Cloning and characterizing repetitive sequences for the study of molecular diversity in Triticeae
by Ipsita Sarkar

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Agronomy
Montana State University

Abstract:
The Triticeae is a tribe of immense economic importance. The large number of species in the group and extensive pattern of hybridization among the species makes classification difficult. Several studies have shown that repetitive DNA sequence may vary among related species. In this study, we assessed the possibility of using this variation to construct a phylogeny of the Triticeae.

Variation in the abundance of repetitive DNA sequences in 25 diploid species covering 12 genomes (A, B, D, F, K, M, Q, R, T, U, V and Z) from the Triticeae was studied. Relationships among 25 diploid species were deduced using phenetic and single degree of freedom contrast analysis.

Abundance of 15 moderately to highly repeated DNA sequence probes (8 were derived from the A genome and 7 were derived from the V genome) varied not only among species but also within species. Phenetic analysis showed inconsistency in results whereas single degree of freedom contrast analysis gave consistent results in indicating relationships. The study supported the amalgamation of Triticum and Aegilops into one genus Triticum. The V genome of Dasypyrum was found unrelated to all other genomes studied instead of its appearance in a cluster with the Q, F, K, R and T genome in the phylogenetic tree.

Our result suggested that probes derived from a genome which is closely related to a group should only be used to indicate relationships among them.

CLONING AND CHARACTERIZING REPETITIVE SEQUENCES FOR THE STUDY OF MOLECULAR DIVERSITY IN TRITICEAE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

ABSTRACT

The Triticeae is a tribe of immense economic importance. The large number of species in the group and extensive pattern of hybridization among the species makes classification difficult. Several studies have shown that repetitive DNA sequence may vary among related species. In this study, we assessed the possibility of using this variation to construct a phylogeny of the Triticeae.

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Our result suggested that probes derived from a genome which is closely related to a group should only be used to indicate relationships among them.

CHAPTER 1

INTRODUCTION

The tribe Triticeae (Poaceae: Gramineae) consists of more than 300 species, including both diploid and polyploid species. The extensive pattern of hybridization among a large number of genomes has made classification of the Triticeae a difficult task and has resulted in nomenclatural problems. However, this type of evolutionary pattern has made the tribe rich in genetic variation.

The Triticeae is of great economic importance, containing not only cereal crops such as wheat, barley and rye but also useful species of other types. These include forage crops (such as species of *Agropyron, Dasypyrum, Triticum*), ornamental species (such as *Critesion jubatum*) and weeds (such as species of *Hordeum, Triticum, Secale*). In addition to the direct importance of several species, many species in the Triticeae contain agronomic traits of interest to plant breeders. These include disease resistance in *Thinopyrum species* (Knott et al., 1977) and *Dasypyrum villosum* (Jan et al., 1986), drought and alkalinity tolerance in *Psathyrostachus species* (Plourde et al., 1990) and perennial habit in approximately 75% of the species in the Triticeae (Dewey, 1984). As stated by Kimber (1984), knowledge of relationships between cultivated species and their wild

relatives may help in the use of the wild species in applied plant breeding. This is mainly because of the wide genetic resources in the tribe. It is assumed that finally all these important characters are genetically mediated. In other words the expression of phenotypic characters is a reflection of genetic characters. This assumption is mainly based on the ubiquitous existence of DNA as a basis for subtle changes in the genotype through the evolutionary process. Thus, it is worth knowing the biological relationships and genetic constitution of various species and their genomes if we want to utilize them through applied plant breeding.

The generic makeup of the Triticeae has historically varied widely. Linnaeus (1753) named seven genera while Bentham (1881) recognized 12 genera. Nevski (1933) described 25 genera which Pilger (1954) and Tzvelev (1973, 1976) followed with some modifications. Melderis et al. (1980) recognized 14 genera by incorporating Nevski's concepts into Bentham's treatment. Recently, a unique treatment was proposed by Love (1982, 1984) and this treatment was supported by Dewey (1982, 1984). Love (1982, 1984) recognized 38 genera entirely on the basis of genomic relationships in which a genus was recognized as a biological unit consisting of species of a given genome or a combination of genomes.

The genomic system of classification is based on Love's taxonomic philosophy that a classification should reflect phylogenetic history and biological relationships. Given that

the genome mediates stable inheritance of characters, it follows that all characteristics of interest are genetically mediated. Phylogenetic inferences are based on inheritance of ancestral characteristics, thus they reflect either directly or indirectly genomic information. Finally, evolutionary relationships represent step by step accounting of fixed mutations in each and every genomic lineage of interest. Consequently, the major argument in favor of genomic classification has been the clarification of evolution of members of the tribe.

Several arguments against the genomic system of classification have come from Baum, Estes and Gupta (1987). For instance, they argued that genomes are merely a single character, thus classification based on the genome alone is unreliable. They also pointed out that genome classification leads to many monotypic genera and classification will change with every new genome combination. Additionally they suggested that the genomic character is an unreliable taxonomic character because of the incongruence in genomic and morphological characters most of the time. According to Kellogg (1989), the most serious objection to the genomic system of classification is that the genome is a continuum rather than a discrete unit. The continuum can range from complete homology to partial homology (homaeology) to nonhomology, and thus is not a very reliable character. On this point, even Dewey (1984) has mentioned the problem of

subjectivity in separating related genomes. However, he mitigated the importance of this negative point by indicating the general agreement on the genome makeup of the Triticeae among cytogeneticists. In any event, the problem of subjectivity cannot overcome the value of using a classification system which represents biological relationships and evolutionary history for a group which is so rich in genetic variation.

