Phylogenetic relationships among *Secale* species revealed by amplified fragment length polymorphisms

T. Chikmawati, B. Skovmand, and J.P. Gustafson

Abstract: Amplified fragment length polymorphism (AFLP) data were utilized to analyze the phylogenetic relationships among 29 accessions representing 14 of the most commonly recognized ranked species or subspecies in the genus *Secale*. We observed 789 AFLP markers of 1130 fragments utilizing 18 P-/M- and E-/M- primer combinations. All polymorphic fragments were used to construct phenetic and phylogenetic trees. The resulting phenogram and cladogram had similar tree topologies. Cluster analysis showed that *Secale sylvestre* was the most distantly related to all other ryes. Annual forms were grouped together, and the perennial forms appeared more closely related to each other. This suggested that life cycle could have played an important role in determining the relationships among *Secale* species. *Secale sylvestre* was considered to be the most ancient species, whereas *Secale cereale* was the most recently evolved species. Amplified fragment length polymorphism analysis clearly separated all *Secale* species into only 3 major species groups, within the genus *Secale*: *S. sylvestre*, *Secale montanum* (syn. *Secale strictum*) for perennial forms, and *S. cereale* for annual forms. This study demonstrated that the AFLP approach is a useful tool for discriminating species differences, and also gave a much better resolution in discerning genetic relationships among *Secale* species as compared with previous studies using other approaches.

Key words: AFLP, *Secale*, phylogenetic relationship.

Résumé : Le polymorphisme de longueur des fragments amplifiés (AFLP) a été employé pour analyser les relations phylogénétiques entre 29 accessions représentant 14 des espèces ou sous-espèces les plus communément reconnues au sein du genre *Secale*. Les auteurs ont obtenu 789 marqueurs AFLP parmi 1130 fragments en employant 18 combinaisons d'amorces P-/M- and E-/M-. Tous les fragments polymorphes ont été employés pour produire des arbres phénétiques et phylogénétiques. Les phénogrammes et dendrogrammes résultants avaient une topologie semblable. Une analyse de groupement a montré que le *Secale sylvestre* était l'espèce la plus éloignée de tous les autres seigles. Les formes annuelles ont été groupées ensemble et les formes pérennes étaient plus apparentées les unes aux autres. Ceci suggère que le cycle vital pourrait avoir joué un rôle majeur dans l'établissement des relations au sein des espèces du genre *Secale*. Le *S. sylvestre* est considéré comme étant l'espèce la plus ancienne, tandis que le *Secale cereale* serait l'espèce la plus récente. L'analyse AFLP a clairement séparé toutes les espèces entre seulement 3 groupes majeurs : *S. sylvestre*, *Secale montanum* (syn. *Secale strictum*) chez les formes pérennes, et *S. cereale* chez les formes annuelles. Cette étude montre que les AFLP constituent un outil utile pour discerner les relations génétiques au sein des espèces du genre *Secale* par comparaison avec les études antérieures faisant appel à d'autres approches.

Mots clés : AFLP, *Secale*, relation phylogénétique.

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Introduction

The genus *Secale* L. is a typical representative of Mediterranean flora. It has a wide distribution from central Europe and the western Mediterranean through the Balkans, Anatolia, Israel and the Caucasus to Central Asia, with an isolated population in South Africa (Sencer and Hawkes 1980). This genus includes perennial or annual, self-incompatible or self-compatible, and cultivated, weedy, or wild species (Vence et al. 1987). Cultivated rye (*Secale cereale* L.) is an

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important source of bread, especially in parts of Northern and Eastern Europe with poor soils and severe winters (Leonard and Martin 1963). In addition, wild and cultivated ryes also have a great potential as a source for value-added trait genes such as those for high protein content, disease resistance, and other morphological and biochemical traits, for wheat (*Triticum* spp. L.) and triticale (×*Triticosecale* Wittmack) improvement.

