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# Reconstruction of the phylogeny of the genus *Triticum* from variation in repeated nucleotide sequences

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**Summary.** The potential of variation in repeated nucleotide sequences as a tool for phylogenetic studies was examined by investigating the phylogeny of 13 diploid species of the genus *Triticum* L. sensu Bowden. Low intraspecific variation in repeated nucleotide sequence families in *Triticum* indicated that restriction fragment profiles of repeated nucleotide sequences in Southern blots are reliable and uniform characteristics of each species. Cloned repeated nucleotide sequences were hybridized with Southern blots of DNAs of the *Triticum* species and the outgroup, *Lophopyrum elongatum* (Host) Á. Löve. The presence or absence of bands in the Southern blot autoradiograms was considered to be a character for phylogenetic analysis. A most parsimonious tree was resolved with the PAUP version 3.0L computer package. The tree was consistent with cytotaxonomic and evolutionary data available on the species.

**Key words:** Genome size – Geographic distribution – Evolution

## Introduction

Although variation in organellar genomes is an outstanding phylogenetic tool, it is desirable to employ a phylogenetic technique based on nuclear genomes when hybridization, allopolyploidy, or intraspecific variation in organellar genomes is indicated (Palmer et al. 1983; Holwerda et al. 1986; Neale et al. 1988; Tsunewaki 1988; Baldwin et al. 1990; Doyle et al. 1990). We report here such a technique, which is based on variation in the nuclear repeated nucleotide sequences. Its utility is demonstrated by inferring the phylogeny of 13 diploid species of the genus *Triticum* L. sensu Bowden. Because these species are closely related to the cultivated wheats, they have been extensively investigated and a wealth of cytogenetic and taxonomic data is available for each taxon. We used this information to scrutinize the phylogenetic inferences drawn from the new technique.

Sequences within repeated nucleotide sequence families tend to evolve in concert by repeated cycles of homogenization (Dover 1982; Strachan et al. 1985). Concerted evolution is a conservative process largely characterized by a slow and gradual turnover of sequences in the families (Birky and Skavaril 1976; Strachan et al. 1985; Dvořák et al. 1987). When a repeated nucleotide sequence cloned from one species is hybridized with a Southern blot of genomic DNA from another species zero to many bands may be observed in an autoradiogram, depending on homology of the cloned sequence with those in the second species and on variation within the repeated sequence family. The signal from unique or rare variants is either undetected or obscured by the signal from the predominant variants in the Southern blots. As a result, bands observed in Southern blots reflect only frequently occurring sequence variants in the genome.

It would be very difficult to determine the nature of a restriction pattern change in a restriction profile of a repeated nucleotide sequence from examination of Southern blot autoradiograms. Scoring must be, therefore, limited to the presence or absence of bands in Southern blots. The ease with which repeated nucleotide sequence profiles can be generated makes it possible to score larger numbers of restriction fragments than is potentially feasible for organellar DNA.

A prerequisite for using repeated nucleotide sequences in phylogenetic studies is that they must be rela-

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**Table 1.** Plant materials used in the study, their geographic origin, and sources

| Species | Number of accessions | Origin | Source |
|---|---|---|---|
| *T. speltoides* (Tausch.) Gren. | 40 | Turkey | G. Waines, University of California, Riverside |
| | 9 | Israel | M. Feldman, Weizman Institute of Science, Rehovot, Israel |
| | 5 | Iraq | G. Waines |
| | 4 | Syria | S. K. Jana, University of Saskatchewan, Saskatoon, Canada |
| | 2 | Unknown | J. Dvořák |
| *T. sharonense* (syn. *Aegilops sharonensis* Eig) | 9 | Israel | M. Feldman<br>G. Waines |
| *T. longissimum* (Sweinf. et. Muschl.) | 14 | Israel | M. Feldman, G. Waines |
| | 1 | Jordan | G. Waines |
| *T. bicorne* Forssk. | 3 | Egypt | G. Waines |
| | 5 | Israel | G. Waines, M. Feldman |
| *T. searsii* (syn. *Aegilops searsii* Feldman et. Kislev) | 17 | Israel | M. Feldman, G. Waines |
| | 2 | Syria | M. Feldman |
| *T. comosum* (Sibth. et Smith) Richter | 4 | Greece | G. Waines |
| | 1 | Turkey | G. Waines |
| *T. uniaristatum* (Vis.) Richter | 1 | Unknown | E. R. Sears, University of Missouri, Columbia |
| *T. caudatum* (L.) Godron et Gren. | 1 | Unknown | E. R. Sears |
| *T. umbellulatum* (Zhuk.) Bowden | 1 | Iraq | G. Waines |
| | 3 | Turkey | G. Waines |
| | 1 | Unknown | G. Waines |
| *T. tauschii* (Coss.) Schmalh. | 1 | Azerbaijan | V. Jaaska, Estonian University, Tartu |
| | 3 | Iran | B. S. Gill, Kansas State University |
| | 2 | Afghanistan | B. S. Gill |
| | 1 | Iran | Kyoto University |
| | 1 | China | Y.-S. Dong, Chinese Academy of Agricultural Science Beijing, China |
| *T. monococcum* L. ssp. *aegilopoides* | 17 | Turkey | S. K. Jana |
| | 1 | Lebanon | G. Waines |
| | 1 | Iraq | G. Waines |
| | 1 | Iran | G. Waines |
| | 2 | Syria | G. Waines |
| *T. urartu* Thum. | 33 | Turkey | S. K. Jana |
| | 1 | Lebanon | G. Waines |
| | 4 | Armenia | G. Waines |
| *T. muticum* (Bois.) Hackel | 1 | Turkey | R.J. Metzger, University of Oregon, Corvallis |
| *L. elongatum* (Host) Löve | 1 | Tunisia | G. L. Stebbins, University of California, Davis |

tively homogeneous within a species. To address this question, the study presented here examined the extent of variation in repeated nucleotide sequence families in a number of diploid *Triticum* species, using identical criteria for restriction fragment band scoring as those employed in the phylogenetic analysis.

## Materials and methods

*Plants*

The names of species employed in the study, numbers of accessions, their geographic origin, and sources are listed in Table 1. A systematic treatment by Bowden (1959) is followed. He combined the genera *Triticum* and *Aegilops* sensu L. into a single genus *Triticum*. In those cases where species names with priority over those used by Bowden were available (Löve 1984) the names with priority were used.