A primary concern should be the way genomic data may be applied to taxonomy, rather than the value of genomic relationships (Dewey, 1984). Classification in the Triticeae should start with a good phylogeny. Since there are several ways to construct a phylogenetic tree, any one of them that is most suitable for a particular experiment may be used. Although various types of characters may be used , some sort of analysis will be needed for the meaningful use of data to produce phylogenies. The most important potential problem at this point may be the selection of character, namely morphological, cytogenetical or molecular.

On this point, Crawford (1990) has suggested the use of characters by plant systematists which have a genetic basis. The basis for this is that phenotypic characters are the reflection of the genetic characters. Genetic characters are responsible for bringing changes in organisms through time and thereby represent evolutionary relationships. Though the argument that taxonomic classification should be based on the

genetic materials is straightforward, the use of molecular data in systematics has caused controversies for many reasons. Some of these reasons are unique to molecular data whereas others are applicable to all types of data.

An understanding of the following two topics may shed some light on the validity of controversies.

Molecules Versus Morphology

There have been disputes regarding the use of morphological or molecular data for systematic studies. Though collection of gross morphological data may be easier than the collection of molecular data, some authors have recommended that morphological characters may be misleading in that environment may influence morphology (Frelin and Vuillenmer, 1979; Sibley and Ahlquist, 1987). Additionally, morphological measurements are several steps removed from direct measurements of genetic materials. A minute change in any single locus of the genetic material might create a new characteristic in function or other, without altering the gross morphology. This kind of change in turn might create a distinct generic rank though the gross morphology remains the same. On the other hand, though molecular data are a direct reflection of genetic information, some taxonomists (eg. Kluge, 1983) see molecular data as comparatively weak. Comparative biological studies have found that morphological

change and molecular divergence have been under different evolutionary selection pressure and thus have followed different rules and paths through evolution (Wilson et al., 1977). Both types of data have unique advantages and disadvantages. Our purpose should be to use a character preferably with a clear genetic basis, which is appropriate to the question asked and which can be collected and used in a meaningful way to answer the question. Since the two above mentioned characters are under separate evolutionary processes, studying both molecules and morphology instead of only one can provide a broader sense of biological diversity through time.

Molecular Homology

The study of evolutionary relationship among organisms using morphological characters has relied on two basic concepts required for this type of study. These two concepts are the concept of homology and its application in comparative biology. On the contrary, concepts of molecular homology have remained undeveloped (Bledshoe and Sheldon, 1990).

Homology in general is the correspondence between characteristics, caused by continuity of information (Van Valen, 1982). In more details, homology between two organisms is represented by the fact that these two organisms have common ancestry. Similarity has sometimes been misinterpreted

as homology. The main difference between homology and similarity is that the former is a hypothetical whereas the latter is an empirical term.

Homology at the molecular level infers genetical relationships caused by modification of the genetic structure at some previous point (Bledshoe and Sheldon, 1990). Since molecularly homologous features are an outcome of modified and inherited genetic materials from a common ancestry, they are direct measure of evolutionary relationships. This may make molecular homology a better tool for studying phylogenetic history. Both paralogy and orthology are to be considered as valid indicators of molecular homology (Bledshoe and Sheldon, 1990). Paralogy can be exemplified as homology in the gene family whereas orthology can be exemplified as homology in a particular gene locus. By its ability to be expressed at various level of organization, which are independent, molecular homology is synonymous to partial homology.

In summary, the researcher assumes that the observed variation in the character is a reflection of genetic variation and thus DNA is the preferred material for studying phylogeny. Within a plant genome the majority of DNA is repeated nucleotide sequences. Flavell et al. (1974) and Nagl et al. (1983) found that repetitive DNA can constitute up to 90% of a total plant genome. In wheat only 12% to 20% of the genome consists of "unique" sequences (Smith and Flavell, 1975; Flavell and Smith, 1976). The remaining 70% to 80%

consists of highly or moderately repeated DNA. Their organization may be localized or interspersed throughout the genome. Though many of these repeated nucleotide sequences from various sources have been characterized on the basis of their length, abundance, distribution in the chromosome or even nucleotide sequence, but no definite function can be attributed to repeated DNA sequences. Since eukaryotic genomes contain both unique and repeated DNA sequences, some differences in the both types of sequences are expected through evolution as subtle changes in genomes take place under selection pressures. Chooi (1971) and Straus (1972) found that different species of *Vicia* with the same chromosome number, exhibit a six-fold range in nuclear DNA content. This kind of variation can be explained largely due to variation in the repeated DNA sequences. The reason behind this is that changes in unique sequences occur less frequently than other genomic changes because of the occurrence of unique sequences in small portion of genome. On the other hand, it seems that changes in repeated sequences take place through frequent events such as amplification, deletion, translocation and base substitution though the exact mechanism is not known (Flavell, 1982). Since changes in repeated nucleotide sequences can affect many loci in contrast to unique sequence, such changes may have pleiotropic effect on genome resulting in intraspecies and interspecies changes. The ubiquitous presence of repeated sequences in a large portion of eukaryotic genome

makes it important to know about their quantity, organization and nucleotide sequences in studying genomic relationships (Ganal et al., 1988). Additionally studying these changes in a group of closely and distantly related species may provide a clue regarding their relationships through evolution and also a possible role in the genome.