Despite its economical importance, taxonomy and phylogenetic relationships within the *Secale* genus have long been the subjects of controversy. Taxonomists have tried to discriminate each species within *Secale* using different approaches; however, identification of many taxa has been difficult because of a lack of diagnostic characters. As a result, many varying classification efforts have been reported. The first reports based on morphological characteristics, life cycle, and geographical distribution (Vavilov 1917, 1926) accepted 4 species in the genus *Secale*, for example, *Secale africanum* Stapf., *S. cereale* L., *Secale fragile* Marsch, and *Secale montanum* Guss. Roshevitz (1947) distinguished as many as 14 species based on their crossability. However, Khush (1962) did not find any cytogenetic support to classify perennial ryes (*S. montanum*, *S. africanum*, and *Secale kuprijanovii* Grossh.) as different species and proposed that they should be taken as subspecies of *S. montanum,* whereas the weedy ryes (*Secale ancestrale*, *Secale afghanicum* Vav., *Secale dighoricum* Vav., and *Secale segetale* Zhuk.) were to be considered subspecies of *S. cereale*. Lastly, Frederiksen and Petersen (1998) made a taxonomic revision of *Secale* based on examination of material in several herbaria and comments on the application of many of the species names that were used. They recognized only 3 species: *Secale sylvestre, Secale strictum* (syn. *S. montanum*), and *S. cereale*. Phylogenetic relationships among *Secale* species have also been studied using many different approaches, including morphological analyses (Frederiksen and Petersen 1997), isozymes (Vence et al. 1987), thin-layer chromatography patterns (Dedio et al. 1969), ribosomal DNA spacer lengths (Reddy et al. 1990), restriction fragment length polymorphism (RFLP) of plastid genome (Murai et al. 1989), and chloroplast DNA variation (Petersen and Doebley 1993), as well as the internal transcribed spacer sequences of the 18S–5.8S rDNA (ITS-1) region of cultivated and wild species (De Bustos and Jouve 2002). In contrast with the classification systems, all phylogenetic studies demonstrated similar results. *Secale sylvestre* showed distinct characteristics and was the most distant species, whereas other taxa have been and continue to be more difficult to distinguish from each other.

Amplified fragment length polymorphism (AFLP) analysis is a technique through which selected fragments from the digestion of total plant DNA are amplified by PCR (Vos et al. 1995). This technique produces DNA fingerprints that provide a large number of genetic markers, which can be used as a satisfactory alternative to morphological and biochemical trait analyses. Amplified fragment length polymorphism markers have been used for phylogenetic studies to uncover closely related taxa that had been impossible to resolve with morphological or other molecular systematic characteristics (Russell et al. 1997; Janssen et al. 1997; Mueller and Wolfenbarger 1999; Zhang et al. 2001; Hodkinson et al. 2000; Beardsley et al. 2003; Laurence et al. 2003). This method could be suitable for the analysis of relatedness, especially because AFLP markers are virtually free of artifacts, which is an acute problem of anonymous markers for relatedness estimation, and because comigration of nonallelic fragments occurs at extremely low levels (Waugh et al. 1997). Previous studies showed that AFLP technology also provided better resolution in discerning phylogenetic relationships as compared with isozymes, nuclear RFLPs and chloroplast DNAs (Sharma et al. 1996). However, the use of AFLPs for studying phylogenetic relationships in the genus *Secale* has not been previously reported. The present research was designed to demonstrate the use of AFLPs in discriminating *Secale* species and to understand the phylogenetic relationships among them.

The objectives of the present study were to (*i*) apply AFLP technology to perform detailed analyses of polymorphism in the genus *Secale*, (*ii*) discriminate *Secale* species, and (*iii*) describe the phylogenetic relationships among *Secale* species based on AFLPs.

Materials and methods

Plant materials and DNA isolation

Twenty-nine accessions of weedy/wild rye and cultivated rye were used (Table 1), representing 14 of the most commonly recognized taxa ranked as species or subspecies in the genus *Secale*. Seeds were kindly provided by the germplasm collections of the Plant Breeding and Acclimatization Institute (IHAR), Poland; Germplasm Resources Information Network (GRIN) of the United States Department of Agriculture (USDA); and International Maize and Wheat Center (CIMMYT), Mexico. Different numbers of accessions per species were used, because that is what was available from the germplasm collections. One accession from each of *Agropyron cristatum* (L.) Gaertn and *Triticum monococcum* L. were used as outgroups (Monte et al. 1995) and were obtained from the USDA-Sears collection, University of Missouri. All of the accessions are carefully maintained in seed form by the various collections and can be obtained from the collection's curator. DNA was extracted from young freeze-dried tissue collected from 5–10 plants of each accession using a cetyltrimethylammonium bromide method (Saghai-Maroof et al. 1994). Five to ten plants were used based on preliminary results, which showed that 5–10 samples per accession represented the diversity within each species.

AFLP analyses

Amplified fragment length polymorphism analyses were carried out according to the technique developed by Vos et al. (1995). From each accession, a 500-ng sample of total genomic DNA was digested with the combination of *Eco*RI and *Mse*I or *Pst*I and *Mse*I restriction enzymes and ligated with the respective adaptors for each enzyme. Preamplification reactions were performed using *Eco*RI and *Mse*I or *Pst*I and *Mse*I primer combinations with 1 nucleotide extension being added to the 3′ end of each primer. The pre-amplification products were used as templates for selective amplification, which involved 3 additional nucleotides being placed at the 3′ end of *Eco*RI or *Pst*I and *Mse*I prim-

Table 1. List of species name, accessions number (existing germplasm bank location), country of origin, type, life cycle of *Secale* species studied.

