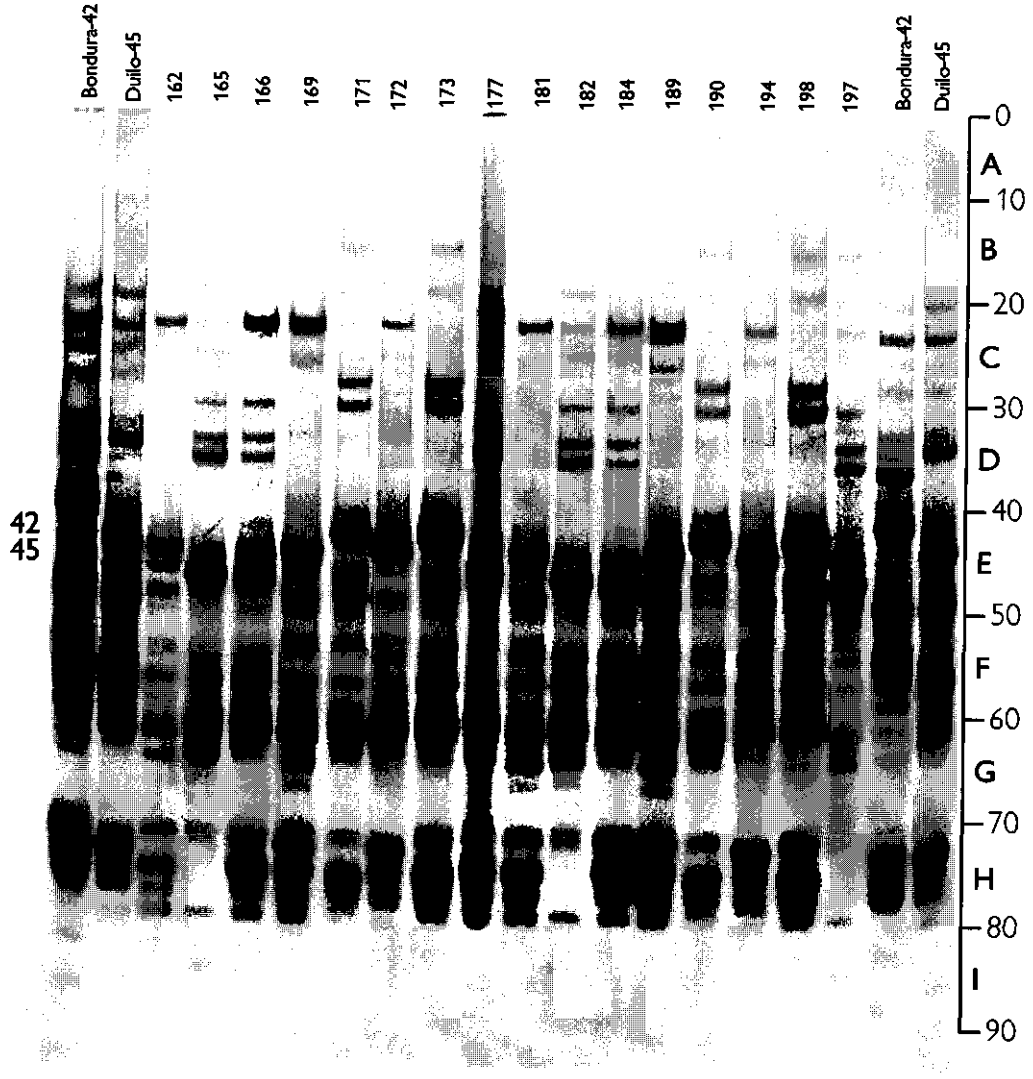

Fig. 6-1 . A-PAGE gliadin protein gel showing profiles of 18 storage protein

two Italian cultivars (Bondura and Duilo) were used as a standard for the γ-42 and γ-45 alleles, respectively.

Gliadin isolation and electrophoresis

The isolation of gliadin protein was done from a single seed by adding 200 mL 70% aqueous ethanol. After brief agitation on a high speed vortex, the suspensions were left to mix on a slow roller mixer

overnight. Next day, 200 mL sucrose solution (20 g sucrose was dissolved in to 50 mL sample dilution buffer), containing 0.2g methyl green was added to 200 mL gliadin extract. After brief vortexing and centrifugation at 13 000 rpm for five min, 10 mL of the supernatant from each sample was loaded to each slot of the 6% acrylamide gel. Acrylamide gel was prepared by mixing 35 mL acrylamide gel solution (Pharmacia 1999), 75 mL ferric sulphate and 120 mL 1% hydrogen peroxide.

Glutenin isolation and electrophoresis

Two wheat seeds from a single plant were crushed to a fine powder using mortar and pestle. The glutenin was isolated according to Singh et *al.* (1991): in the first step, gliadin was discarded by treating 30 mg flour in 1 mL 50% propanol for 30 min at 65 ºC with intermittent vortexing and centrifugation. This step was repeated two times. The remaining gliadin was further removed by washing the residue with 0. 5 mL 50 % propanol and finally the remaining liquid (drops) was removed by aspiration.

Glutenin was extracted from the residue by adding 0.2 mL solution of 50 % propanol plus 0.08 M Tris-HCl, pH 8.0. After initial brief vortexing, 1% freshly prepared dithiothreitol solution was added and incubated for 30 min at 65 ºC with 5 min centrifugation. Finally 1.4 % 4-vinylpyridine solution was added to the supernatant and incubated at 65 ºC for 15 to 30 min. After centrifugation for 2-5 min, 0.1mL supernatant was transferred to a new tube containing 0.1 mL sample buffer prepared by mixing: 2 %SDS, 40 % glycerol, 0.02 % bromophenol blue and 0.08 M Tris-HCl, pH 8.0. After the run the gel was polymerised in the gel cassette for about 1h to overnight, the stacking gel was poured on top of the running gel. Gel loading was done by pipetting 30 µL from each sample to each lane of the gel. The running 8.3% SDS-PAGE gel was prepared by mixing 11 mL acrylamide/bisacrylamide, 10 mL Tris buffer pH 8.8, 400 µL 10% SDS, 18 mL water, 400 µL 10% APS and 60 µL TEMED. A stacking gel solution was prepared by mixing 1.4 mL acrylamide/bisacrylamide, 2.5 mL Tris buffer pH 6.8, 100 mL 10% SDS, 6.4 mL water, 75 mL 10% APS, and 40 mL TEMED. The gel was stained in Coommassie brilliant blue staining solution (Pharmacia). After 1 h of shaking the gel was destained by boiling in fresh water for 15 min, and cleaning with fresh water. Destaining solution which contains 200 mL methanol, 10 mL 37% acetic acid and 800 mL water was used to make the gels firm.