Rose and Doolittle (1983) suggested that turnover of repeated DNA sequences plays an important role in the evolution and speciation of eukaryotes. Saul and Potrykus (1984) and Scheweizer et al. (1988) have used repeated sequences in identifying hybrids from somatic protoplast fusion. Dvorak et al. (1988) and Talbert et al. (1991) have utilized them for the detection of diploid ancestors of polyploid species and Appel and Moran (1984) and Zhang and Dvorak (1990) in the detection of introgression of foreign chromatin into crops. Besides these, the use of repeated DNA as chromosomal marker (Appel, 1982; Rayburn and Gill, 1987; McIntyre et al., 1988) has become quite common. The use of repeated sequences in phylogenetic studies have been conducted not only in plants (Bendich and Anderson, 1983; Ranade, Gupta and Ranjekar, 1985; Sivaraman, Gupta and Ranjekar, 1985; Rayburn and Gill, 1988; Evans, James and Barnes, 1983) but also in animals (McLain, Rai and Fraser,1986).

The present study examined variation in the abundance of repeated DNA sequences in 25 diploid species (Table 1) from the tribe Triticeae covering 12 genomes (A, B, D, F, K, M, Q,

R, T, U, V and Z). It is hoped that these studies at molecular level will provide us with an accurate picture of changes that have taken place in repetitive DNA in the Triticeae during evolution. It is understood that the above mentioned knowledge will finally help us to exploit the rich genetic variation in the tribe for agronomic purposes.

Genome relationships were deduced between 25 diploid species in the Triticeae using phenetic analysis and single degree of freedom contrast analysis on molecular data. Usually polyploidy increases incongruence and thus complicate hierarchy (Funk, 1985). To avoid this only diploid species were considered in our analysis. The molecular data used for this study was the intensity of hybridization of random repeated DNA sequences to the genomic DNA of 25 diploid species. The repeated DNA sequence probes were derived from the A genome (*Triticum monococcum*) and V genome (*Dasypyrum species*). There were two reasons behind the selection of the A genome to derive repeated DNA sequence probes. First, it is important in plant breeding due to its presence in the bread wheat (*T. aestivum L.*). Second, we wish to enlighten the controversial relationships of the A genome (all *Triticum species*) to the B, D, M, Z and U genome (all *Aegilops species*) and thereby focusing on the controversy of amalgamation of *Triticum species* and *Aegilops species* into one genus *Triticum*. It is worth mentioning here that though some authors (eg.Kihara, 1954; Hammer, 1980; Gupta and Baum, 1986; Kellogg,

1989) have separated *Triticum* and *Aegilops* as two genera but others (eg. Stebbins, 1956; Bowden, 1959; Morris and Sears, 1967; Love, 1982) have placed them into a single genus. The selection of the V genome to derive repeated DNA sequence probes was based on the observation of its close association with the A genome in the phylogenetic tree obtained using morphological data (Kellogg, 1989). Since the closely related genomes are supposed to indicate similar relationships, it was possible to shed more light on the relationships of *Triticum monococcum* to *Dasypyrum species* using the V genome derived repeated DNA sequence probes depending on what results were found. DNA hybridization distances were used in an algorithm, which gave an unrooted tree, more commonly known as network. Based on the observation of the clustering of the genome in these networks, single degree of freedom contrasts were done on the molecular data to interprete the results in a more meaningful way. With the increase in congruency among results from experiments using different parameters, the phylogenetic history of that group tends to be more profound. For this reason the proposed relationships were compared with relationships inferred from other parameters such as morphological (Kellogg, 1989) and isozyme (McIntyre, 1988) data.

CHAPTER 2

EXPERIMENTAL PROCEDURE

Plant Material

Twenty-five diploid taxa covering 12 genomes were selected for this study. The nomenclature, genome designations and sources have been listed in Table 1. The seeds were sown in 6" pots and grown in the green house.

DNA Isolation

Genomic DNA was isolated from green leaf tissues of 25 taxa for three different replications by the procedure given by Dellaporta et al.(1983). After isolation DNA was resuspended in 100ul TE and treated with 2ul RNAase (1ug/ul) for 1 hour at 37^0C. Following incubation 10ul of 3M sodium acetate (pH 7.0) and 220ul of ethanol were added, mixed thoroughly and kept at -20^0C for 20 minutes to a maximum period of overnight. The clump of DNA which appeared after this incubation was centrifuged for 60 seconds. The supernatant were poured off and the DNA pellets were dried and redissolved in 100ul of TE. DNA concentration was determined spectrophotometrically using a Varion Techtron series 635 spectrophotometer at 260A in uv light.

DNA Dot Blotting

RNAase-treated genomic DNAs were dot-blotted using the Hybri-Dot Manifold (Bethesda Research Laboratories, MD). DNAs from different taxa for each replication were randomized using the SAMPLE program in MSUSTAT (Lund, 1988).

