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Analysis of phylogenetic relationships in the Triticeae tribe using RFLPs

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Abstract. The use of restriction fragment length polymorphisms in combination with other approaches is very useful for the reconstruction of evolutionary events revealing phylogenetic relationships. A set of 21 cDNA probes hybridizing to different chromosome arms in hexaploid wheat was used in a series of experiments designed to estimate the phylogenetic relationships among and within 16 species of the Triticeae tribe. A high degree of polymorphism was found both between and within the species examined. The RFLP data were used to generate a cladogram and a phenogram in order to compare the two different methods of constructing phylogenetic trees. The results of both methods were consistent with each other and with the general taxonomic information provided by earlier morphological studies, meiotic pairing analysis, isozyme tests, and sequence alignment in the *Ter*, *NOR*, and 5*s* DNA loci. In addition, several correlations were found between the geographical origin of accessions from the same species and their phylogenetic relationships as shown by the cladogram and phenogram.

Key words: Cladogram – Phenogram – Phylogenetic tree – Restriction fragment length polymorphisms – Triticeae tribe

Introduction

The tribe Triticeae represents a group of plant species which have had a significant impact on the cultural and economic history of human development. Today, cereals like wheat (*Triticum* ssp.), and barley (*Hordeum vulgare* L.) are major world food crops. In addition to these very well known species, other species in the Triticeae tribe are useful as forages or possess agronomically-valuable traits for wheat and barley improvement (e.g., disease-resistance genes, perennial habit, salt and drought tolerance, and dwarfing genes).

The main genetic characteristic of this plant group is a basic chromosome number of seven. The general evolution of the tribe, as revealed by meiotic pairing analysis, has been defined by divergence at the diploid level from a common diploid ancestor, and convergence at the polyploid level involving the diverged diploid genomes (Kimber and Feldman 1987; West et al. 1988). The goal of many taxonomic studies has been to determine the phylogenetic distance among the diploid genomes and their contribution to the polyploid forms. Early efforts in this direction involved classification studies based on morphological characters. These markers, however, could be affected by environmental fluctuations and stage of development (Wang and Tanksley 1989). Thus, further studies on the phylogenetic variation among the various species have been conducted at the genetic level to reveal the extent to which morphological phylogenies reflect the course of evolution. The study of meiotic chromosomal pairing in hybrids of the different species has been a very useful approach (Kimber and Yeng 1990), but this technique is not very informative when large phylogenetic distances are involved between species. Isozyme studies in the Triticeae tribe have provided valuable insights into the phylogeny among the genera and species (Asins and

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Table 1. Germplasm utilized in the present study

Species	Genome	Accessions	Geographical
Triticum monococcum L.	A	A1	Turkey
		A2	Iran
		A4	USSR
		A5	USSR
Triticum speltoides (Tausch) Godron	B	B1	Turkey
		B2	Israel
		B3	Iraq
		B4	Rumania
Triticum tauschii (Coss.) Schmalh.	D	D1	Afghanistan
		D2	USSR
		D3	USSR
		D5	Turkey
Secale cereale L.	R	R1	Poland
		R2	USA
		R3	Ecuador
		R4	USA
		R5	Mexico
Hordeum vulgare (L.)	I	I1	USA (Nebraska)
		I2	Canada (central)
		I3	Netherlands
		I4	Canada (Ontario)
Critesion bogganii (Wilensky) Love	H	H1	USSR
		H2	Afghanistan
		H3	Iran
		H4	PRC
Critesion brevisubulatum (Trin.) Love s. lat.	H	H5	Iran
		H6	Iran
		H7	USSR
		H8	USSR
Agropyron cristatum (L.) Gaetner	P	P1	USA
		P2	Germany
		P3	USSR
		P4	USSR
Agropyron desertorum (Fischer ex Link) Schultes	PP	P5	Denmark
		P6	Portugal
		P7	Turkey
		P8	USSR
Dasypyrum villosum (Cosson and Duieau) T. Durand	V		USSR
Psathyrostachys juncea (Fischer) Nevski	N	N1	USSR
		N2	USSR
		N3	Canada
		N4	USA
Psathyrostachys fragilis (Boiss.) Nevski	N	N5	Iran
		N6	Iran
		N7	Iran
		N8	Iran
Pseudoroegneria spicata (Pursh) Love	S	S1	USA
	S	S2	Canada
		S3	USA
		S4	USA
Pseudoroegneria libanotica (Hackel) D.R. Dewey	S	S5	Iran
		S6	Iran
		S7	Iran
		S8	Iran
Thinopyrum elongatum (Host) D.R. Dewey	E	E1	France
		E2	France
Thinopyrum bessarabicum (Savul and Rayss) Love	J	J1	USSR
		J2	USSR
		J3	France
		J4	USSR
		J5	USSR