Characterization of the 160 OTUs as to the application for bread and alcohol production

Based on Ethiopian commonly used criterions for selecting wheat seeds for making bread and alcohol the 160 OTUs were grouped into two groups. Three Ethiopian informants, two ladies and one man with different backgrounds: house wife, agronomist and extension agent, respectively, were involved in grouping the OTUs into the "bread" and "alcohol" groups. The criterions for characterization was mainly based on seed texture, *i.e.*, size and colour, vitreousness and grindability. Seeds that were hard and not or little vitrous, relatively small in size, and generally purple were grouped as appropriate for alcohol and other beverage production, whereas seeds that were pale, with little or no purple pigment, and vitrous were considered good for bread making.

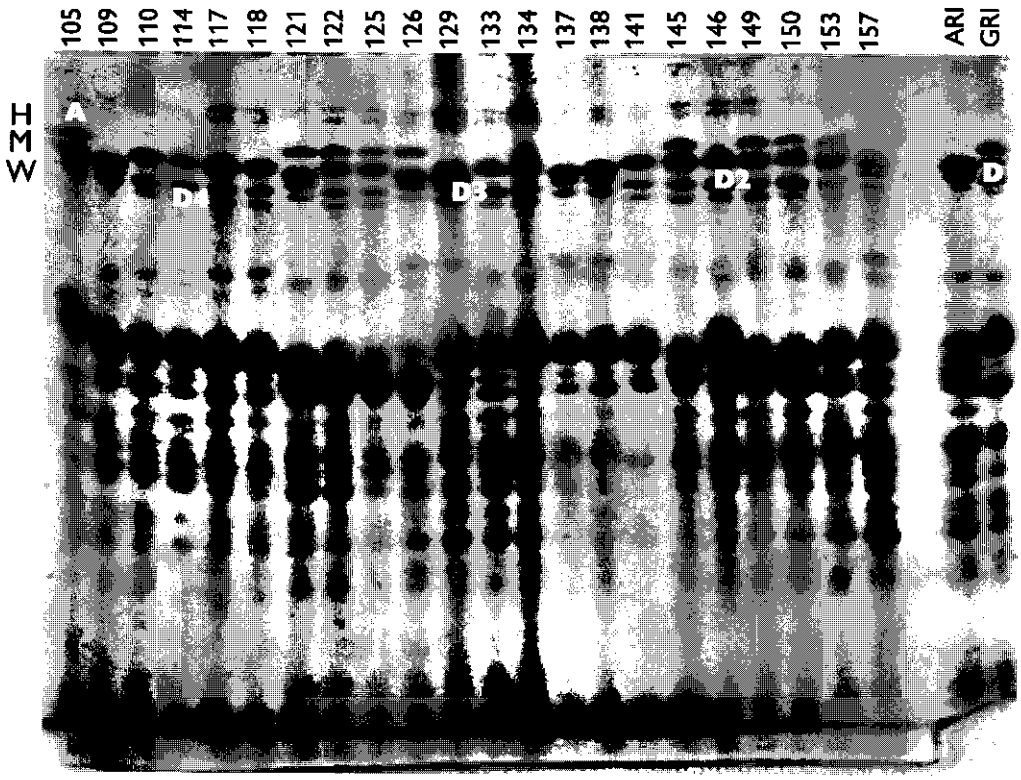

Fig. 6-2. SDS-PAGE HMW glutenin gel depicting the profiles of 24 OTUs.

Data acquisition and analysis

In both gliadin and glutenin gels the migration distance of individual bands was measured from photographs (Fig 6-1 and Fig. 6-2). Bands were designated based on their relative mobility compared to a reference band. Band scoring is similar to the nomenclature followed by Kosmolak et al. (1980). The relative reference protein component used to standardise the relative mobility was band 42 and 45, which are present in the Italian durum wheat cultivars (Bondura γ-42 and Duilio γ-45). The binary, 1 (present) and 0 (absent) data matrix over all OTUs was established. Band intensity differences were not scored.

Data analysis

Detailed description of data analysis followed for this chapter is given in chapter 2.

Results

The 160 Operational Taxonomic Unit (OTUs) representing the 32 accessions for the two storage proteins HMW (high molecular weight) and LMW (low molecular weight) glutenin and gliadins showed a

total of 49 polymorphic bands, out of which eight were from HMW glutenin and fourty one were from gliadin and/or LMW glutenin. Rare alleles (bands) were found in nine OTUs (allele-D3 in OTUs 6022-254,5917-134, allele-K in OTUs 5909-222, 6000-118, 6000-117, allele-ZF in OTUs 5441-53, 5441-57, 5921-65, and allele-AZ in OTU 5908-129 (data not shown).

Genetic diversity

Accession

The total mean Shannon-Weaver diversity value for the 26 accessions is 0.77 (Table 6-1). Ten accessions showed significant high diversity values ($P<0.05$).

Table 6-1. Storage protein Shannon-Weaver diversity for accessions and geographical areas

	Ht	Hs	Dst	Gst
Diversity for all accessions	0.77	0.44	0.33	0.43
Variation within and between altitude class	0.77	0.73	0.04	0.05
Variation within and between accessions per altitude class:				
1600-2000	0.74	0.35	0.39	0.53
2000-2400	0.75	0.35	0.40	0.53
2400-2800	0.74	0.39	0.35	0.47
2800 and >	0.70	0.32	0.38	0.54
Variation within and between subregions	0.77	0.67	0.10	0.13
Variation within and between accessions per subregion:				
East	0.74	0.32	0.42	0.57
North	0.72	0.30	0.42	0.58
South	0.48	0.10	0.38	0.79
West	0.75	0.30	0.45	0.60

Durum and turgidum

Shannon-Weaver trait diversity for durum and turgidum revealed that durum is slightly more diversed than turgidum. The SW total trait diversity average value for durum was 0.77, while for the turgidum it was 0.29. The Gst value for the differentiation of durum / turgidum is 0.31, which is higher than for

microsatellite and out of the same order of magnitude than AFLPs.

Durum and turgidum variation by number of bands

Two bands in LMW glutenin were specific to durum (ZF and ZG, table 6-2), while 47 bands out of 49 polymorphic bands were common for both durum and turgidum. Although the D3 band is not absent in durum, it seems more abundant in turgidum. The two bands that are unique to durum occurred at low frequency and can therefore not be considered durum specific (table 6-2).

Altitude and subregion variation

Table 6-1 presents storage proteins Shannon-Weaver diversity index values calculated for the total accessions, altitudes and subregions.