*Probes*

Forty-six clones of repeated nucleotide sequences were used in this study (Table 2). The clones were isolated according to Dvořák et al. (1988) and Zhang and Dvořák (1990a). All clones, except for two (Table 2), were *Mbo*I DNA fragments inserted in the pUC18 vector at the *Bam*HI site. Seven clones were from *T. urartu* ($A^u$ genome), 18 were from *T. kotschyi* nom. nud., $2n = 4x = 28$, genomes $S^lS^lUU$, (syn. *Aegilops kotschyi* Eig) (Zhang et al. 1991), 14 were from *T. longissimum* ($S^l$ genome), four were from *T. speltoides* (S genome), one clone was from *T. monococcum* ssp. *aegilopoides* ($A^m$ genome), and one clone (pEleAcc 2) was isolated from the lambda library of genomic DNA of *Lophopyrum elongatum* (McIntyre et al. 1988). Because of the large numbers of cloned repeated sequences and investigated species the copy numbers of the repeated sequences per genome were not determined. From a comparison of the overall hybridization intensities of the probes with the genomic DNAs relative to those for which the copy numbers were determined (Dvořák et al. 1988), it appeared that the copy numbers of the sequences employed here ranged approximately from $10^3$ to $10^5$ copies per genome.

*DNA isolation and hybridization*

Nuclear DNAs were isolated from single plants according to Dvořák et al. (1988) and digested with restriction endonucleases. The DNAs were fractionated in 1.6% agarose gels and blotted

**Table 2.** The origin of probes, the restriction enzymes used for digestion of genomic DNAs, and the numbers of bands scored at each enzyme × probe combination in the phylogenetic studies

| Clone[a] | Species | Length (bp) | Restriction enzymes[c] (number of bands scored) | Source |
|---|---|---|---|---|
| pTbUCD1 | *T. mon. aegilop.* | 290 | H(12) S(12) Dd(18) T(19) | Dvořák et al. (1988) |
| pTuUCD16 | *T. urartu* | 150 | Dr(15) E(1) | Authors |
| pTuUCD17 | *T. urartu* | 350 | T(2) Dr(2) | Authors |
| pTuUCD18 | *T. urartu* | 130 | T(4) | Authors |
| pTuUCD19 | *T. urartu* | 150 | T(14) H(22) Mb(3) Dr(2) E(11) | Authors |
| pTuUCD20 | *T. urartu* | 380 | Dr(3) T(2) | Authors |
| pTuUCD21 | *T. urartu* | 450 | T(5) H(5) E(5) Mb(11) | Authors |
| pTuUCD22 | *T. urartu* | 300 | Mb(10) E(7) H(2) T(3) | Authors |
| pTlUCD1 | *T. longissimum* | 250 | Mb(20) S(2) T(1) A(12) | Zhang et al. (1992) |
| PTlUCD3 | *T. longissimum* | 270 | Mb(4) S(2) Ms(9) T(4) A(14) D(4) H(5) | Zhang et al. (1992) |
| pTlUCD4 | *T. longissimum* | 330 | Mb(11) Ms(2) | Zhang et al. (1992) |
| pTlUCD5[b] | *T. longissimum* | 540 | H(6) | Zhang et al. (1992) |
| pTlUCD6[b] | *T. longissimum* | 280 | A(20) | Zhang et al. (1992) |
| pTlUCD7 | *T. longissimum* | 290 | A(14) B(12) H(6) Mb(8) S(12) Ms(15) T(6) | Zhang et al. (1992) |
| pTlUCD8[b] | *T. longissimum* | 870 | A(14) | Zhang et al. (1992) |
| pTlUCD9[b] | *T. longissimum* | 330 | H(8) | Zhang et al. (1992) |
| pTlUCD10 | *T. longissimum* | 220 | Dd(9) H(18) S(10) Ms(8) | Zhang et al. (1992) |
| pTlUCD11 | *T. longissimum* | 540 | A(17) B(18) H(13) Mb(2) Ms(27) S(22) | Zhang et al. (1992) |
| pTlUCD12 | *T. longissimum* | 430 | A(5) Dd(11) H(1) B(2) | Zhang et al. (1992) |
| pTlUCD14 | *T. longissimum* | 360 | A(2) Dd(10) B(9) Mb(2) S(22) | Authors |
| pTlUCD15[b] | *T. longissimum* | 210 | Ms(2) | Authors |
| pTlUCD16[b] | *T. longissimum* | 310 | Ms(4) | Authors |
| pTlUCD17[b] | *T. longissimum* | 230 | A(18) | Authors |
| pTsUCD2 | *T. speltoides* | 150 | T(4) | Dvořák and Zhang (1990) |
| pTsUCD4 | *T. speltoides* | 440 | H(1) Dd(8) Mb(5) | Dvořák and Zhang (1990) |
| pTsUCD5 | *T. speltoides* | 210 | T(20) S(24) | Dvořák and Zhang (1990) |
| pTsUCD6 | *T. speltoides* | 200 | Mb(10) Dd(7) S(5) H(1) | Authors |
| pTkUCD1 | *T. kotschyi* | 480 | A(5) Dd(22) H(9) Ms(3) T(7) | Zhang et al. (1991) |
| pTkUCD2 | *T. kotschyi* | 290 | Mb(6) Ms(6) T(7) | Zhang et al. (1991) |
| pTkUCD3 | *T. kotschyi* | 480 | Mb(9) S(13) Ms(15) T(11) A(7) B(22) | Zhang et al. (1991) |
| pTkUCD4 | *T. kotschyi* | 510 | A(7) Dd(13) b(1) H(8) T(1) | Zhang et al. (1991) |
| pTkUCD5[b] | *T. kotschyi* | 210 | T(5) | Zhang et al. (1991) |
| pTkUCD6[b] | *T. kotschyi* | 850 | Ms(1) | Zhang et al. (1991) |
| pTkUCD7 | *T. kotschyi* | 180 | A(12) | Zhang et al. (1991) |
| pTkUCD8 | *T. kotschyi* | 150 | Mb(3) S(7) Ms(4) T(5) Dd (16) B(1) H(4) | Zhang et al. (1991) |
| pTkUCD9[b] | *T. kotschyi* | 330 | S(1) | Zhang et al. (1991) |
| pTkUCD10[b] | *T. kotschyi* | 310 | A(2) | Zhang et al. (1991) |
| pTkUCD12 | *T. kotschyi* | 230 | S(3) A(7) Dd(7) H(5) Mb(4) | Authors |
| pTkUCD13 | *T. kotschyi* | 520 | A(8) H(4) Dd(7) | Authors |
| pTkUCD14 | *T. kotschyi* | 500 | A(5) Dd(13) H(6) Ms(2) | Authors |
| pTkUCD15[b] | *T. kotschyi* | 490 | Dd(25) | Authors |
| pTkUCD16[b] | *T. kotschyi* | 220 | Dd(3) | Authors |
| pTkUCD17 | *T. kotschyi* | 480 | Mb(5) S(11) | Authors |
| pTkUCD19[b] | *T. kotschyi* | 210 | A(10) | Authors |
| pTkUCD20[b] | *T. kotschyi* | 850 | Ms(3) | Authors |
| pElAcc2 | *L. longatum* | 600 | S(17) | McIntyre et al. (1988) |