Carbonell 1986; McIntyre 1988). However, the number of loci that can be examined are limited and the data obtained can be tissue- and developmental-stage-specific. The use of DNA markers in plant systematics represents a new approach that is capable of overcoming most of the above limitations (Crawford 1990). Restriction fragment length polymorphism (RFLP) analysis has two distinct advantages over other approaches: (1) the absence of tissue and developmental stage problems, and (2) a much larger number of loci are available to be tested. Probably the most accurate and reliable method of studying phylogenies would be by DNA sequence alignment of orthologous loci, but this method is very laborious and expensive and few genes from Triticeae species have been isolated and sequenced to-date.

Two previous taxonomic studies based on RFLPs have been conducted, one in the genus *Brassica* (Song et al. 1988) and the other in *Oryza sativa* L. (Wang and Tanksley 1989). In *Brassica,* 57 species and subspecies were screened with 21 probe/enzyme combinations, while 70 varieties were tested with 50 probe/enzyme combinations in rice. The cladogram constructed in *Brassica* was successful in suggesting a pattern of evolution in which particular species evolved from others that appeared to be more ancestral. In *O. sativa,* a phenogram was built and it showed sufficient resolution to discriminate among the varieties tested.

The present study of the Triticeae tribe analyzed the relationships between 16 diploid and autotetraploid species representing the A, B, D, H, I, J, N, P, R, S and V genomes. Several accessions per species were tested with 63 probe/enzyme combinations. Whenever possible, the accessions within a species were chosen from different geographical locations in order to capture any variability caused by local ecological adaptations.

Materials and methods

Plant material

Sixteen species representing 11 genomes of the Triticeae tribe and several accessions per species were selected (species, accessions, and their sources are listed in Table 1).

Table 2. Size and chromosomal location of the probes utilized in the RFLP experiments

Probe number	Chromosome arm	Insert length (kb)
Psr 162	1L	2.00
Psr 161	1S	0.70
Psr 101	2L	0.60
Psr 135	2S	1.00
Psr 156	3L	0.90
Psr 123	3S	1.00
Psr 144	4AS, 4BL, 4DL	1.30
Psr 163	4AL, 4BS, 4DS	0.50
Psr 118	5S	0.70
Psr 128	5L	0.50
Psr 154	6L	0.40
Psr 167	6S	0.70
Psr 129	7L	1.20
Psr 152	7S	0.80
Psr 106	Group 6	0.40
Psr 113	Group 6	0.56
Psr 134	Group 6	0.55
Psr 141	Group 6	0.80
Psr 142	Group 6	0.80
Psr 149	Group 6	0.30
Psr 168	Group 6	1.00

Restriction enzymes

The restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I; were used in Southern-blot hybridization experiments (Sambrook et al. 1989) because they have been reported to produce high levels of polymorphisms in *Triticum aestivum* L. em Thell. (Chao et al. 1989). After several hybridizations of the enzymes *Eco*RI, *Eco*RV, and *Hin*dIII showed the highest levels of polymorphisms in the species selected (data not shown), and therefore, they were chosen for the RFLP experiments.

RFLP probes

A set of 14 low-copy-number cDNA probes, one of which hybridizes to each of the homoeologous chromosome arms of hexaploid wheat, plus seven available probes representative of homoeologous group six, were used (the sizes and chromosomal location of the probes are shown in Table 2). All these probes were obtained from the Cambridge Laboratory, John Innes Centre for Plant Sience Research, Norwich, England (Sharp et al. 1989).