For the accessions, altitude classes and subregions the within accession components of variation are high and very similar (Table 6-1). The total diversity values for the four altitude classes are almost similar and almost no between altitude class variation is observed. For subregions a similar pattern for altitude groups is observed.

Variation by banding pattern

Gliadin

According to the presence and absence of band 42 and 45, we classified the OTUs into four groups, *i.e.*, with only γ42 (group-I), with only band γ45 (group-II), both bands (group-III) and neither band (group IV). The highest number of OTUs 74 (46%) were in group-II, followed by 56 (35%) in group-IV, the number of OTUs in group-I and III were low 16 (10%) and 14 (9%), respectively (Table 6-3).

Geographical distribution of the four classified groups showed that group-II and group-IV are distributed over all altitudes (1600 to 3020 m) and subregions (East, West, North and South), while group I and III were only within altitude range from 2300 to 2600 m and in two sub-regions, North and West (Table 6-3).

Glutenin

Out of the 160 OTUs one (5976-309) was different by lacking a band (D1) in the HMW glutenin.

Identical OTUs

In eight cases, two OTUs from the same accession (5908-122, and 5908-125; 6038-210 and 6038-209; 6158-234 and 6158-230; 5977-150 and 5977-149; 5180-278 and 5180-281; 6022-257 and 6022-258; 5909-225 and 5909-226; 5976-306 and 5976-305) and in two cases two OTUs from different accessions (5454-166 and 7118-169, 5908-120 and 7977-146) showed similar banding patterns.

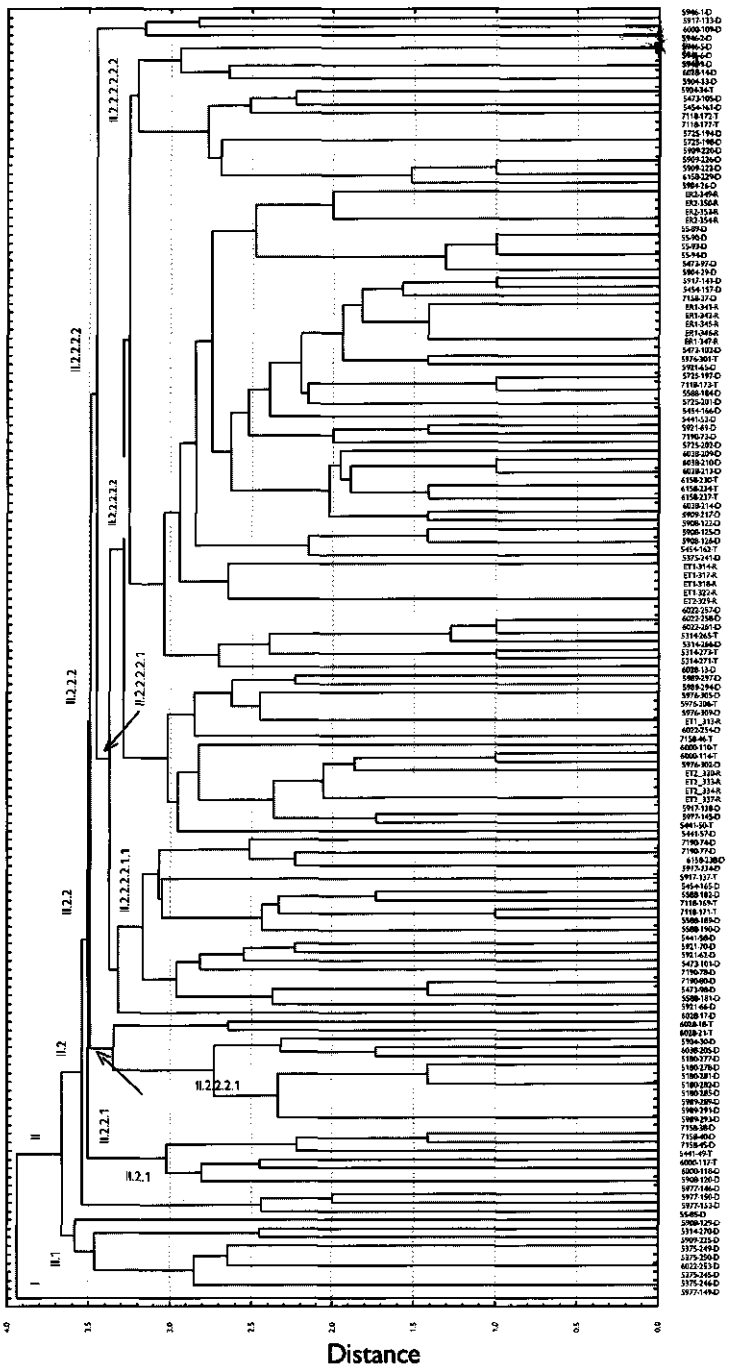

Fig. 6-3. UPGMA dendrogram of the storage proteins for all 160 OTUs.

Table 6-2. Durum and turgidum variation by the number of bands

Cultivar groups	Randomly selected bands											
	A1	C	D	D3	E1	F1	K	P	Q	R	ZF	ZG
Durum	13	46	59	2	40	41	2	15	47	8	3	1
Turgidum	1	30	29	10	13	3	1	2	7	2	0	0

Quality assessment

The quality assessment based on the information obtained from the respondents was related with the four classified gliadin groups (Table 6-3). Based on the respondent information, the percentage of OTUs possessing bread-making quality was high in group-II, followed by group-I. For bread making quality (27 and 6%) and for alcohol production (48% and 19%) was durum superior compared to turgidum. OTUs classified in all groups showed low bread making quality and high for alcohol.

Phenetic relationships

Cluster analysis

Fig 6-3 presents a similarity relation of the 160 OTUs, based on UPGMA dendrogram. The dendrogram shows two groups. In the dendrogram OTUs similarities within accession were generally higher than between accessions.

Fig. 6-4 shows the 30 accessions and cultivars similarity relation using a UPGMA dendrogram. The dendrogram has two groups. The first group (I) contains two accessions which clustered together, while the second group (II) had 30 accessions. In this dendrogram, except in few cases, there was no specific grouping observed by geographical area (altitude or region).

Discussion

Our results indicate that the two storage protein groups (gliadin and glutenin) are highly informative and suitable for estimating variation within and between accessions, between OTUs and within the two cv groups (durum and turgidum). In general the storage protein profiles appeared to be useful to assess genetic variation and relationships in Ethiopian tetraploid wheat.

The high accession variation observed in our storage protein experiment can be attributed to the selection for different uses like for making broad bread, leavened bread, diversity of alcohols, cereals, whole roasted seeds, whole boiled seeds, porridge, etc. The rare alleles only occurring in certain OTUs can be considered recent mutant or since these OTUs are only grown in the high and low extreme altitudes, the rare alleles might associate with stressful environment conditions like adaptability to drought or to high frost tolerance (Sozinov 1986).