[a] All clones were *Mbo*I fragments inserted in the *Bam*HI site of pUC18 except for pTbUCD1 and pElAccI, which were *Mbo*I and *Eco*RI-*Acc*I fragments inserted in pBS M13 and pUC18, respectively

[b] Additional 15 probes used for the reconstruction of the tree in Fig. 5b

[c] A, B, Dd, Dr, E, H, Mb, Ms, S, and T indicate *Alu*I, *Bam*HI, *Dde*I, *Dra*I, *Eco*RV, *Hae*III, *Mbo*I, *Msp*I, *Sst*I, and *Taq*I, respectively

onto a Zeta-Probe membrane according to the specifications by the manufacturer (BioRad). The inserted fragments of repeated sequences in the plasmids were liberated by restriction endonuclease digestion, fractionated in an agarose gel, and electroeluted from the gel. The inserts were labelled with $^{32}P$ using a random hexamer primer labelling kit (Amersham). The membrane was prehybridized and hybridized with the probe according to Zhang and Dvořák (1990a) and autoradiographed.

*Intraspecific variation*

Accessions were selected to represent the geographic distribution of each species. DNAs were isolated from a single plant per accession and digested with two restriction endonucleases randomly selected from *Hae*III, *Mbo*I, *Taq*I, *Dde*I, *Msp*I, *Alu*I, and *Sst*I. The immobilized DNAs on the blots were hybridized with repeated nucleotide sequence probes (see Table 3). Two were

**Table 3.** Intraspecific variation (V) in repeated nucleotide sequence families in the k accessions of ten diploid species of *Triticum* investigated in the specified number of restriction enzyme × probe (E × P) combinations

| Species | Accessions (k) | Sequence families | E × P combination | Bands (*n*) | Variable bands | V |
|---|---|---|---|---|---|---|
| *T. speltoides* | 60 | 8 | 16 | 56 | 10 | 0.003 |
| *T. sharonense* | 9 | 8 | 16 | 94 | 1 | 0.001 |
| *T. longissimum* | 15 | 8 | 16 | 64 | 1 | 0.001 |
| *T. bicorne* | 8 | 8 | 16 | 104 | 0 | 0.000 |
| *T. searsii* | 19 | 8 | 16 | 54 | 2 | 0.002 |
| *T. comosum* | 5 | 5 | 10 | 103 | 0 | 0.000 |
| *T. umbellulatum* | 5 | 5 | 10 | 103 | 3 | 0.006 |
| *T. tauschii* | 5 | 5 | 10 | 126 | 14 | 0.022 |
| *T. mon.* ssp. aegilopoides | 22 | 2 | 4 | 25 | 0 | 0.000 |
| *T. urartu* | 38 | 2 | 4 | 33 | 1 | 0.001 |

from *T. urartu*, 3 from *T. speltoides*, 3 from *T. kotschyi*, and 2 from *T. longissimum*. The presence or absence of each restriction fragment band in the autoradiogram was visually determined.

Since the DNA fragments present in a single band in a Southern blot may originate from a single chromosomal site or from a number of sites on different chromosomes (Dvořák et al. 1988; Dvořák and Zhang 1990; Zhang and Dvořák, 1990 a, b), the standard methods of expressing population diversity could not be used. To express variation in the repeated nucleotide sequence profiles within a species, repeated sequence variation V was calculated according to equation:

$$V = \frac{1}{n} \sum_{i=1}^{n} v_i,$$

where n is the number of bands investigated, and $v_i$ is the variation of the ith band in the population of DNAs. Band variation $v_i$ was calculated as $v_i = d_i/k$, where d is the number of DNAs that differ in the ith band from the majority of DNAs and k is the number of DNAs investigated. The repeated nucleotide sequence variation V varies from zero, if all investigated DNAs have identical profiles, to 0.5, if $v_i$ is 0.5 for all investigated bands.

### *Phylogenetic analysis*

On accession was selected randomly from each *Triticum* species and *Lophopyrum elongatum*. *Lophopyrum elongatum* was used as an outgroup because it is closely related to *Triticum* (Dvořák et al. 1986; H.-B. Zhang and J. Dvořák, unpublished). DNA of a single plant per accession was digested with the restriction endonucleases listed in Table 2. The DNAs digested with a specific restriction endonuclease were electrophoresed together in an agarose gel, blotted, and hybridized with 31 probes listed in Table 2. From 1 to 7 enzyme × probe combinations were used per probe (Table 2). Nine hundred and fifty-five bands were scored in the species, except for *T. monococcum* ssp. *aegilopoides* and *L. elongatum*, in which only 263 and 831 bands were scored, respectively, because of the limited number of lanes available in the gels. The numbers of investigated bands per enzyme × probe combination ranged from 1 to 27 (Table 2); the mean was 8.3. Presence versus absence of bands was scored as "1" or "0" to create a data matrix (for an illustration see Figs. 2 and 3). The presence or absence scoring of bands was relative and relevant only to the specific hybridization and autoradiography conditions employed. The scoring did not imply that a specific restriction fragment was absent from a genome, but merely that it was not observed under the specific experimental conditions that were the same for all investigated DNAs. Bands which were too faint or obscured by other bands or a smear were neglected. Each band was considered an independent character for the phylogenetic analysis. The most parsimonious phylogenetic tree of the shortest topology was constructed using the Wagner parsimony method. The construction of the phylogenetic tree was accomplished with the MacIntosh computer package PAUP version 3.0 L (Swofford 1990). The confidence of each branch in the tree was estimated with Felsenstein's bootstrap method with branch-and-bound search and 100 replications utilizing the PAUP package.