RFLP assays

Seeds from the selected species and accessions were grown in growth chambers and greenhouses on the University of Missouri-Columbia campus. Plant genomic DNA was extracted from young, green leaves by a sap extractor using a technique described in Clark et al. (1989). Genomic DNA of each plant sample was digested with the restriction enzymes *Eco*RI, *Eco*RV, and *Hin*dIII, separated by electrophoresis in agarose gels, transferred to nylon filters by Southern blotting, and hybridized with the 21 P^{32}-labeled probes using standard techniques (Sambrook et al. 1989).

RFLP data analysis

The principles and methodology used in analyzing the RFLP data have been previously described for cladistic (Felsenstein 1982; Fink 1986; Sytsma and Gottlieb 1986; Song et al. 1988) and phenetic approaches (Rogers 1972; Wang and Tanksley 1989). Each fragment detected by Southern analysis for each probe/enzyme combination tested was treated as a character, and its presence or absence was scored assuming that common restriction fragments among different taxa were indicative of homologies in genomic DNA sequences. The heuristic approach of the computer program "Phylogenetic Analysis Using Parsimony", version 3.0, (personal communication: David L. Swofford, Illinois Natural History Survey, 607 E. Peabody Drive, Champaign, Illinois 61820) was used on a Macintosh II ci computer to generate the cladogram. The Wagner parsimony method that treats traits as binary characters, and permits free irreversibility of character states, was chosen for analyzing the data. The genus *Critesion* Rafinesque was used as an outgroup because it represented a plant group that has been shown to be well separated within the tribe by previous studies and therefore was useful for cladistic comparison (Appels et al. 1989). The phenogram was constructed according to the modified Rogers' genetic distance (Rogers 1972) using the average-linkage algorithm in the clustering procedure of SAS (SAS Institute 1988). We prefered this measure of genetic distance due to the uncertainties concerning the relative importance of the forces involved during the evolution of the tribe Triticeae. The methodology applied to taxonomical analysis is limited by the statistical assumptions made. Phenetics looks at the polymorphisms among the different end products of evolution and assumes that similarity among the characters measured with a genetic distance equation indicates a closer phylogenetic relatedness. However, cladistics take in to consideration the evolutionary pathways in which the characters scored were derived from more ancient ones by choosing the most parsimonious tree to explain the evolution of the taxa with the minimum number of evolutionary steps. Each method has advantages and disadvantages. For instance, phenetics ignores evolutionary parallelisms and may cluster together taxa which are in reality taxonomically more separated; on the other hand, evolution does not necessarily occur in the most simple manner as cladistic parsimony analyses assume. Despite the different biases that may arise in the use of these methods, they do provide important insights into the evolution of taxonomic groups.

Results and discussion

A total of 2,647 DNA fragments were recorded from the Southern hybridization experiments. Only 79 fragments (2.98%) were common among all species and accessions. The remaining 2,568 fragments (97.12%) were phylogenetically informative and useful for generating a cladogram and a phenogram (Figs. 1 and 2). Theoretically the highest number of probe/enzyme combinations used in the RFLP analysis should result in the most accurate phylogenetic tree, since a larger portion of the genome would be compared among the taxa analyzed. Song et al. (1988) compared *Brassica* phylogenetic trees based on subsets of 5, 10, 20, and 30 probes chosen at random. Their results suggested that when more than 20 probes were used to construct a phylogenetic tree, each additional probe provided less information than when only ten probes were used. They found no significant differences for the separation of the large diverse groups among the phylogenetic trees using either 10, 20, or 30 probes. These