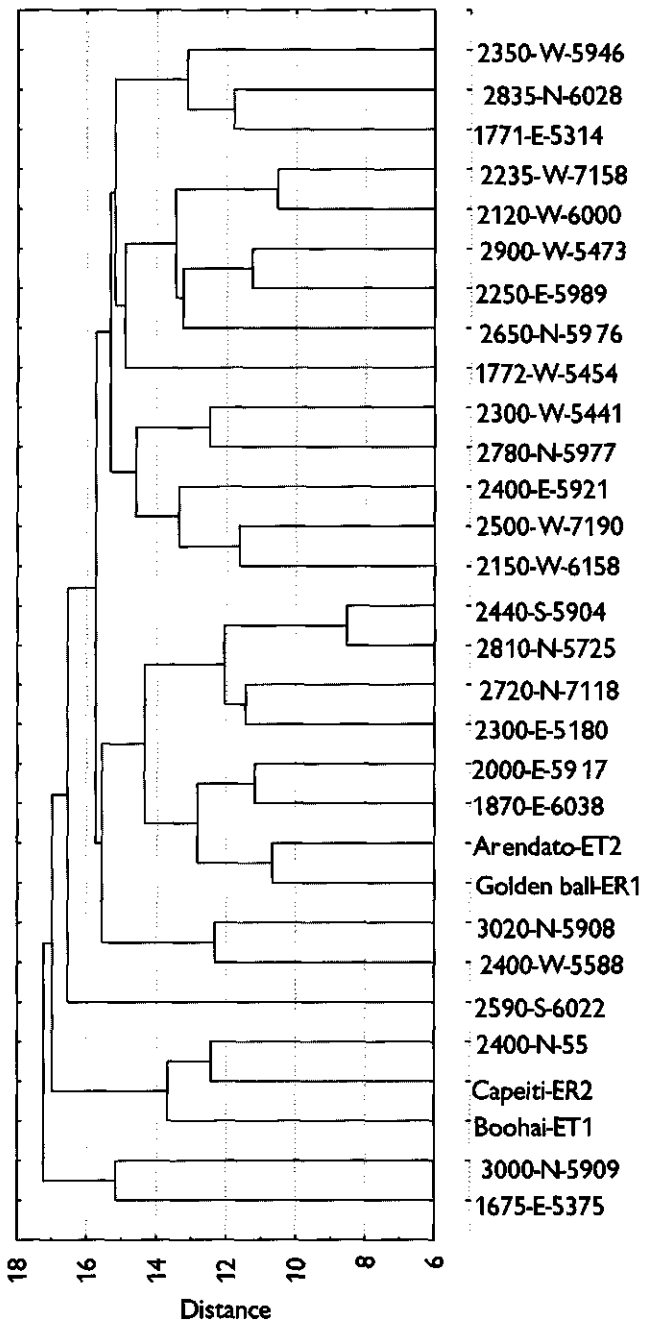

Fig. 6-4. UPGMA dendrogram of the storage proteins for the 30 accessions.

Table 6-3. Gliadin alleles (γ-42/γ-45) distribution by OTUs and geographic areas

γ-42	γ-45	OTUs		Traditional uses (no of OTUs)				OTUs distribution	
				bread		alcohol			
		Durum	Turgidum	D	T	D	T	Altitude	Sub-region
+	-	16	0	6	0	10	0	2300-2600	west; north
-	+	55	19	23	3	32	16	all ranges	all regions
+	+	7	7	1	2	6	5	2300-2600	west; north
-	-	41	15	13	5	28	10	all ranges	all regions

Turgidum is inferior to durum because of its hard, mainly low vitreous seed and low grain weight. Despite this no strong selection for specific bands or for a strong difference in variation level is found.

Phenetic relations

The phenetic relationship revealed that out of the 160 OTUs, in four cases, pairs of OTUs from the within accession and in two case pairs of OTUs from different accession were identical. The cultivars do not form a specific cluster indicating the overall protein pattern of these cultivars do not differ from the landraces. The observation that some durum and turgidum OTUs showed close clustering in the OTUs dendrogram (Fig. 6-3) indicate that there are intermediate types that have been developed through gene exchange, most probably while growing as a mixture in the same field. According to Bozzini (1988) and Schmidt and Heslop-Harrison (1998), the tetraploid wheats are easily inter-crossed and fertile and also they all have the same chromosome structure and perfect pairing at meiosis.

Quality assessment

Based on the two γ-42 and γ-45 bands it was possible to subdivid the 160 OTUs in to four groups, which can further be categorised into different quality groups (bread making and alcohol). The storage protein electrophoratic observation compared to respondent analysis based on quality assessment criteria showed that out of the 55 OTUs possessing γ-45 only 23 durum and three turgidum were recommended by respondent as good for bread making, indicating that not all durum types possessing the γ-45 are good for bread making. This observation agrees with the report made by Autran et *al.* (1993) that although there are some genotypes approaching the bread making quality of common wheat not all durum wheat possessing γ-45 has good bread making characteristics. Another possible explanation is that the γ-45 band might not be the only marker for identifying bread making quality in durum. There may be more genetic components linked to γ-45 that may have specific properties required for bread making (Pogna et *al.* 1988). Likewise, gluten protein specifically low-molecular-weight (LMW) and

high-molecular-weight (HMW) glutenin may have a role in durum bread making quality (DuCros et al 1982; Pechanek et *al.* 1997; Pogna and Mellin 1988; *cf. Liu et al.* 1996). According to Pechanek et *al.* (1997) experiments on bread making quality showed a higher correlation to bread volume with the percentage of HMW flour protein, while the reverse is true for LMW protein.

The presence of two alleles (γ-42 and γ-45) in a locus may interfere and impose a negative impact for bread making quality. DuCros et *al.* (1982), Taha and Sagi(1987) and Damidaux et *al.* (1980) in their comparative study indicated a dosage effect of both bands (γ-42 and γ-45), band γ-42 allele displaying a stronger negetive effect on bread making quality compensating for the positive effect of γ-45. This may explain why almost all Ethiopian OTUs that had both bands showed poor quality in spite the presence of band 45. Much more information is required to better understand the relationships between the presence and absence of γ-45 and quality related performances of the Ethiopian tetraploid wheats.

7.