To assess the effects of multiple enzyme digests and the number of characters on the topology of the phylogenetic tree and the bootstrap confidence of the branches, data obtained by the analysis of the hybridization of 15 additional repeated nucleotide sequence clones were added to those obtained by the hybridization of the initial 31 probes (Table 2).

## Results

### *Intraspecific variation*

To determine intraspecific variation in repeated nucleotide sequences, Southern blots of restriction digests of genomic DNAs from 5 to 60 accessions per species were hybridized with randomly selected clones of repeated nucleotide sequences (Table 1, Fig. 1). The estimates of the intraspecific variation V ranged from zero in *T. monococcum*, *T. bicorne*, and *T. comosum* to 0.022 in *T. tauschii* (Table 2). The allogamous and widely distributed *T. speltoides* showed variation equally as low (e.g. Fig. 1) as the remaining 9 autogamous species. Higher intraspecific variation in *T. tauschii* was largely due to a single accession from a disjunct population in China. The low levels of intraspecific variation in these 10 species indicated that repeated nucleotide sequences can be used in phylogenetic analysis of the genus *Triticum*.

### *Methodology of phylogenetic analysis*

Some bands were observed in the DNAs of all 13 *Triticum* species and *L. elongatum* whereas others were

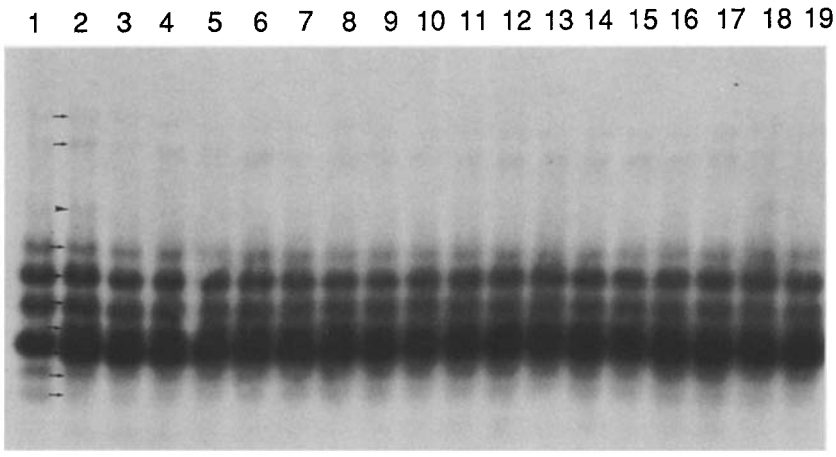



**Fig. 1.** Southern blot hybridization of the pTsUCD2 insert isolated from *T. speltoides* with DNAs of 18 accessions of *T. speltoides*. The sample in *lane 1* is *T. aestivum*. The accessions in *lanes 2–7* and *13–15* are of the awned var 'liqustica', and those in the *remaining lanes* are of the awnless var 'aucheri'. Accessions in lanes 2–8, 11–14, 17, 18, and 19 were collected in Turkey, 9, 10, and 16 in Israel, 5 in Syria, and 15 in Iraq. There are ten distinct bands in the profiles of the restriction fragments, nine (arrows) of which are invariable among the accessions, whereas one (*arrowhead*) is variable. Note that most of the bands are also in the profile of *T. aestivum*, to which *T. speltoides* is ancestral, illustrating the slow rate of evolutionary change in this repeated sequence family

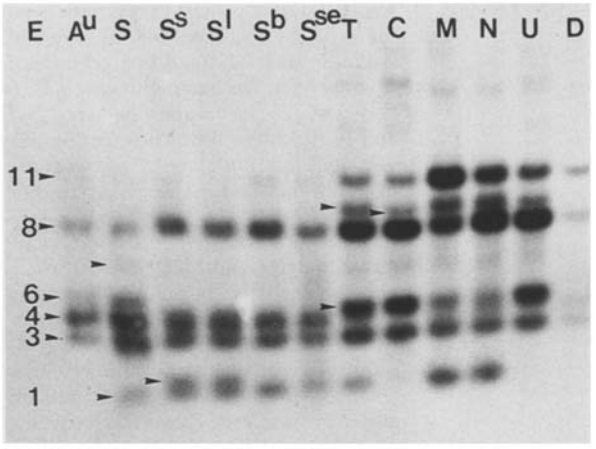
observed only in a subset of DNAs (Figs. 2 and 3). Additionally, the intensity of a band often varied among the DNAs, and in extreme cases it ranged from high intensity to bare visibility for the same band (e.g., band 11 in Fig. 2). This quantitative variation was rendered qualitative by scoring as "1" DNAs of species in which a specific band was observed, irrespective of its intensity, and as "0" DNAs of species in which it was not observed under identical conditions (Figs. 2 and 3). If a band was too faint in all species, such as the bands in the upper area of the autoradiogram in Fig. 2, or if a band was obscured by other bands or a smear, as in the upper area of the autoradiogram in Fig. 3, they were not analyzed.

The data matrix of 955 bands obtained from the analysis of profiles generated by the hybridization of 31 repeated nucleotide sequences was analyzed with PAUP using the Wagner parsimony method. A single most parsimonious tree was obtained (Fig. 4). Most of the branches in the tree showed a high bootstrap confidence. As pointed out earlier it was impossible to determine whether the absence of a band in a specific DNA profile was due to a site mutation followed by reproduction of the mutation by homogenization or by deletion of a repeated sequence subfamily located in a single chromosomal site. In the latter case, if more than 1 enzyme × probe combination was analyzed per probe (Table 2) a single evolutionary event could be repeatedly scored. As a result, the data matrix could be biased, and the resulting tree could show an incorrect topology. An important attribute of such a bias would be that the bootstrap confidence would be inflated upward. As we were aware of this danger, only several enzyme × probe combinations per each probe were used to generate the data matrix of 955 bands (Table 2). Additionally, a large number of bands were analyzed to reduce this potential bias.