Five ug of DNA per sample in a total volume of 0.5ml of 0.4M NaOH and 10mM EDTA were heated for 10 minutes at 100^0C. These were transferred into ice. A nylon membrane was immersed in distilled water and the Hybri-Dot Manifold apparatus was assembled with the membrane under vacuum. The wells were rinsed with 0.5ml TE and vacuum was applied until the wells were empty. A volume of 0.5ml of DNA solution was added into appropriate well according to the randomized design without vacuum. Vacuum was applied until the wells were dry. Again the wells were rinsed with 0.5ml of 0.4M NaOH and vacuum was applied until the wells were dry. The apparatus was disassembled. The membrane was rinsed in 2 X SSC for 10 minutes and air dried between two paper towels at room temperature usually over night. Next morning, the membrane with the DNAs was vacuum dried at 80^0C for 30 minutes. The membrane was stored dry between two pieces of paper towels in a plastic bag at room temperature until hybridization.

Cloning

Triticum monococcum (PI190945) and *Dasypyrum species* (HA04) total genomic DNAs were digested with *Taq*I and ligated into *Acc*I - cut pUC12 and pUC18, respectively. Both pUC12 and pUC18 contain a lacZ gene in *Acc*I restriction site which shows b-galactosidase activity in the presence of X-gal and IPTG. These ligated plasmids were used to transform the bacterial strain NM522. The ligation and transformation were as described in Maniatis et al. (1982). The transformants were plated on a medium of Luria broth containing ampicillin, X-gal and IPTG and incubated at 37^0C overnight. Plates were then transferred to 4^0C. The transformants harboring pUC12 or pUC18 containing inserts were identified as white colonies indicating the presence of pUC12 or pUC18 with an interruption in lacZ region. The white colonies were used to make partial genomic libraries of *Triticum monococcum* (A genome) and *Dasypyrum species* (V genome).

Isolation Of Recombinant Plasmids

For *T.monococcum*, the miniprep method of plasmid DNA isolation was followed as described by Vieira and Messing (1982).

For *Dasypyrum, the polymerase chain reaction* (PCR) miniprep method of Gussow and Clackson (1989) was followed

with some modifications. The white colonies were grown overnight in 750ul of Luria broth with ampicillin. The next morning each overnight culture was mixed thoroughly with 300ul of sterile glycerol in an eppendorf tube. One ul of each well-mixed glycerol was added in 48ul of PCR mix (0.01M Tris-HCl pH 8.3, 0.05M KCl, 0.025mM nucleotides, 5mM $MgCl_2$, 0.05ug/ul of each universal and reverse primer, 1 unit of Taq polymerase and water to make a final volume of 50ul) in 0.5ul microfuge tubes and mixed well. One drop of mineral oil was added on top and a thermocycle program in a Coy Tempcycler was run as follows -- 94^{0}C for 4 minutes; followed by 33 cycles of 94^{0}C for 1 minute, 55^{0}C for 1.1 minute and 72^{0}C for 1.5 minutes. This was followed by 72^{0}C for 7 minutes and finally the tubes were stored at 4^{0}C. At the end of the program, the reaction mix was pipetted out carefully from under the mineral oil and collected in an eppendorf tube.

For *T. monococcum*, the recombinant plasmid were digested with *Eco*RI and *Hin*dIII following the plasmid miniprep to liberate the insert. On the contrary, for *Dasypyrum* following the PCR miniprep only the inserts were amplified and thus no restriction digestion was needed. All these inserts were separated on 1.2% agarose gel with ethidium bromide (EtBr) which were run in 1 X TBE buffer. Electrophoresis was generally carried out for 3 to 4 hours at 111 volts. DNAs were visualized using a UV transilluminator. DNAs were transferred to nylon membrane by Southern Blot method.

Detection And Generation Of Repeated Sequence Probes

Probes for the hybridization were labeled by nick - translation of total genomic DNA of *T. monococcum* and *Dasypyrum species* following the procedure of Rigby et al. (1977). Southern blot hybridizations were carried out as described by Chandler, Rivin and Walbot (1986) except that after hybridization followed by formamide wash the blots were washed in 500ml of blot wash buffer (0.1% SSC and 0.1% SDS) three times at 58^0C water bath with 30 to 45 minutes for each wash. Following the wash after hybridization, the blots were wrapped in Saran Wrap (Dow Brands) and exposed to Kodak X-OMAT film for 72 hour at -70^0C. After the film was developed , the repeated sequence probes for this study were identified on the basis of strong hybridization to labeled total genomic DNA from which the sequences were isolated. The selected repeated sequence inserts were purified from PCR preps through phenol/chloroform/ethanol precipitation to use as probes on the dot blots.

Dot Blot Hybridization

Probes for dot blot hybridization were labeled by a random primer reaction of inserts (Feinberg and Vogelstein, 1983). Each probe was hybridized to 3 replicate blots of the 25 diploid species.

Variation in abundance of repeated DNA sequence were measured by differences in the dot intensity which were quantified through densitometric tracing using a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments). Depending on the intensity of a particular dot, a peak of corresponding size was obtained.