General Discussion and Recommendations

In this thesis I have described my study on diversity assessments of 150 OTUs (Operational Taxonomic Units) representing 26 Ethiopian landraces and two cultivars, together with an Italian and a South African cultivar using morphological characteristics, chromosome morphology, AFLPs, microsatellites and storage proteins as markers. This study has four major objectives:

- Estimation of the magnitude of genetic diversity within and between the 26 Ethiopian tetraploid wheat accessions
- Detection of duplications within and between accessions
- Identification of the pattern of genetic diversity within geographical areas
- Obtaining information that can prioritise marker technologies for estimating the genetic variation in Ethiopian tetraploid wheat landraces.

Data analysis

To describe genetic diversity Shannon-Weaver indexes for the within and between accessions, and also for geographical areas were calculated. Four UPGMA dendrograms based on 150 OTUs (Operational Taxonomic Units) for the four markers were constructed and from these dendrograms the number of duplicate OTUs were scored for each marker (see chapter 2, 4, 5 and 6). In addition, the UPGMA dendrograms for accessions and cultivars were obtained to show their genetic relationships.

1. General discussion

1.1 Marker polymorphisms

Comparison of the four markers by the number of traits classes and number of polymorphic loci indicate that microsatellites (96), and AFLPs (84) showed relatively high polymorphism rates compared to morphology (64) and storage proteins (49). The chromosome analysis based on ten OTUs from six accessions revealed only twelve polymorphic C-bands and two more polymorphisms for tandem repeats. More cases of chromosome polymorphisms might be detected but would require time consuming experiments. Cytogenetics was therefore not further involved in the comparison of marker polymorphisms.

When the different markers measure the same genetically determined diversity, it is expected that the Shannon-Weaver (SW) values for individual accessions are strongly correlated. However, as shown in table 7-1 the correlation of SW values between morphological markers and the molecular and storage protein markers is very limited.

Table 7-1. Correlation matrix of the four markers calculated based on the mean Shannon-Weaver indexes per accession for all traits or bands. All correlations were significant.

Markers	Morphology	AFLP	Microsatellites
AFLP	0.18		
Microsatellites	0.16	0.38	
Storage proteins	0.15	0.13	0.43

Table 7-2. Correlation matrix of the four marker types calculated from their respective 150 OTUs UPGMA matrixes. All correlations were significant.

Markers	Morphology	AFLP	Microsatellites
AFLP	0.31		
Microsatellites	0.30	0.57	
Storage proteins	0.07	0.42	0.23

The low correlation values obtained between distance matrixes of the different markers (Table 7-2) indicate that the genetic similarities measured by the different markers is also relatively low. The highest correlation was found between the two neutral molecular markers. It might be possible that the genetic basis underlying the morphological variation is not always related to a large variation in neutral molecular markers. It might be possible that especially for the morphological traits selection is applied by the farmers compared to neutral traits.

The high polymorphism rate shown for microsatellites compared to AFLP can be ascribed to the mechanism of producing variation for this type of marker. The rate of mutation in microsatellites due to the DNA polymerase slippage is considered to occur more frequently compared to point and insertion/deletion mutations responsible for generating AFLP polymorphisms. A high level of polymorphism for microsatellites is reported by different authors (Plaschke *et al.* 1995; Edwards *et al.* 1991, 1992; Allen *et al.* 1995; Scribner *et al.* 1994; Chakraborty 1984). Microsatellites are considered to be superior to other molecular techniques such as, RFLP, RAPD and isozymes (Korzun *et al.* 1999; Röder *et al.* 1999), because it has a potential to produce sufficient variation, in the range of 60 to 90% polymorphic bands for variation within species.

The four marker sets used for our diversity assessment analysis for the 26 tetraploid accessions and for the four cultivars effectively detected variation within and between accessions and allowed a measure of similarity to be derived between any pair of OTUs (Operational Taxonomic Units) and accessions.

The level of diversity varied between accessions. However, for all markers, except for the morphology, the average within accession variation was higher than the between accession variation (Table 7-3).

Table 7-3. The within and between accession variation based on the total Shannon-Weaver index (Ht) and the coefficients of gene differentiation (Gst).

Markers	Ht	within accession variation	between accession variation
Morphology	0.80	0.44	0.56
AFLP	0.50	0.66	0.34
Microsatellites	0.55	0.65	0.35
Storage proteins	0.77	0.57	0.43

1.2 Duplicates

Among the landraces the number of observed duplicates differed considerably depending on the marker systems (Table 7-4). The observation that molecular markers revealed much less duplications than morphological analysis, indicates larger discriminative power of the molecular markers.

The lowest number of them were found with AFLPs where duplicates were found only six times involving twelve plants, which were all identified within the same accession. Two of these six duplicate OTUs were also identical for microsatellites and morphology. Some of the OTUs that have identical microsatellites could be distinguished on the basis of their AFLP and protein patterns. However, when the microsatellites were identical the differences for the other markers were very small. This indicates that the plants with identical microsatellites are also closely related on the basis of the other markers.

It should be noted that also the cultivars, which are considered to be pure lines and which also look identical for their morphological traits, show some differences for some molecular and storage protein markers. Apparently no true duplicates are present between the accessions. That accessions hardly contain duplicates is a surprise since in these mixtures of mainly pure lines the number of genotypes might

be limited. It indicates that landraces are rather heterogenous mixtures of lines in which a low level of cross pollination may increase the genetic variation between plants. Indications for some heterozygosity probably due to such cross pollinations were obtained in the microsatellite analysis (Chapter 4).

Table 7-4. Number of OTU duplicates within and between accessions and between and within 150 OTUs

markers	Number of identical OTUs within accession	Number of cases	Number of accessions with identical OTUs	Number of cases
morphology	2 3	4 1	2 3	20 1
AFLP	2	6	0	0
Microsatellites	3 5 2	1 1 2	2 3	1 2
Storage proteins	2	4	2	2

1.3 Durum and turgidum variation

The diversity within the durum and turgidum OTUs revealed that for the four marker types the total diversity for durum was higher than for turgidum. Among the markers the highest diversity within the durum group was observed for storage proteins, followed by AFLP and microsatellite markers (Table 7-5). The high diversity value for durum relative to turgidum might reflect the larger variation in the use of durum types compared to turgidum (Chapter 6).

Table 7-5. Comparison of genetic diversity within and between the durum and turgidum cv. groups for the four marker types

	durum	turgidum	Gst
Morphology	0.77	0.68	0.10
AFLPs	0.48	0.16	0.36
Microsatellites	0.52	0.40	0.15
Storage proteins	0.77	0.29	0.31

Despite the fact that four markers tested have differences in the potential of detecting diversity, each marker showed the potential of discriminating diversity in these closely related cv groups. The large overlap between both cultivar groups, shown by the low Gst value, implies that the groups are not sepa-

rated and that gene flow occurs frequently. The latter may be promoted by growing these types together in Ethiopian agricultural practice, which may lead to cross pollination.