To determine how significant this problem really was, bands scored in a single enzyme × probe combination per each of the 31 probes were combined to generate a new data matrix. This matrix involved 231 bands. A single most parsimonious tree was obtained by the analysis of these data with PAUP using the same options as earlier (Fig. 5a). The topology of the tree was similar to that obtained earlier except for a change in the position of *T. caudatum* and *T. umbellulatum*.

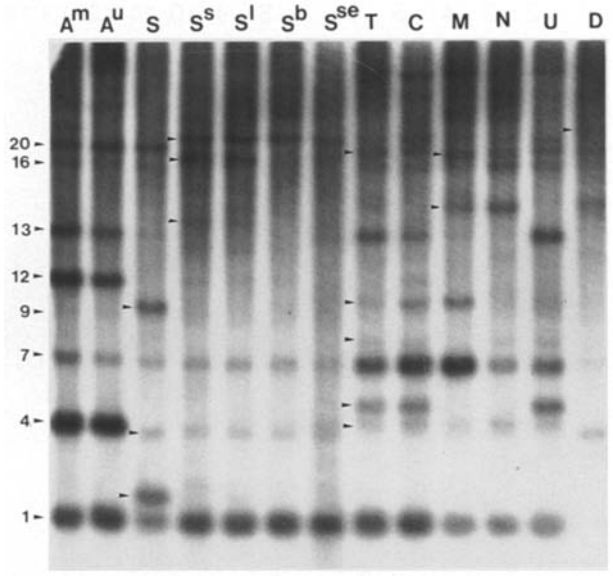
If a significant number of bands in the matrix violated the assumption of independence, the bootstrap confidence should be biased upward and be higher than the bootstrap confidence on the same branches obtained by the analysis of single enzyme × probe combinations. There were ten branches common between the tree in Fig. 4 and that in Fig. 5a. These ten bootstrap confidences were averaged. While the mean bootstrap confidence of the tree in Fig. 4 was 87.5%, that of the tree in Fig. 5a was 78.9%. The difference between the means was tested by a paired *t*-test using the differences between the paired bootstrap estimates in the two trees as variables in estimating variance. Because this test is conservative, a 10% probability level was used as the level of statistical significance to reduce the likelihood of rejecting a hypothesis when it is true. The calculated *t* value of 1.09 was not statistically significant.

In addition to having slightly, although not significantly, reduced bootstrap confidence the phylogenetic tree obtained by the analysis of 231 bands differed from the tree obtained by the analysis of 955 bands in the position of *T. umbellulatum* and *T. caudatum*. Since it was possible that both these effects were caused by the reduced sample size, additional data obtained by the analysis of single enzyme × probe combinations of 15 more probes (see Table 2) were added to the data matrix. This




| | E | $A^u$ | S | $S^s$ | $S^l$ | $S^b$ | $S^{se}$ | T | C | M | N | U | D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| 8 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| 6 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| 4 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| 3 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |

**Fig. 2.** Southern blot hybridization of the pTlUCD12 insert with the panel of *Dde*I-digested DNAs of diploid *Triticum* species and *Lophopyrum elongatum*. Three micrograms of DNA of single plants of *L. elongatum* (*E*), *T. urartu* ($A^u$), *T. speltoides* (*S*), *T. sharonense* ($S^s$), *T. longissimum* ($S^l$), *T. bicorne* ($S^b$), *T. searsii* ($S^{se}$), *T. muticum* (*T*), *T. caudatum* (*C*), *T. comosum* (*M*), *T. uniaristatum* (*N*), *T. umbellulatum* (*U*), and *T. tauschii* (*D*) were loaded per lane. Bands that were scored are indicated by *arrowheads* and some are *numbered* (*left side*) for orientation. The *bottom part* of the figure indicates the scoring of the bands among the DNAs. A score of *0* indicates the absence of a visible band in the specific profile, and a score of *1* indicates the presence of the band in the profile. Note that not all bands were scored, such as the faint bands in the *upper part* of the autoradiogram




| | $A^m$ | $A^u$ | S | $S^s$ | $S^l$ | $S^b$ | $S^{se}$ | T | C | M | N | U | D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 20 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| 12 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 |
| | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 |
| 7 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| 4 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |

**Fig. 3.** Southern blot hybridization of the pTlUCD11 insert with a panel of *Sst*I-digested DNAs. The designations of species are the same as in Fig. 2 except that *L. elongatum* DNA was replaced with *T. monococcum* DNA. The *bottom part* of the figure indicates the visibility of each band by a *0* or *1* score as explained in Fig. 2

increased the number of bands to 353. A single most parsimonious tree was obtained by the analysis of these data (Fig. 5b). The tree was identical to the tree obtained by the analysis of 955 bands (Fig. 4). The mean bootstrap confidence for the ten branches statistically analyzed above was 91.0% in this tree, which was not significantly different from the mean of 87.5% obtained for the tree in Fig. 4 ($t = 0.526$). This indicated that the differences between the phylogenetic trees in Figs. 4 and 5a could be accounted for by the reduced number of the sampled characters. The fact that the analysis based on a single enzyme × probe combination per probe did not result in reduced bootstrap confidence indicated that the bias due to the use of several enzyme × probe combinations in the data matrix used for the construction of the tree in Fig. 4 was negligible.

### *Phylogenetic inferences*

The hybridization of probes with the DNA of the outgroup *Lophopyrum elongatum* was usually weak or absent, although the same probes hybridized strongly with

the DNAs of all the *Triticum* species (Fig. 2). The DNAs of the *Triticum* species often showed common bands (Figs. 2 and 3). These observations suggested that *Triticum* is a homogeneous group relative to *L.* elongatum and supported the hypothesized monophylesis of the genus.