Statistical Methods

Analysis of variance among accessions was accomplished using the AVFT program in MSUSTAT (Lund, 1988) considering the area under each peak as the dependent variable. The probes which revealed significant variation among accessions ($P < 0.05$) were considered for the purpose of making phylogenetic trees.

The average of area values of a particular accession over three replications were used to construct a distance matrix. Utilizing this distance matrix phylogenetic trees were made using PHYLIP program (Fitch and Margoliash, method version 3.3, 1990). Consensus trees were made using Consens Algorithm of PHYLIP program (Fitch and Margoliash, method version 3.3, 1990).

Individual phylogenetic trees were analyzed visually in search of a general tendency in association of the A and V genome with other genomes used for the study.

Based on the observation on association of the A and V genome with others, hybridization data were analyzed using single degree of freedom contrast (Table 2 and 3) with the help of the COMPARE program in MSUSTAT (Lund, 1988). The purpose of the study was to shed more light on the associaton of the genome observed in the phylogenetic tree.

CHAPTER 3

RESULTS

Cloning And Selection Of Repeated Sequence Probes

To determine the relationship between repetitive DNAs of a group of species in the tribe Triticeae, we made partial genomic libraries from *Triticum monococcum* (A genome) and *Dasypyrum species* (V genome), respectively. Genomic DNA of *T. monococcum* and *Dasypyrum species* were cloned into pUC12 and pUC18, respectively, and used to transform the *E. coli* strain NM522. A total of 102 and 179 recombinants were randomly selected from *T. monococcum* (A genome) and *Dasypyrum species* (V genome), respectively. For the A and V genome respectively, 30 inserts out of 102 and 63 inserts out of 179 appeared to contain repeated DNA sequence. A strong hybridization signal of the inserts with the radioactively labeled total DNA were the basis for considering the inserts as repeated DNA. However, we also cannot dismiss the possibility that the strongly hybridizing inserts are organeller. Ten inserts from both genomic libraries were selected at random for use as probes for dot blot hybridization.

Dot Blot Analysis

Ten selected probes from each library were used on the dot blots. Each probe was hybridized to 3 replicate blots of the diploid species listed in Table 1. Four repeated sequence probes (PA40, PA94, PV21 and PV110) hybridized to all diploid taxa tested (eg. Fig. 1). On the other hand, 6 repeated sequence probes (PA50, PV15, PV35, PV47, PV49 and PV105) were only abundant in a few taxa (eg. Fig. 2). Other repeated sequence probes showed considerable variation in their abundance among the species (eg. Fig. 3).

The abundance of these repeated sequence probes were quantified as intensity of the hybridization using a scanning densitometer.

Statistical Analysis

Analysis of variance on the dot-blot data revealed 8 repeated sequence probes from *T. monococcum* (PA11, PA12, PA14, PA37, PA41, PA48, PA50 and PA94) and 7 repeated sequence probes from *Dasypyrum* (PV38, PV47, PV49, PV67, PV73, PV105 and PV110) which showed significant variation ($P < 0.05$) among accessions. On the other hand, 2 probes from *T. monococcum* (PA13 and PA40) and 3 probes from *Dasypyrum* (PV15, PV21 and PV35) did not exhibit significant variation ($P > 0.05$) among accessions. The probes which did not show significant

variation among accessions were not considered for further study.

Data from blots which showed significant variation among accessions were used to construct a distance matrix. The distance matrix was constructed using the average of area values (as given under the peak by densitometric readings) over 3 replications for the same probe. Using these distance matrices, phylogenetic trees (Fig. 4 to 18) were produced using the PHYLIP program (Fitch and Margoliash, method version 3.3, 1990). Two consensus trees (Fig. 19 and 20) were constructed using the Consens Algorithm of the PHYLIP program (Fitch and Margoliash, method version 3.3, 1990). One tree was based on 8 phylogenetic trees obtained using 8 different A genomic repetitive DNA probes (Fig. 19) and the second was based on 7 phylogenetic trees obtained using 7 different V genomic repetitive DNA probes (Fig. 20). From these 2 consensus trees it was not possible to reach a profound conclusion regarding the clustering of species indicating genomic relationships because of inconsistency in the data. The inconsistency of various probes were not only among species but also within species (Fig. 19 and 20).

Individual trees were visually analyzed in search of general tendency in association of the A and V genome with the other genomes used in the experiment. From the analysis of 8 phylogenetic trees from the A genomic probes, 5 out of 8 times the A genome of *Triticum monococcum* was found in a loose group

containing the A (the *Triticum species*), B, D, M, Z and U (all of which are *Aegilops species*) genome, though there was considerable species variation in the group (Fig. 4, 6, 7, 9 and 11). In the same study, the V genome was in close association with the Q, F, T, K and R genome 5 out of 8 times. Three out of 8 times, no such clustering was found (Fig. 5, 8 and 10). The analysis of 7 phylogenetic trees from the V genome showed that 5 times the V genome was in very close association with the Q, F and K genome (Fig. 12, 14, 15, 16 and 17) whereas 3 times was also in close association with both the R and T genome (Fig. 12, 14 and 16) and once only with the R genome (Fig. 17). Besides these, the V genome was also found in occassional association with the D, Z, U and M genome. No definite association was found with the A genome and the other genomes using the V probe except in one case (Fig. 12). The remaining 2 out of 7 times the clustering of the genomes in the tree appeared to be at random (Fig. 13 and 18). There was always intraspecies variation in all the above mentioned clusters.