1.4 Geographical diversity

Table 7-6 shows that the variation within altitude and subregion groups are very similar, suggesting that there is no restriction to a more limited number of suitable genotypes in some agro-ecological regions defined by these altitude and subregion classes. The low coefficients of gene differentiation (Gst) for geographical areas, altitude and sub-regions indicate that the four markers are not capable of pinpointing diversity by geographical areas.

The accession dendrograms (in the chapters 2, 4, 5 and 6) also showed that accessions from different altitudes and regions are often related and, however, do not form obvious clusters. This indicates that exchange of seed samples within the central region of Ethiopia is common practice and no specific restrains exist for agricultural practices. This implies that genetic isolation is limited.

Table 7-6. Shannon-Weaver indexes for altitude and subregion variation by marker type

markers	Altitude classes							Subregions						
	I	II	III	IV	Hs	Ht	Gst	E	N	S	W	Hs	Ht	Gst
Morphology	0.32	0.35	0.32	0.41	0.70	0.80	0.13	0.34	0.31	0.46	0.37	0.68	0.80	0.15
AFLPs	0.47	0.52	0.53	0.49	0.49	0.50	0.02	0.40	0.51	0.42	0.52	0.46	0.50	0.08
Microsatellites	0.49	0.50	0.52	0.48	0.50	0.55	0.09	0.51	0.49	0.36	0.52	0.47	0.55	0.15
Storage prot.	0.74	0.75	0.74	0.70	0.73	0.77	0.05	0.74	0.72	0.48	0.75	0.67	0.77	0.13

2. Comparison and evaluation of marker systems for diversity assessments

To attain the objective of diversity assessment in genetic resources for organisations like gene banks, only analysing and recording the diversity produced by one marker system is not sufficient. When comparing different marker systems the technical efficiency of the marker in estimating diversity and similarity needs to be also considered.

2.1 Technical efficiency

For morphological markers, the ease at which morphological traits can be assessed and the large samples that can simultaneously be studied provides an advantageous to use this marker type compared to the molecular markers and the storage proteins.

AFLPs diversity detection is based on polymorphisms of genomic restriction fragments. The advantage of AFLP technique is that it is not influenced by environmental factors and most restriction fragments detected by AFLP correspond to unique positions on the genome. The technique can generate

fingerprints of any DNA, no matter its origin or complexity in size (Vos et *al.* 1995), because the choice of primers allows variation in the number of bands that are amplified. The disadvantage of AFLP markers is that the interpretation is based on migrations of DNA bands and non-homologous but in size similar AFLP fragments can be scored as identical bands. The second drawback is that, because the AFLP technique is based on scoring the size of restriction fragments the allele identity or the type of locus is not considered, specifically when the locus is not mapped on the genetic map or other relevant information is unavailable. The third problems is that AFLPs are dominant markers, when scored as present / absent, and do not allow the detection of heterozygosity. The number of restriction fragment detected by AFLP is unlimited when different primers are used. However, to obtain scoreable bands the number of bands in AFLP gels based on a primer combination is usually restricted to yield 50 to 100 bands per gel, of which a limited number can be polymorphic.

The fact that makes the microsatellite technique a very useful marker for studying variation in landraces is that the technique can detect many alleles at a single locus and is not affected by environmental fluctuations. The other advantage of the microsatellite markers is that they display mostly co-dominant markers, in which the products of both alleles are visible as bands with different mobility within a gel. This allows the detection of heterozygous genotypes. Stability of inheritance in microsatellite and specificity to a given genome (A, B and D) in wheat (Chee et *al.* 1999) and reproducibility make microsatellites an ideal marker tool for efficient estimation of diversity in landraces. One of the drawbacks of microsatellite markers is, because of high species specificity, it often cannot be used to compare closely related species (Röder et *al.* 1999).

Concerning the storage proteins the marker technology is based on co-dominant alleles and they are strictly genetically determined (Benedettelli et *al.* 1990). Storage protein analysis produces bands for different alleles for a limited number of loci. The latter implies that the information obtained by storage proteins covers a small part of the genome (Lafiandra et *al.* 1993).

2.2 Financial and technical considerations

Some of the markers are relatively expensive and technically demanding, so that the development of cheaper and less demanding techniques with high reproducibility is required. It means that, in addition to comparing the potential of each marker in estimating diversity, it is important to consider financial features before deciding which marker system to adopt. This is because in the organisations such as gene banks financial considerations play a major role.

PCR-based molecular markers like AFLPs (see table 7-7) and microsatellites require PCR primers and sometimes radioactive detection procedures, which means high costs for the diversity assessment (Karp et *al.* 1997). Also the supply of these materials is often difficult. Microsatellites also require an appropriate expensive infrastructure during the developmental phases. However, once a set of microsatellites have been developed, only a supply of primers for application is required. The cost for radioactive or nonradio-active (fluorescent) reagents makes AFLPs more expensive per assay compared to microsatellites. In addition, the establishing and management of radioactive substances and waste do

not invite to adopt markers requiring radioactivity like AFLP (Milbourne 1997). The cost needed for running a sample for storage protein analysis compared to the AFLP and microsatellite is less. There are no requirements for sophisticated laboratory infrastructures or laboratory equipment and reagents. Morphological analysis can be conducted in the field as well as in the green house conditions. In the field, mainly in the rain fed regions the costs for regulating the conditions in the greenhouses are relatively low. Under field conditions in small areas, diversity for a large number of samples can be assessed within a short period of time depending on the plant type. Compared to molecular markers the time and cost for assessing a similar number of samples for morphological analysis is low (Table 7-7).

Table 7-7. Comparison of morphological, AFLPs, Microsatellites and storage protein technologies for diversity studies (after Karp et *al.* 1997; Newbury and Ford-Lloyd 1997; this study)

Technical and cost criterions	Morphology	Chromosome morphology	AFLP	Microsatellites	Storage proteins
Development costs	Low / No	Low	Low	High	Low
Level of polymorphisms	High / medium	High/Low	Medium/High	High	Low/Medium
Different bands/trait classes **	64	< 150 C-band many FISH bands	84	96	49
Number of traits/assay	14	not relevant	9	12	2 (gels)
Reproducibility	Low/Medium	Medium	Medium/High	High	Medium
Cost per assay	Very low	Medium	Medium	Low	Low
Radio-active requirement	No	No	Yes / No*	Yes / No*	No
Samples per day	-	1-5	50	40 - 80	40 - 80

*) alternative detection methods (fluorescent labelling, silver nitrate, ethidium bromide) are now available.