A basal node in the tree shown in Fig. 4 separated *T. tauschii* from the rest of the species. The remaining species were divided into two clades. Clade A included two einkorn wheats, *T. monococcum* ssp. *aegilopoides* and *T.*

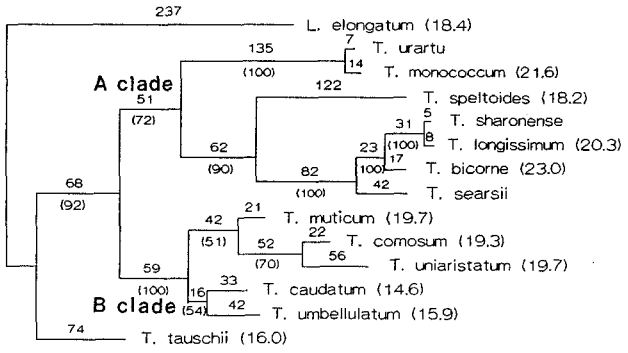



**Fig. 4.** Rooted phylogenetic tree of the genus *Triticum* reconstructed using the Wagner parsimony method (Swofford 1990). The polarity of the tree was determined by global parsimony to *L. elongatum*. The genome sizes in pg/3c (Bennett 1972) are given in parentheses following the species names. The *numbers above* the horizontal branches indicate the numbers of characters that have been altered since divergence from the preceding node. In total, 955 characters were investigated except for *T. monococcum* ssp. *aegilopoides*, in which only 263 characters were investigated, and *L. elongatum*, in which only 831 characters were investigated. The bootstrap (Felsenstein's bootstrap approach) confidence estimates on groups in the most parsimonious tree are given *below* the branches

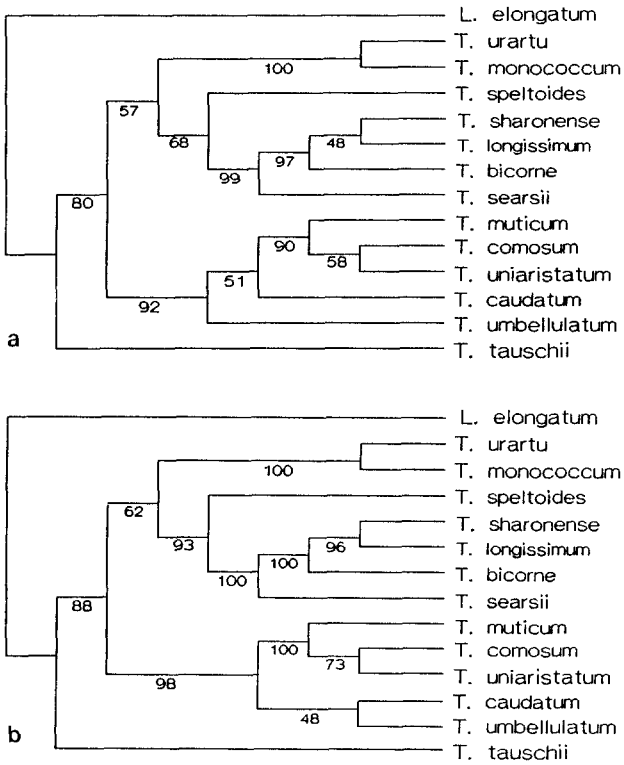



**Fig. 5a, b.** Rooted phylogenetic trees of the genus *Triticum* reconstructed using the Wagner parsimony method as described in Fig. 4. Tree *5a* was reconstructed from a data matrix of 231 bands that were scored in a single enzyme × probe combination for each of the 31 repeated nucleotide sequence probes used for the reconstruction of the tree in Fig. 4. Tree *5b* was reconstructed from the data matrix of 353 bands using the same method as above. The data matrix for the tree included the scores of the matrix used for the reconstruction of tree *5a* plus scores of additional 122 bands of single enzyme × probe combinations per 15 additional repeated nucleotide sequence probes. The *numbers below* the horizontal lines are bootstrap confidence estimates on the branches using the Felsenstein's bootstrap method

**Table 4.** Pairwise distances between species in the genus *Triticum* and *Lophopyrum elongatum*. Above the diagonal are mean distances computed from only those characters for which both members of a pair had a nonmissing value, and below the diagonal are actual distances in the numbers of characters for which a pair of species differed. Note that fewer characters were scored in *T. monococcum* and *L. elongatum* than the rest of the *Triticum* species. The distances were calculated with PAUP version 3.0L

| | *Elong.* | *Urart.* | *Spelt.* | *Sharon.* | *Longis.* | *Bicorne* | *Searsii* | *Mutic.* | *Caudat.* | *Comos.* | *Uniaris.* | *Umbel.* | *Tausch.* | *Monoc.* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Elong.* | – | 0.390 | 0.357 | 0.413 | 0.416 | 0.402 | 0.374 | 0.405 | 0.370 | 0.401 | 0.374 | 0.373 | 0.329 | 0.078 |
| *Urartu* | 362 | – | 0.348 | 0.349 | 0.355 | 0.337 | 0.322 | 0.324 | 0.301 | 0.332 | 0.321 | 0.307 | 0.316 | 0.023 |
| *Spelt.* | 331 | 323 | – | 0.268 | 0.274 | 0.252 | 0.263 | 0.349 | 0.317 | 0.362 | 0.341 | 0.325 | 0.337 | 0.145 |
| *Sharon.* | 383 | 324 | 249 | – | 0.014 | 0.051 | 0.105 | 0.297 | 0.261 | 0.305 | 0.292 | 0.284 | 0.312 | 0.125 |
| *Longis.* | 386 | 329 | 254 | 13 | – | 0.054 | 0.110 | 0.305 | 0.268 | 0.312 | 0.300 | 0.292 | 0.322 | 0.130 |
| *Bicorne* | 373 | 313 | 234 | 47 | 50 | – | 0.082 | 0.283 | 0.249 | 0.291 | 0.276 | 0.270 | 0.301 | 0.125 |
| *Searsii* | 347 | 299 | 244 | 97 | 102 | 76 | – | 0.281 | 0.240 | 0.267 | 0.246 | 0.255 | 0.277 | 0.124 |
| *Muticum* | 376 | 301 | 324 | 276 | 283 | 263 | 261 | – | 0.116 | 0.102 | 0.139 | 0.110 | 0.265 | 0.129 |
| *Caudat.* | 343 | 279 | 294 | 242 | 249 | 231 | 223 | 108 | – | 0.167 | 0.148 | 0.081 | 0.246 | 0.115 |
| *Comos.* | 372 | 308 | 336 | 283 | 290 | 270 | 248 | 95 | 155 | – | 0.084 | 0.182 | 0.260 | 0.123 |
| *Uniarist.* | 347 | 298 | 316 | 271 | 278 | 256 | 228 | 129 | 137 | 78 | – | 0.154 | 0.230 | 0.126 |
| *Umbel.* | 346 | 285 | 302 | 264 | 271 | 251 | 237 | 102 | 75 | 169 | 143 | – | 0.235 | 0.121 |
| *Tausch.* | 305 | 293 | 313 | 290 | 299 | 279 | 257 | 246 | 228 | 241 | 213 | 218 | – | 0.093 |
| *Monoc.* | 72 | 21 | 135 | 116 | 121 | 116 | 115 | 120 | 107 | 114 | 117 | 112 | 86 | – |

*urartu*, and *Triticum* sect. *Sitopsis*, which includes *T. speltoides*, *T. searsii*, *T. bicorne*, *T. longissimum*, and *T. sharonense*. Clade B included *T. uniaristatum*, *T. comosum*, *T. umbellulatum*, *T. caudatum*, and *T. muticum*.