No definite conclusion could be reached from the consensus tree, though visual analysis revealed some consistent clusterings of the genome. To test further these associations, our hybridization data were analyzed using single degree of freedom contrast with the help of the COMPARE program in MSUSTAT (Lund, 1988). This study was based on the observation of clusters in visual analysis of individual

trees. The purpose was to shed more light on the relationship among the A genome of *Triticum monococcum*, genomes of *Aegilops species* (the B, D, M, Z and U genome) and the V genome of *Dasypyrum species*. It was possible to come to more meaningful conclusions regarding the tendency of association of the A and V genome observed in the tree.

As shown in the Table 2 and 3, comparison of the 2 accessions of the A genome (Aa and Ab) while using the A probe hybridization data revealed similarity 5 out of 8 times between these 2 accessions. On the contrary, while using the V probe hybridization data, the 2 accessions of the A genome always appeared to be similar. This result might have occured for two reasons --- either these 2 accessions of the A genome are similar or the V genome is far from the A genome that it cannot distinguish the minor differences between the 2 accessions of the A genome. The reason behind the latter possibility is that because of the distant relationships or low homology and consequently the same intensity of hybridization is expected. The latter reason was consistent with results obtained while comparing the A and V genome. Comparison of the A and V genome revealed both accessions of the A genome as different from the V genome 6 out of 7 times using the V probe hybridization data and 5 out of 8 times using the A probe hybridization data.

Since the A genome was found in a loose cluster of the A, B, D, M, Z and U genomes in the majority of the tree and also

all of them are considered as *Triticum* by some authors instead of separating them into *Triticum species* (the A genome) and *Aegilops species* (the B, D, M, Z and U genome) as it has been done by another group of authors, the A genome was compared to the group of the B, D, M, Z and U genome. In this comparison while using the V probe hybridization data, there was always similarities between both accessions of the A genome and the B, D, M, Z and U genome. The same comparison using the A probe hybridization data, showed that 3 out of 8 times both accessions of the A genome were similar to the B, D, M, Z and U genome, while 5 out of 8 times one of two accessions of A genome appeared similar to the B, D, M, Z and U genome while the other one did not. The results obtained using the V probe hybridization data are considered to be unreliable for the purpose of identifying relationships between the A and the B, D, M, Z and U genome. The reason behind this was that the V probe tended not to hybridize with them. All these nonhybridized DNA dots which did not give a peak in densitometric reading were considered as same values for AOV, COMPARE and PHYLIP program. Consequently, all the nonhybridizing DNAs appear similar statistically but this may not reflect the relationship between DNAs of those different genomes. Rather, it shows the relationship of those genomes to the genome which has been used as probe. It is worth mentioning that the chance of occurrence for this type of error is less when a probe hybridize to DNAs from all genomes.

Among our V probes, only 1 probe (PV110) hybridized to all taxa whereas 3 probes (PV47, PV49 and PV105) hybridized to only a few taxa. So this data was only used to predict that the V genome is not close to the A, B, D, M, Z and U genome (i.e. the *Triticum* and *Aegilops species*). It has already been mentioned that the A (the *Triticum species*) and V genome differed from each other the majority of the time. To find out the relationship between the V and the B, D, M, Z and U genome (i.e. the *Aegilops species*), the V genome was compared to the above mentioned. The V genome was found different from the B, D, M, Z and U genome 6 out of 7 times using the V probe hybridization data and 4 out of 8 times using the A probe hybridization data. Again the V probe hybridization data was a better choice than those from the A genome to identify the relationship between the V and the B, D, M, Z and U genome. It is worth mentioning here that V genome's relationships to the B, D, M, Z and U genome (i.e.the *Aegilops species*) was consistent to its relationships to the A genome (the *Triticum species*) except at one case (PA48). This observation again indicates that the A genome (the *Triticum species*) is close to the B, D, M, Z and U genome (i.e. the *Aegilops species*) whereas the V genome is not. This statistical observation was in support of observation in the visual analysis. Thus, more emphasis was on the A probe hybridization data to identify the relationship between the A and B, D, M, Z and U genome. Moreover, among the A probes only 1 out of 8 (PA50) hybridized

to a few taxa. These data majority of the time showed no significant differences between the A and B, D, M, Z and U genome though there was variation among accessions within the same genome. Thus the A genome of *Triticum monococcum* was considered to be close to the B, D, M, Z and U genome i.e. the *Aegilops species*.