**) in the present study.

3. Conclusions and recommendation

The diversity detected by using the AFLP and microsatellite markers showed little difference (see tables 7-1, 7-2 and 7-6). Table 7-7 shows a comparison of all four markers based on diversity estimating potentials, technical efficiencies and cost effectiveness. Either the marker is efficient by one of the criterions or

not efficient in others, for example, microsatellite and AFLP detect more variation and also showed high level of reproducibility but they are not cost effective. Chromosome and storage protein analyses are relatively cheap. Storage protein analysis gives high reproducible results and can be applied routinely in large landraces collections. However, the number of polymorphic bands has an upper limit, whereas with molecular markers the number of markers is almost unlimited, when one increases the number of primer combinations. Storage proteins have been used extensively for the analysis of diversity in durum wheats (Pflüger *et al.* 2001). In the case of morphology the analysis is cost effective and easy to apply. However, some of the traits like plant height, ear and awn colour, and ear density may be strongly affected by environmental influence. Despite this drawback, it is possible to use morphological traits for diversity estimates, when environmental factors can be kept low.

Morphological traits should always be included as markers for diversity assessment, because they are valuable indicators of genetical diversity between and within the geographical and ecological zones, and are easy to assay. The integration of morphological data with molecular and biochemical markers avoids duplicate lots within the collections as could occur when single marker systems are used, and thereby also limits genetic erosion. Due to the limited number of loci, it is ambiguous that the storage protein alone is sufficient and reliable to estimate the genetic diversity in the landraces. However, because of the high total number of bands (different alleles and different loci) among the Ethiopian landraces, we recommend that the storage protein in conjunction with or as a complementary to other markers like morphology can be useful for the gene bank in estimating diversity. Molecular markers like microsatellites that detect neutral genomic differences might be used for additional characterisations. Chromosome analyses are important to distinguish large scale genomic rearrangements like translocations and inversions and are required to establish ploidy levels and aneuploidy.

Chase (1998) mentioned that using a single marker for diversity assessment could misinform the degree of the diversity and advises to incorporate at least more different types of markers. The other argument to use integrated markers for estimating diversity in the landraces is that (Lafiandra *et al.* 1993, Milbourne *et al.* 1997; Tsegaye *et al.* 1996; Russell *et al.* 1997; Bekele 1985) markers might have specifc distribution patterns, which may differ between markers. Moreover, different markers represent diversity from within different regions of a genome.

Landraces are the result of long evolutionary processes under the influence of environmental conditions and human selection pressure and as a result of which they are well adapted to different environmental complexes (Tesfaye 1991b). Genetic resources such as the Ethiopian tetraploid wheats are valuable sources of genes useful for many programmes aiming at breeding for resistance to biotic and abiotic stresses. Using the integrated diversity assessment work the gene bank can provide genetic background information by which specific genotypes can be identified. Maxted *et al.* (1997) suggest that a gene bank should collect and preserve the possible range of the available diversity within the country. For this, detailed information on the range and amount of genetic diversity, and its geographical distribution needs to be included. However, even if more marker types are applied to detect diversity in the landraces, this does not mean that the magnitude of the variation within that particular population is

totally studied and resolved. Moreover, high diversity can be reached in one part of the genome and may not in the part designed for the particular genome region. According to Maxted *et al.* (1997) and Ayad et *al.* (1997) to ensure and achieve the conservation goals, in addition to morphological and molecular more investigation and diversity assessment work like, eco-geographical, breeding systems needs also to be done.

In conclusion, different but integrated approaches that includes morphological, storage proteins, together with microsatellite and in some cases, chromosome analyses are recommended as tools for effective diversity assessment work in the gene bank. One of the outstanding problems in using integrated marker systems is the cost for dealing with the very useful molecular markers. To alleviate the cost inquired by the molecular techniques a small subset (5 to 10%) of the accessions representing the maximum diversity of the entire collections needs to be comprehensively evaluated and preserved for further improvement and utilisation purposes through frequent multiplication or rejuvenation processes. The 90% of the total accessions can be preserved for long term and in case of future requirements. According to Frankel (1970) and Anderson and Fairbanks (1990), this approach is most cost effective and might help to effectively promote the collection and conservation strategy in the gene bank.

8.

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9.

Summary

In this thesis the diversity analysis for a total of 150 OTUs (Operational Taxonomic Units) representing 26 Ethiopian landraces (accessions), together with four cultivars, is described using morphological traits, chromosome band polymorphisms, AFLPs, microsatellites and storage protein markers (gliadins and glutamins).

The aims of this study are:

- To estimate the magnitude of genetic diversity within and between the 26 Ethiopian tetraploid wheat accessions
- To identify the pattern of genetic diversity within and between geographical areas
- To detect duplications within and between accessions.
- To prioritise markers useful for estimating genetic diversity in Ethiopian tetraploid wheat landraces

To detect diversity, similarity relationships between individual plants and between accessions the data obtained from each marker were independently calculated per marker. The extent of diversity was calculated using Nei's and Shannon-Weaver diversity indices. The similarity within the tested OTUs and within the 30 accessions was revealed by constructing UPGMA dendrograms.

The within and between accession variation

Analysis of the diversity for the 26 Ethiopian tetraploid wheat accessions revealed that the accessions differed in the extent of their diversity when compared between the four marker types (morphology, AFLP, microsatellites and storage proteins). The analysis of variation among the accessions indicated that the high variation is due to the within component of accession rather than the between component. The diversity estimated between the morphologically distinguishable cultivar groups durum and turgidum showed that durum had more variation than turgidum. The durum and turgidum relationships analysed by Principal Component Analysis (PCA) based on morphological descriptions separated the

two cv. groups most clearly in a scatterplot of the second and the third component. Five morphological characters for awn and lower glume shape (AWC, AWL, LGSW, LGSS and GBKS) that showed high factor loading values were considered for the separation of the two cultivar groups.

The use of chromosome banding and Fluorescence *in situ* Hybridization of spread metaphase complements have demonstrated few cases of chromosome polymorphism within and between accessions. However, the observed variation of chromosome morphology was insufficient and the techniques too laborious to merit large scale application of this marker technology for diversity estimations.

Although the four marker types differed in their ability to detect diversity, they all could demonstrate diversity within and between accessions. The Shannon-Weaver relative coefficient of gene differentiation (Gst) for the 26 accessions per marker revealed that for morphology the variation for the between component of accessions is relatively high, whereas for the AFLP, microsatellite and storage protein markers the within accessions components were higher.

The correlation coefficient statistics calculated to determine the level of proximity between combinations of markers indicated that the highest correlation values were displayed between AFLP and microsatellite and also between storage protein and microsatellite markers. Morphology had the lowest relation with storage proteins, followed by AFLP and microsatellite. The AFLP, microsatellite and storage protein markers showed comparable correlations to morphological marker.