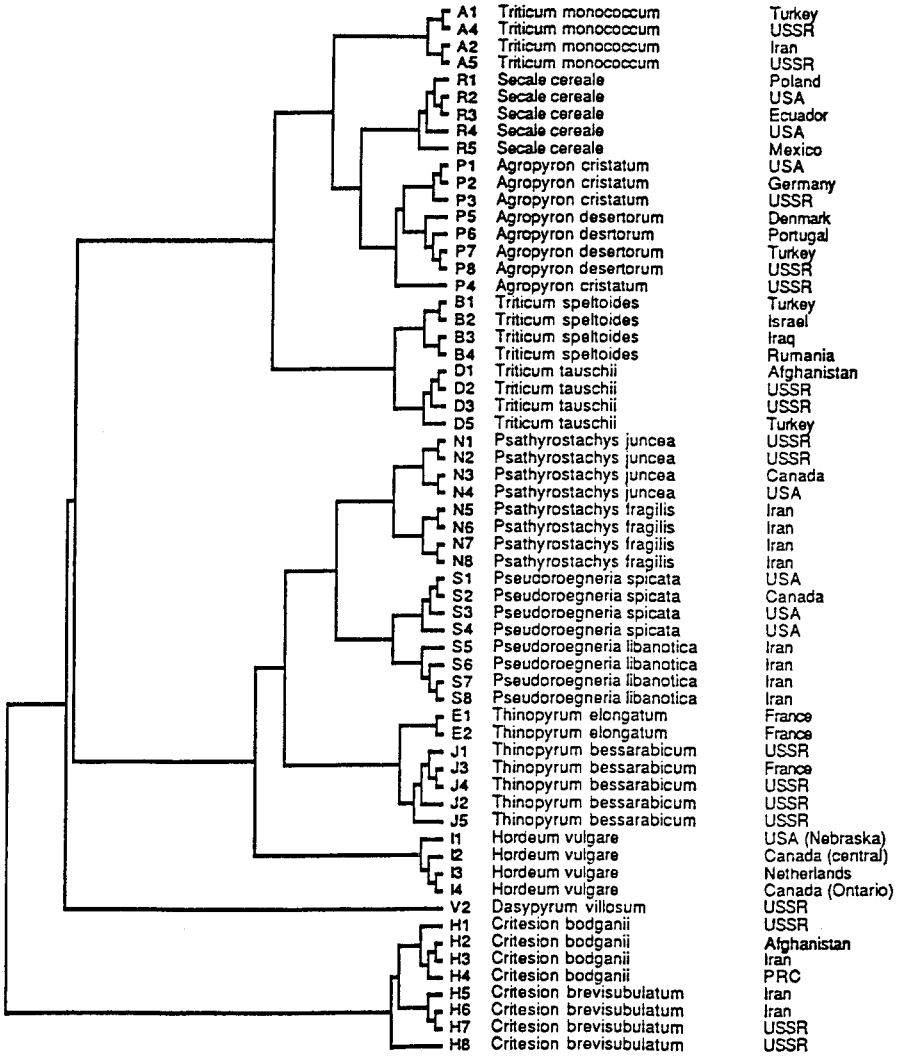

Fig. 1. Triticeae tribe cladogram resulting from the PAUP package analysis of the RFLP data set

results indicate that fewer probes are needed if great diversity exists among the selected taxa. This seems to be the case in the present work. Trees constructed using random sets of ten or more probes showed similar grouping to the results obtained utilizing 21 probes (data not shown), while trees based on sets of five probes showed important deviations. The 21 probes used in this work were cDNA clones rather than genomic probes which were mainly used in the *Brassica* analysis. Even though cDNA clones are likely to detect less polymorphisms in wheat than genomic probes (Devos et al. 1991), most of the probes used in the present study were located on different chromosomes of the seven homoeologous groups, of the tribe Triticeae which enhanced the ability to detect variability in sequences representative of different genomic areas. It also decreased the risks of scoring polymorphisms from sequences located only in very restricted portions of the genome and of counting the same mutations/polymorphism/DNA regions more than once.

Both phylogenetic trees (Figs. 1 and 2) showed very close associations between the genera *Secale* L. and *Agropyron* Gaertner (R-P), and the E and J genomes of the genus *Thinopyrum* Love. The cladogram and the phenogram also clustered *Secale* and *Agropyron* together with the genomes of the three *Triticum* species (R-P-A-B-D). The associations between *Triticum tauschii* (Coss.) Schmalh. and *Triticum speltoides* (Tausch) Godron (D-B) and among *Secale, Agropyron,* and *Triticum monococcum* (L.) Love (R-P-A) were more intense in the cladogram (Fig. 1) than in the phenogram (Fig. 2). In contrast, *Secale, Agropyron* and *T. speltoides* (R-P-B), and *Thinopyrum, Hordeum,* and *Dasypryrum* (E-J-I-V) appeared to be more closely associated in the phenogram (Fig. 2). Finally, the cladogram clustered together *Psathyrostachys* and *Pseudoroegneria* Love with *Thynopyrum* and *Hordeum* (N-S-E-J-I), showing a close association between the N and S genomes. Differences between the cladogram and the phenogram were expect-