Since the V genome appeared in a loose cluster of the Q, F, T, K and sometimes R genome in the majority of the trees, the V genome was compared with the above mentioned using single degree of freedom contrast (Table 2 and 3). This comparison revealed the V genome as similar to the Q, F, T, K and R genome 7 out of 8 times using the A probe hybridization data and 1 out of 7 times using the V probe hybridization data. This result may be explained in the following way. If the A genome is not close to the V and the Q, F, T, K and R genome, it cannot indicate relationships between them but just clusters them together. The dissimilarities between the A and the Q, F, T, K and R genome came from the analysis of comparison between them using the A probe hybridization data. Five out of 8 times both accessions of the A genome and only once 1 accession of the A genome appeared different from the Q, F, T, K and R genome. The V probe hybridization data was not considered for this purpose because of the observed difference between the A and V genome. The A genome showed similarities to the Q, F, T, K and R genome only when it showed similarities with the V genome. This data may be

interpreted as that the V genome is comparatively closer to the Q, F, T, K and R genome than the A genome to the same. To identify the relationship between the V and the Q, F, T, K and R only the V probe hybridization data was considered. Since in this data 6 out of 7 times the V genome showed significant variation to the Q, F, T, K and R genome, it seems that the V genome is not close to either the Q, F, T, K and R genomes or to the A, B, D, M, Z and U genomes. Thus, this statistical observation regarding the relationship of the V genome was not in support of the observation of cluster formation by the V genome in the phylogenetic trees.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

In this study, we used repetitive DNA sequences from the genomes of *Triticum monococcum* (A genome) and *Dasypyrum species* (V genome) as probes on dot-blotted genomic DNA from 25 diploid taxa in the Triticeae to identify relationships. The phenetic analysis data suggested that these sequences vary in abundance not only among closely related species but also among accessions within species. Though phenetic analysis of data failed to give any consistent cluster of either species or genome, visual analysis of individual phylogenetic trees provided some loose clustering of genomes although intraspecies variations were observed. Single degree of freedom contrast analysis of hybridization data not only shed more light on the genomic relationships but also pointed out a potential problem in the utilization of this kind of data for the purpose of phylogenetic tree making. However, genomic relationships based on analysis of repetitive DNA sequences showed considerable variation from those based on morphological data (Kellogg, 1989), but only slightly from those based on isozyme data (McIntyre, 1988).

Inconsistencies in the apparent phylogenetic relationships of species in the Triticeae using different parameters are well known. McIntyre (1988) mentioned

nonidentical results regarding genomic relationships using morphological and chromosome pairing, DNA - DNA hybridization, DNA analysis of NOR - loci, 5S DNA sequence information and isozyme electrophoresis. However, they cited constant clustering of the S, E, J, A, B and D genome with the H and I genome as outlier and the R genome as an intermediate of this cluster in all of the above mentioned studies.

The study reported here was undertaken due to some preliminary observations (data not shown). In a previous study 6 repeated sequence probes (namely pCom11, 18, 24, 31, 36 and 39) from *Triticum comosa* (M genome) and 7 repeated sequence probes (namely pTach24, 46, 86, 92, 110, 129 and 130) from *Triticum tauschi* (D genome) were hybridized with dot-blotted genomic DNA from 18 diploid taxa. These 18 diploid taxa covered 18 different genomes. Though majority of them showed similarities in result but only one (pTach24) was analyzed in details. The result showed a closely knitted group of the A, B, D, M and Z genome. This group was closely associated with another group containing the U, J, K, T genome and comparatively distantly associated with another group containing the V, F, Q, H and P genome (Fig. 21). This data was more or less consistent with the constant cluster data obtained from other studies as previously mentioned. This data also showed similarities with the data obtained from our present study.

Though the present study using phenetic analysis failed to come to a conclusion indicating genomic relationships, visual analysis of individual trees could indicate a general tendency in association of the A and V genome with others. The analysis of the hybridization data using single degree of freedom contrast analysis not only shed more light on the relationship of the A and V genome with others but also indicated a potential error for calculation which can create a false clustering of the genome in the phylogenetic tree. Consideration of the nonhybridized DNAs (which did not give a peak in the densitometric readings) as the same value for the purpose of statistical analysis may generate this type of error. The chance of occurrence for this type of error is more when a genome which has been used to derive a probe is distantly related from other genomes (among which the relationship has to be identified). Thus, it was possible to suggest from this kind of statistical analysis that a closely related genome is a better choice to use for derivation of probes to indicate relationships among other genomes. However, the data obtained from the analysis using single degree of freedom contrast differed from the proposed clustering obtained from visual analysis of individual trees in indicating relationships of the V genome to others. This additional achievement was that the V genome was not only distinct from the A, B, D, M, Z and U genome (i.e. the *Triticum* and *Aegilops species*) but also from the Q, F, T, K

and R genome. However, it appeared in loose cluster with the Q, F, T, K and R genome in the tree. This difference in result was due to the error in utilizing densitometric reading data for the purpose of phylogenetic tree making. The nonhybridizing DNA on the dot blots which did not give a peak in densitometric reading was considered as the same value to make distance matrices. Consequently, they formed a cluster in the phylogenetic tree, though this did not reflect relationships among the genome of the cluster. From this type of cluster the only interpretation might be drawn that the group of genomes forming the cluster are distantly related to the genome used to derive the probe. Though our data was consistent with the constant cluster data mentioned by McIntyre (1988) but still there were some incongruence with the morphological and isozyme data. There were three major differences from those using morphological data (Kellogg, 1989) --- (i) the A genome was in close association with the B, D, M, Z and U genome compared to its distant relationships with the same as mentioned by Kellogg (1989); (ii) the V genome appeared distantly related to the A genome as opposed to close association of the A and V genome in the tree based on morphological data (Kellogg, 1989); (iii) the V genome was not only distantly related to the A genome but also it was distant from the F, R and T genome as opposed to their close association mentioned by Kellogg (1989). The major difference between our data and isozyme data (McIntyre, 1988) was that

the R genome was not so close to the A genome as it appeared in the phenogram based on the isozyme data.