Geographical diversity

Altitude

The relative coefficient of gene differentiation (Gst) for the within and between altitude class variation calculated for the four altitude classes (1600-2000m, 2000-2400m, 2400-2800m and above 2800m) per marker revealed that, the within altitude class component had a stronger effect than the between altitude component. For the four markers the highest the within variation value for the four altitude classes were observed for storage protein, followed by morphology and microsatellite markers.

Subregions

The relative coefficient of gene differentiation (Gst) values for each marker for the four sub-regions disclosed the within component of variation had relatively high compared to the between sub-region variation. This result shows that certain agro-ecological conditions favour specific genotypes and that large variation within each region overshadowed differences between regions.

Duplications

The number of duplicate OTUs was determined using the UPGMA dendrograms constructed for each of the four markers. Based on the OTUs relationships on the dendrogram the number of duplicate OTUs was established for each of the markers. Relatively large numbers of duplicates were found in

morphological markers, whereas AFLP and storage protein markers showed less in number of duplicate OTUs.

Prioritising markers

The integrated application of morphological, microsatellite and storage protein markers is recommended. Especially the combination of morphology and protein storage markers are prioritised for effective and efficient diversity estimations of Ethiopian tetraploid wheat landraces.

Samenvatting

In dit proefschrift is de diversiteitanalyse beschreven van een totaal van 150 OTUs (operational taxonomic units), die 26 Ethiopische landrassen (accessies) en vier cultivars weergeven, gebruikmakend van morfologische eigenschappen, chromosoombandpolymorfieën, AFLPs, microsatellieten en zaadopslag-eiwitmarkers (gliadinen en glutaminen).
Het doel van deze studie is:

- de omvang van de genetische diversiteit binnen en tussen de 26 Ethiopische tetraploïde tarwe-accessies te bepalen
- het patroon van de genetische diversiteit binnen en tussen geografische gebieden vast te stellen
- duplicaties binnen en tussen accessies te vinden
- markers te prioriteren die bruikbaar zijn voor het schatten van genetische diversiteit in dit materiaal

Om diversiteit en verwantschapsrelaties tussen individuele planten en tussen accessies vast te stellen, werden de data van elke markerset onafhankelijk berekend. De mate van diversiteit werd berekend op basis van Nei's en Shannon-Weaver's diversiteitsindexen. UPGMA dendrogrammen werden gemaakt om de mate van gelijkheid tussen de geteste OTUs en binnen de 30 accessies duidelijk te maken.

De variatie binnen en tussen accessies

Analyse van de diversiteit voor de 26 Ethiopische tetraploïde tarwe accessies bracht aan het licht dat de accessies verschilden in hun mate van diversiteit, afhankelijk van het markertype. De analyse toonde verder aan dat de hoge variatie meer veroorzaakt wordt door de binnen-accessie component dan door de tussen-accessie component. De geschatte diversiteit tussen de morfologisch onderscheidbare durum en turgidum typen van dit materiaal toonde een hogere waarde voor de durum planten. Principale componenten-analyse van de morfologische kenmerken scheidden de durum en turgidum cultivargroepen het best in een scatterplot van de tweede en derde component. Vijf morfologische kenmerken voor kafnaald en onderste kelkkafje (AWC, AWL, LGSW, LGSS en GBKS), die relatieve hoge factorladingswaarden toonden, werden verantwoordelijk gehouden voor de scheiding tussen durum en turgidum.

Toepassing van chromosoombandering en Fluorescentie *in situ* Hybridisatie op gespreide metafasecomplementen bleek in staat om chromosoompolymorfie tussen en binnen accessies aan te tonen. De variatie in chromosoommorfologie was evenwel te gering en de technieken te arbeidsintensief om dit markertype uitgebreid op het materiaal toe te passen.

Hoewel de overige vier markertypen (morfologische, AFLPs, microsatellieten en zaadopslageiwit markers) verschilden in de mate van diversiteit, konden allen diversiteit binnen en tussen accessies aantonen. De Shannon-Weaver relatieve coëfficiënt voor gendifferentiatie (Gst), berekend voor alle 26 accessies, toonden aan dat de variatie voor de tussen-accessie component voor morfologie relatief hoog is, terwijl voor de AFLP-, microsatelliet- en opslageiwitmarkers de binnen-accessie component hoger is.

Correlatiecoëfficiënten berekend tussen iedere combinatie van de verschillende datasets (morfologie, AFLP, microsatelliten en opslageiwitten) gaven de hoogste correlatiewaarden voor AFLPs en microsatellieten, en voor opslageiwitten en AFLPs. Een opvallende geringe correlatie werd gevonden tussen de Morfologie- en eiwitopslagmarkers, gevolgd door AFLP en microsatellieten. De AFLP, microsatellieten en opslageiwitten vertoonden vergelijkbare correlaties met de morfologische marker.

Geografische diversiteit

Hoogteklasse

De relatieve coëfficiënten voor gen differentiatie (Gst) voor de variatie binnen en tussen hoogteklassen (1600-2000m , 2000-2400m, 2400-2800m en boven 2800 m) berekend voor de vier hoogteklasses per marker toonden aan dat de binnen-hoogteklasse component een groter effect had dan de tussen hoogteklasse component. Van de vier markers vonden we de hoogste binnen en de laagste tussen Gst waarde voor de opslageiwitten, gevolgd door morfologie en microsatellietmarkers.

Subregios

De relatieve Gst waarde voor elke marker voor de vier subregios (Noord, Oost, Zuid en West) bracht aan het licht dat de binnen subregio variatiecomponent was relatief hoog vergeleken met de tussen subregio component. Hierbij hadden Morfologie en Opslageiwittende relatief hoogste binnen subregio Gst en de laagste tussen subregio variaties vergeleken met die van AFLP en microsatellieten. Het blijkt dus dat er geen specifieke genotypen zijn in bepaalde agro-ecologische omstandigheden en dat de gevonden verschillen overschaduwd worden door de grote variatie in ieder gebied.

Duplicaties

Het aantal duplicaties binnen de OTUs was vastgesteld op basis van de UPGMA dendrogrammen die voor elk van de markers waren geconstrueerd. Deze analyse toonden verschillende aantallen duplicaties bij de verschillende markers. Relatief grote aantallen duplicaties vonden we in de morfologische dataset, terwijl AFLPs en opslag-eiwitten een gering aantal duplicaties gaven.

Het prioriteren van de markers

De geïntegreerde toepassing van morfologische, microsatelliet- en eiwitopslagmarkers wordt aanbevolen. Vooral de combinatie van morfologie en opslageiwitten wordt geprioriteerd voor effectieve diversiteitsschattingen in de Ethiopische tetraploïde tarwe landrassen.

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