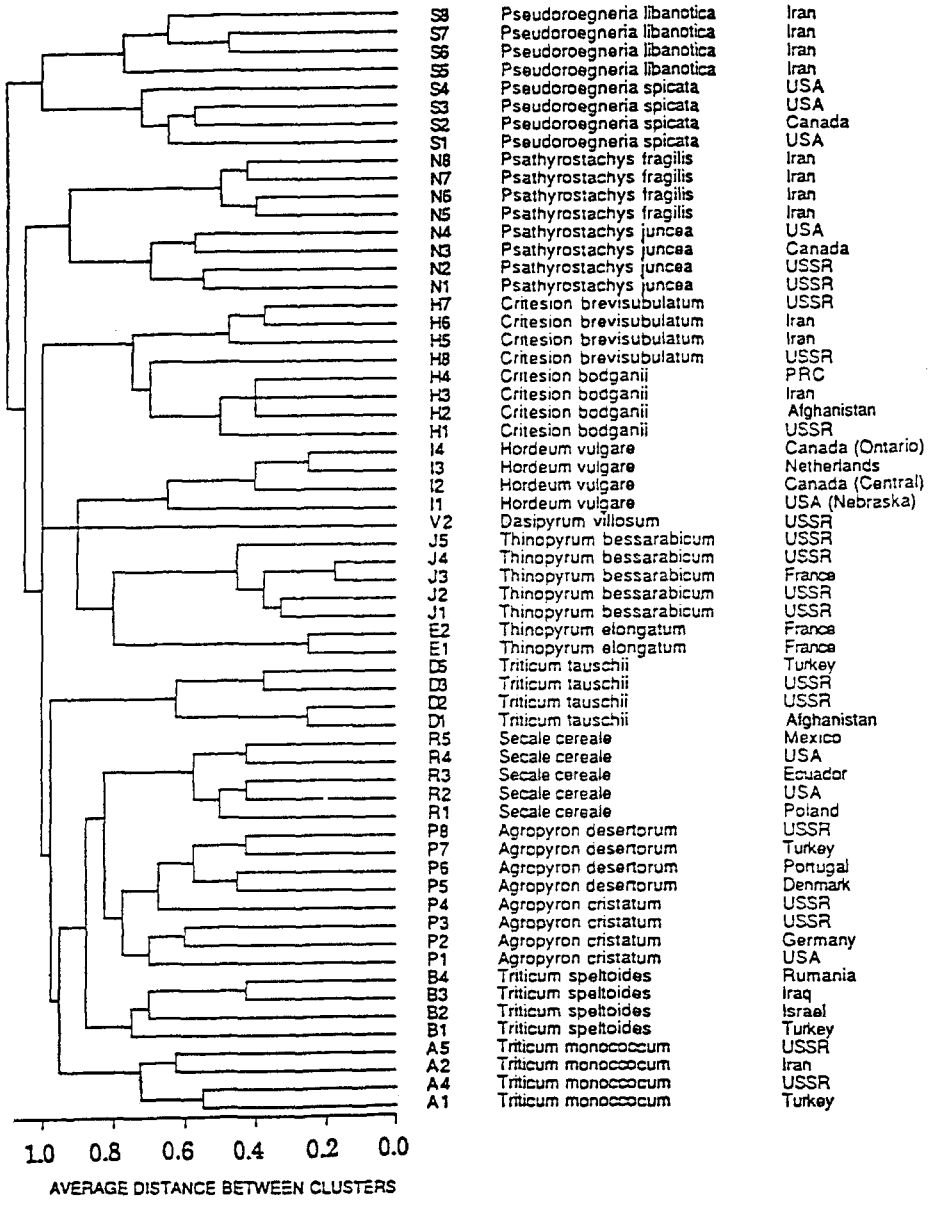

Fig. 2. Triticeae tribe phenogram based on Rogers' Genetic Distance

ed since two different phylogenetic systems were applied to analyze polymorphic data among the taxa.