Most branches in the tree showed a high bootstrap confidence. However, the branching within clade B was a notable exception. The 5 species formed a group in which most of the branches were supported by a low bootstrap confidence (Fig. 4).

The branch lengths (Fig. 4) and pairwise distances calculated from the data with PAUP 3.0 L (Table 4) were uneven in the tree. The variation in the distance from the basal *T. tauschii* to each ingroup species, i.e., the outgroup species *L. elongatum* was excluded, was tested with $\chi^2$ using the mean distance as the expected value. The $\chi^2$ value was 42.9 (10 *df*, $P < 0.01$), indicating that the numbers of characters that have been altered since the divergence from *T. tauschii* in individual clades were uneven.

## Discussion

The analysis of variation in 955 repeated nucleotide sequence bands employing multiple enzyme digests per probe yielded a single most parsimonious tree that had the same topology as the tree based on the analysis employing single enzyme digests per probe. The branches in the two trees had a similar bootstrap confidence. Even though this indicated that the use of several enzyme × probe combinations per probe did not bias the results, the use of too many enzymes per probe, in an effort to increase the number of bands for analysis, should be avoided, particularly in taxa with small genomes.

The chromosomal distribution of repeated nucleotide sequence bands in Southern blots has been investigated for several repeated nucleotide sequence families. Most bands appeared to be associated with more than a single chromosome (Appels and Moran 1984; Dvořák et al. 1988; Zhang and Dvořák 1990; Dvořák and Zhang 1990). In interspersed nucleotide sequences, which may show regional heterogeneity in genomes (Zhang and Dvořák 1990a, b), the bands originating from regional variation are usually faint because of low numbers of copies of those variants and are overshadowed by the signal from bands that are common to several or all chromosomes (Zhang and Dvořák 1990a, b). Bands which result from DNA fragments located in two or more chromosomal sites cannot be lost in a single evolutionary event and would appear as independent characters when several restriction enzyme digests are analyzed per probe.

The assumption of character independence would be also violated if probes would be different fragments of the same repeated nucleotide sequence. The hybridization of such probes would result in the same or related restriction fragment profiles in which the presence or absence of a band would reflect the same evolutionary cause. Since cross hybridization among the clones may not detect a spatial relationship between cloned fragments we compared the hybridization profiles of the cloned repeated nucleotide sequences and eliminated those clones that showed similar profiles.

The phylogenetic tree obtained was consistent with many cytotaxonomical data on species relationships in the genus. It clustered (1) *T. monococcum* with *T. urartu* which have been shown cytogenetically to have a common genome (Dvořák 1976; Chapman et al. 1976; Dhaliwal and Johnson 1982) and (2) the species of *T.* sect *Sitopsis*, which also appear to have a common genome (Kihara 1940, 1949, 1954; Tanaka 1955; Roy 1959; Feldman 1978). Within the section *Sitopsis* the separation of *T. speltoides* from the remaining four species was consistent with the classification by Eig (1929), who placed *T. speltoides* into the subsect. *Truncata* Eig and the other sect. *Sitopsis* species into the subsect. *Emarginata* Eig on morphological grounds. This division of *T.* sect. *Sitopsis* was also consistent with the characteristics of the 5S RNA loci (Dvořák et al. 1989) and 18S–26S rRNA loci (Dvořák and Appels 1982). The species of *T.* subsect. *Emarginata* form fertile hybrids, but their hybrids with *T. speltoides* are sterile (Waines et al. 1982). The clustering of *T. longissimum*, *T. sharonense*, and *T. bicorne* is also consistent with cytotaxonomical data (Roy 1959; Waines et al. 1982).

The placement of the species in clade B was also consistent with cytotaxonomical data, even though the confidence of the branches was low. While the outgroup species, *L. elongatum*, and the basal species, *T. tauschii*, have karyotypes with seven metacentrics or submetacentrics, *T. comosum* and *T. uniaristatum* have related karyotypes with subacrocentrics (Senyaninova-Korchagina 1932; Chennaveeraiah 1960). The karyotypic studies indicated that *T. caudatum* and *T. umbellulatum* have related karyotypes with acrocentric chromosomes that are different from those of *T. uniaristatum* and *T. comosum* (Senyaninova-Korchagina 1932; Chennaveeraiah 1960). While *T. comosum*, *T. uniaristatum*, *T. caudatum*, and *T. umbellulatum* have karyotypes that differ from those of other species, the relative sizes and arm ratios of the homoeologous chromosomes in the A (*T. monococcum* and *T. urartu* clade), S (*T.* sect. *Sitopsis* clade), and D (*T. tauschii*) genomes are almost identical and closely resemble those of the homoeologous chromosomes in *L. elongatum* (Dvořák et al. 1986), in spite of significant differences in the absolute sizes of these genomes (Bennett 1972). This is compatible with the basal position of *T. tauschii* in the tree and terminal position of *T. comosom*, *T. uniaristatum*, *T. caudatum*, and *T. umbellulatum*. The distribution of genome sizes (Bennett 1972) among the

*Triticum* species (Fig. 4) was also consistent with the tree and indicated a general tendency for the genomes to become larger during the adaptive radiation of the genus.