The inconsistencies in genomic relationships inferred with different parameters may reflect the complicated phylogenetic history of the tribe. The incongruence between our results and isozyme data may be explained as McIntyre (1988) suggested it as nonconstant rate of evolution of DNA sequence and protein in a particular lineage. Moreover, rate of change through evolution may be different not only in the different portion of the genome but also within different types of the DNA, namely nuclear, mitochondrial or chloroplastidial (Palmer, 1985). Furthermore, different parameters used for phylogenetic studies have not evolved in concert, rather they have diverged through evolution being under different selection pressure. Since morphological characters bear the influence of the environment too, the discrepancies between morphological data and our molecular data may be explained. This type of discrepancy in proposed phylogenies derived by different parameters have not only reported for plants (Sytsma and Schaal, 1985a, b; Sytsma and Gottleib, 1986) but also for animals (McLain, Rai and Fraser, 1983).

The inconsistency within our data from different probes can be explained as follows. While selecting repeated sequence probes, we selected them at random. We did not select probes from a particular family of repeated DNA sequence. Different

repeated DNA sequence families have evolved through rapid amplification, deletion most probably by unequal crossing over, disproportionate replication or even by sequence rearrangement or damage (Posakony et al., 1981; Marseca et al., 1984). Thus, the chance of obtaining identical results by the members of different repeated sequence family are slight. Moreover, even within a particular family of repeated DNA sequence, different sequence variants can be found depending on the proportion of constant sequences in that family (McLain, Rai and Fraser, 1983). Dover et al. (1982) found that these sequence variation in repeated DNA sequence of a particular family is less within species as compared to among species. Thus, selection of repeated sequences at random might have been a reason for failure to cluster all the closely related species within a genome.

It is worth mentioning that similar hybridization intensity on dot blots using repeated DNA may be due to similar abundance in copy number or similarity in sequence. At this point, we do not know whether the different hybridization intensity are due to difference in abundance or sequence.

The lack of similarity among the closely related species indicate that the species have diverged through evolution. Each of these evolutionary lines have been under different selection pressure to serve different purposes which has finally reflected in their sequence divergence. As a consequence, not only closely related species with the same

basic genome but also different accession of the same species contain different repeated DNA sequence. This assumption is congruent with the finding of Talbert, Kimber and Magyar, 1991 (submitted for publication) that an M genomic probe hybridized with different intensity not only to DNA from different species with the M genome but also to different accession of the same species.

Moreover, it has been found that average rate of divergence of nuclear DNA and mitochondrial DNA may or may not be similar within a particular lineage (Palmer, 1985). Since our probes were not determined as only of nuclear origin, it might be possible that some of the probes actually consist of organellar DNA whereas others are not. This might have given nonidentical results.

One more important point is that the inconsistency while making consensus tree might be due to large number of taxa (25) used. Swofford and Olsen (1990) have mentioned that more than 8 to 12 taxa may tend to give inaccurate result while finding the best tree.

As a conclusion, though there were apparent inconsistencies in the phylogenetic tree, the result from single degree of freedom contrast analysis were consistent. Not only the latter analysis gave consistent results but it also indicated a potential error while utilizing the densitometric reading data for the purpose of phylogenetic tree making. This type of error contributed to the

inconsistency in the phenetic analysis results besides the role of other potential problems. With the use of single degree of freedom contrast analysis it was also possible to find out how to avoid the potential error which may give a misleading cluster of a group of genomes. It was suggested that the probe derived from a genome which is closely related to the group should only be used. On the contrary, the use of a distantly related genomic probe to identify relationships among a group of genomes has more chances either not to hybridize to them at all or to hybridize with the same intensity. As a consequence, the group of genomes cluster together indicating their distant relationships from the genome of the probe used and not indicating relationships among them.

The genomic relationships deduced in this study were similar regarding the occurrence of clusters of some genomes to those obtained using other parameters as mentioned by McIntyre (1988). The major discrepancy with our study was to that obtained using morphological data by Kellogg, 1989. Besides these, this study also supported the amalgamation of *Triticum species* and *Aegilops species* into a single genus *Triticum*.

CHAPTER 5

SUMMARY

The findings in the present study were supported by a majority of the previous investigations but showed incongruence with those obtained using morphological data. This study may also be used to shed light on the controversial relationships of *Triticum species* and *Aegilops species*. This study not only indicated genome relationships in the 25 diploid taxa in the Triticeae but also pointed towards a potential problem in using molecular data which may give misleading results. It was also possible to suggest how this type of error might be avoided. Thus, this study may be used against the arguments of molecular data being weak. The problem with molecular data in this case was more procedural than theoretical. This study not only supported the use of molecular data in systematics but also the use of repeated DNA sequence as probes to identify genomic relationships in the Triticeae. One new and interesting finding on this point was that a closely related genome is a better choice to derive a probe which may be used to identify relationships among a group of genomes.

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APPENDIX