Appels et al. (1989) built a consensus phylogenetic tree for the tribe Triticeae taking into account previous taxonomic studies made on this plant group. They compared all the existing trees utilizing classical taxonomic characters, isozyme analysis, and sequence alignments in the NOR, 5 s DNA, and Ter loci. Their compiled consensus tree showed a close relatedness among the genomes of the two *Triticum* species tested and *Thinopyrum* (B-D-E-J). The trees generated in the present study (Figs. 1 and 2) agreed with the close genomic associations within the *Triticum* (A-B-D) and *Thinopyrum* (E-J) genera as described by Appels et al. (1989), even though the present results indicate that *Triticum* appears to be closer to *Secale* and *Agroypron* (A-B-D-R-P), and *Thinopyrum* to *Hordeum* (E-J-I). A strong association between *Secale* and *Agropyron* (P-R) was observed (Figs. 1 and 2), consistent with the Ter alignmnet analysis, even though the rest of the characters used in the consensus tree construction clearly separated these genomes (Appels et al. 1989). This result has been explained by assuming that the Ter sequence was an ancient element retained by these two genera (Appels et al. 1989). However, the results obtained comparing 21 cDNA probes in the present RFLP assays support the idea that the phylogenetic distance between the P and R genomes could be smaller than was previous estimated. The cladogram also supports the data obtained with isozyme analysis (McIntyre 1988) in which the genera *Psathyrostachys* and *Pseudoroegneria* (N-S) appear closely related, in opposition to the previously obtained data (Appels et al. 1989).

One of the objectives of the present study was to attempt an association between the intraspecific variation and the geographical origin of each accession. Unfortunately, it was not always possible to obtain accessions from well-separated geographical regions. However, in most of the cases the polymorphisms among accessions in both the cladogram and the phenogram seem to be related to their physical location. The geographical distances involved in the accessions of *Triticum monococcum, Triticum tauschii, Psathyrostachys fragilis* (Boiss.) Nevski, *Pseudoroegneria spicata* (Pursh) Love, *Pseudoroegneria libanotica* (Hackel) D. R. Dewey, and *Thinopyrum* and *Critesion* species were not sufficiently discriminative to make geographical correlations. Even though most of the species used in this study were wild, the accessions of *H. vulgare* L. and *Secale cereale* L. were selected from cultivars. Both genera showed high levels of polymorphism, probably as a result of intense human manipulation for agronomic improvement under different local conditions. Nevertheless, the clustering pattern shown in *S. cereale* presented discrepancies with the geographical origin of the accessions; for example, one accession from Poland (R5) appears to be closer to another from the United States (R4) than the one from Mexico (R5). These results may stem from the influences of plant breeding programs where seeds have been artificially exported for use in different regions of the world. One of the problems that arises in studies such as the present one is created by the pattern of seed dispersion caused by human beings, making the results difficult to be completely understood. In *Agropyron cristatum* (L.) Gaertner, one accession from the United States (P1) clustered together with the one from Germany (P2) in contrast with the two accessions from a closer location in the Former USSR (P3 and P4) (Figs. 1 and 2). In this case, it is believed that the US accession was imported from western Europe.

The clustering pattern of *Agropyron desertorum* (Fischer ex Link) Schultes is even more interesting in that the trees show it as being derived from the Soviet accession P4 of *A. cristatum* (Fig. 1). *A. desertorum* is an autotetraploid species that most likely has been derived from a diploid ancestor by doubling its chromosome complement. The RFLP data seem to reflect this evolutionary event Branches among the different accessions of these two species suggest a dispersion of the P4 ancestor through central or northern Europe to the north-west limit of the continent (P5, Denmark). A further dispersion took place to southern Europe (P6, Portugal), followed by a final dispersion through the Mediterranean region coming back in an easterly direction to Turkey and southern Russia (P7 and P8) (Fig. 3).