The branching of the tree parallels the sizes of the geographic distributions of resulting species as reported by Eig (1929) and Kihara (1954). *Triticum tauschii*, which appears to have diverged first from the common lineage, occupies the largest area ranging from Turkey to China and Pakistan. Einkorn wheats (*T. urartu* and *T. monococcum*) and *T. speltoides* occupy the next largest geographic areas. The species of *T.* subset. *Emarginata* of *T.* sect. *Sitopsis* have limited distributions on the southwestern fringe of the distribution of the diploid *Triticum* species. In *T.* subsect. *Emarginata, T. searsii* and *T. bicorne* occupy a greater area than *T. longissimum* and *T. sharonense*. In the B clade, *T. muticum*, *T. comosum*, and *T. uniaristatum* occupy smaller areas than *T. umbellulatum* and *T. caudatum*.

*Triticum muticum* holds a puzzling position in the tree. *Triticum muticum* and *T. speltoides* are the only allogamous taxa in the genus. Because most perennial relatives in the tribe Triticeae are allogamous, it appears that allogamy is a primitive character (Stebbins 1957). Morphologically *T. muticum* resembles the perennial relatives (for a review see Ohta 1991). All its chromosomes are metacentric or submetacentric, whereas the 4 other species in the B clade have derived karyotypes (Senyaninova-Korchagina 1932; Chennaveeraiah 1960). Yet the tree does indicate that *T. muticum* is a result of recent speciation in the genus. This dilemma can be reconciled if it is assumed that *T. comosum, T. uniaristatum, T. caudatum*, and *T. umbellulatum* radiated from a species morphologically closely resembling *T. muticum*; i.e., if the dichotomies in clade B are viewed as side branches from the lineage leading to the modern *T. muticum*. The limited distribution of *T. muticum* and some of its specialized molecular characters, such as a single 5S RNA locus and derived lengths of the spacers separating the 5S RNA genes (Dvořák et al. 1989), indicate that the genome of the extant *T. muticum* is advanced.

The clade leading to the extant *T. tauschii* resulted in a single species, whereas both the A and the B clades indicated several speciation events within them. Considering the wide distribution of *T. tauschii* and the diversity of its habitats the lack of speciation in this clade is striking.

While the tree reported here is in many aspects consistent with previous cytotaxonomical and morphological work, it is inconsistent with phylogenetic inferences based on restriction enzyme analysis of cpDNAs (Ogihara and Tsunewaki 1988). The tree based on cpDNAs separated *T. speltoides* from *T. longissimum* and *T. bicorne* by a basal dichotomy, which conflicts with the previous taxonomic and cytogenetic work on these species (Eig 1929; Kihara 1940, 1949). It also grouped *T. caudatum* with *T. tauschii* and *T. umbellulatum* with *T. muticum*, which contradicts the karyotypic and cytogenetic data that grouped *T. umbellulatum* with *T. caudatum* (Senyaninova-Korchagina 1932; Chennaveeraiah 1960). The tree was not outgroup-rooted and was constructed by the unweighted pair-group clustering method (Sneath and Sokal 1973), a phenetic algorithm that is greatly influenced by variation in evolutionary rates. Additionally, the assumption of the absence of intraspecific variation in cpDNA may not be true in the genus. The cpDNAs of the awned and awnless varieties of *T. speltoides* greatly differed (Ogihara and Tsunewaki 1988), but profiles of repeated nucleotide sequences failed to show a distinction between the two varieties (Fig. 1), indicating that the differences in their cpDNAs are probably indicative of natural variation within the species. If *T. speltoides* is not exceptional, intraspecific variation could exist in other species of *Triticum*, as exists in barley (Holwerda et al. 1986; Neale et al. 1988), and could persist during speciation. Such variation could confound phylogenetic inferences in cpDNA studies.

Several other attempts have been made to reconstruct the phylogeny of diploid *Triticum* species. Asins and Carbonell (1986) used variation in peroxidases and phosphatases to infer the phylogenetic relationships among wheats and selected diploid *Triticum* species, mostly from *T.* sect. *Sitopsis*. Their results showed poor correspondence with those reported here since they affiliated *T. searsii* with *T. tauschii* and did not recognize the close affinity between *T. longissimum* and *T. sharonense*.

An attempt to reconstruct phylogenetic relationships in *T.* sect *Sitopsis* from quantitative analysis of metaphase I (MI) chromosome pairing in interspecific hybrids (Kimber and Yen 1990) yielded results that also showed a poor correspondence with those reported here. According to those analyses *T. bicorne* appeared more closely related to *T. speltoides* than to *T. longissimum*. This disagreed not only with present results, which closely affiliated *T. bicorne* with *T. longissimum* and *T. sharonense* and only distantly with *T. speltoides*, but also with classical taxonomical studies (Eig 1929), hybridization and seed protein studies (Waines et al. 1978), and 5S and 18S–26S rRNA data (Dvořák and Appels 1982; Dvořák et al. 1989). Similar criticisms can be raised against the suggested equidistant relationship of *T. sharonense* and *T. searsii* to. *T. longissimum* and *T. speltoides* (Yen and Kimber 1990).

Another attempt to determine the phylogeny of *Triticum* from MI chromosome pairing in interspecific hybrids was reported by Ohta (1991). His method also generated results that did not agree with the present phylogenetic tree. Ohta placed *T. muticum* into *T.* sect. *Sitopsis* as the closest relative of *T. speltoides*. The branch by which clade B branched off from clade A that leads to *T. speltoides* had 100% bootstrap confidence in the present

tree, indicating a high likelihood not only that *T. muticum* and *T. speltoides* are not a result of terminal dichotomy in the tree, but also that *T. comosum*, *T. uniaristatum*, *T. umbellulatum*, and *T. caudatum* diverged from *T. muticum*, not from *T. speltoides* as suggested by Ohta (1991).

In contrast to these studies, the present phylogenetic tree is similar to the phylogenetic trees of *T.* sect. *Sitopsis* reconstructed from variation in shoot proteins assayed by 2-dimensional isoelectric focussing and electrophoresis (Thiellement et al. 1989).

The fact that the tree based on repeated nucleotide sequences reflected many relationships revealed by classical taxonomy, cytotaxonomy, and cytogenetics in the genus *Triticum* indicated that repeated nucleotide sequences can be used in phylogenetic studies of closely related taxa. Whether the utility of this tool is limited to species with large genomes, which contain large quantities of repeated nucleotide sequences, or is generally applicable to plants and animals needs investigation. It is also not known if repeated nucleotide sequences can be used to investigate the phylogeny of higher taxonomical categories, such as tribes.

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