Psathyrostachys juncea (Fischer) Nevski is a native species of the Eurasian interior that was imported into North America to re-vegetate arid rangelands. In this

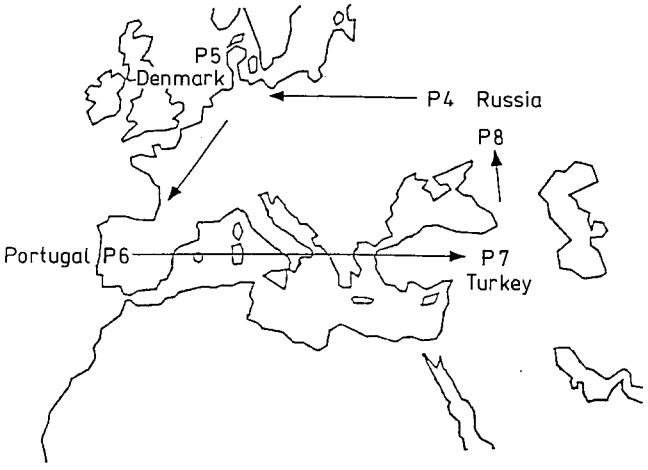

Fig. 3. Dispersion pattern of *Agropyron desertorum* as suggested by the cladogram

case the trees reflect how the American accessions cluster together (N3 and N4) in constrast with the European ones (N1 and N2). This may be due to adaptations to local ecological conditions.

Another positive correlation is shown by the cluster pattern of *H. vulgare*. The accession from the Netherlands is close to one from Ontario (Canada), less related to another from central Canada, and far away from the one released in Nebraska (United States). The climate in Ontario is cold and humid, similar to the Netherlands. The climate in central Canada is cold and dry, while the climate in Nebraska is not as cold. Again, the local environmental adaptations are apparently reflected in the phylogenetic trees.

An accession of *Triticum speltoides* from Rumania clusters with another from Iraq (B3 and B4), in opposition to one from Turkey that is close to another from Israel (B1 and B2). The negative geographical correlation in this species could probably be better understood with more data concerning the local ecological conditions where these populations were found.

Conclusions

In combination with other approaches (i.e., classical morphological comparisons, meiotic analyses, isozyme tests, and sequence alignment) the use of RFLPs in plant systematics is capable of providing a source of information for analyzing taxonomic characters. RFLPs are very useful for the reconstruction of evolutionary events to reveal phylogenetic relationships. The consensus results form analyzing the tripe Triticeae, using both a phenogram and a cladogram, support a closer association between the genomes of *Secale* and *Agropyron* (R-P) than was previously estimated. The cladogram supported the

phylogenetic proximity of *Psathyrostachys* with *Pseudoroegneria* (N-S) in concordance with previous isoenzymatic analysis (McIntyre 1988). The present results also confirmed the proximity of the three *Triticum* genomes (A-B-D) which are believed to be the procursors of hexaploid wheat and closely associates them with the P and R genome (A-B-D-P-R), The other common cluster in the trees associated *Thinopyrum* and *Hordeum* (E-J-I). Discrepancies with other phylogenetic trees previously constructed (Appels et al. 1989) were not completely unexpected since different sources of genetic variability were tested in the present study.

A positive correlation between the geographical origins of some accessions within species and their branch-distribution patterns in the phylogenetic trees was detected. In the case of *A. desertorum*, the branch-clustering patterns suggested a particular geographical pattern that could provide interesting insights into the dispersion of these crops in the Eurasian continent. The fact that many of the phylogenetic relationships scored using RFLPs were consistent with previous taxonomic studies, provides valuable evidence for reconstructing the evolution of the tribe Triticeae.

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Book reviews

Enrique Sanchez-Monge: Flora Agricola: Taxonomía de las Magnoliofitas (Angiospermas) de interés agricola, con excepción de las aprovechamiento exclusivamente ornamental o forestal. Ministerio de Agricoltura, Pesca y Alimentación. Madrid, 1991, 1296 (I volume) and 680 (II volume) pages.

A flora of interest to agriculture and in search of an English translation.

The content. This is an ecumenical summary describing the identification of species of plants of interest to agriculture. For each species are given: specific name, synonyms, taxa, botanical description, chromosome number, utilization, type of reproduction, area of diffusion, name in several languages. For the genera it is included the botanical description, the basic chromosome number and the dichotomic key to be used to classify the species. Similar informations are summarized for the families, including the dichotomic key to divide the genera. The basis for the inclusion of a genus in a family is the existence of at least one species reproduced artificially by man, or, even if not so, at least systematically exploited as source of food, forage or industrial products. The review covers all angiosperms with the exclusion of the forestal species and of those used as ornamentals.

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