No.	Accession No.*	Species†	Country of origin	Type‡	Life cycle
1	31209 (IHAR)	*S. anatolicum* Boiss.	Poland	Wi	P
2	31082 (IHAR)	*S. afghanicum* (Vav.) Roshev.	Afghanistan	We	A
3	31319 (IHAR)	*S. africanum* Stapf	South Africa	Wi	P
4	31344 (IHAR)	*S. ancestrale* (Zhuk.) Zhuk.	Turkey	We	A
5	CIse 107	*S. ancestrale* (Zhuk.) Zhuk.	Japan	We	A
6	PI 283971 (USDA)	*S. ancestrale* (Zhuk.) Zhuk.	Algeria	We	A
7	31322 (IHAR)	*S. chaldicum* Fed.	Russia Federation	Wi	P
8	31323 (IHAR)	*S. ciliatoglume* (Boiss.) Grossh.	Poland	Wi	P
9	CIse 1	*S. cereale* L.	Sweden	C	A
10	PI 534929 (USDA)	*S. cereale* L.	Italy	C	A
11	PI 534962 (USDA)	*S. cereale* L.	US (Mississipi)	C	A
12	PI 534965 (USDA)	*S. cereale* L.	US (Florida)	C	A
13	PI 535008 (USDA)	*S. cereale* L.	Canada (Alberta)	C	A
14	30186 (IHAR)	*S. cereale* L.	Poland	C	A
15	31211 (IHAR)	*S. dighoricum* (Vav.) Roshev.	Poland	We	A
16	31083 (IHAR)	*S. dighoricum* (Vav.) Roshev.	Poland	We	A
17	31328 (IHAR)	*S. kuprijanovii* Grossh.	Poland	Wi	P
18	31351 (IHAR)	*S. segetale* (Zhuk.) Roshev.	Russian Federation	We	A
19	CIse 105	*S. segetale* (Zhuk.) Roshev.	Italy	We	A
20	PI 283982 (USDA)	*S. segetale* (Zhuk.) Roshev.	Former USSR	We	A
21	31359 (IHAR)	*S. sylvestre* Host	Poland	Wi	A
22	31366 (IHAR)	*S. turkestanicum* Bensin	Turkey	C	A
23	31367 (IHAR)	*S. vavilovii* Grossh.	Poland	Wi	A
24	PI 573649 (USDA)	*S. vavilovii* Grossh.	Afghanistan	Wi	A
25	PI 383756 (USDA)	*S. montanum* Guss.	Turkey	Wi	P
26	PI 383757 (USDA)	*S. montanum* Guss.	Turkey	Wi	P
27	PI 205222 (USDA)	*S. montanum* Guss.	Turkey	Wi	P
28	PI 401404 (USDA)	*S. montanum* Guss.	Iran	Wi	P
29	PI 440654 (USDA)	*S. montanum* Guss.	Hungary	Wi	P

*Accession numbers followed by existing germplasm bank location.

†Botanical names of accessions that originated from Poland were verified using *Secale* monographs (Hammer et al. 1987; and Kobylyanskyi 1989). Botanical names of accessions that originated from GRIN were based on old names.

‡Wi, wild; We, weedy; A, annual; C, cultivated; and P, perennial.

ers. The *Eco*RI or *Pst*I primers were labeled with ^{33}P prior to amplification. The amplification products were resolved on a 5% polyacrylamide sequencing gel in 1× Tris–borate–EDTA buffer for 2.5–3 h. The gels were dried for 2 h and exposed to X-ray film for 48 h.

Data analysis

Polymorphism was scored manually. DNA fragments located on the same migration position were read as 1 locus and scored 1 for present or 0 for absent. The data matrices for each primer combination were converted into Nei–Li's or Dice similarity matrix and then compared among each other using Mantel's test (Mantel 1967). The data matrices showing good relationships were then combined and used for cluster analysis. To detect potentially weakly supported lineages, 2 different algorithms were used in the data analysis. The 1st approach utilized phenetic analysis involving various similarity coefficients and clustering methods to obtain the shortest tree. Similarity coefficients and clustering methods were tested using similarity of qualitative data (SIMQUAL), sequential agglomerative hierarchical nested (SAHN) clustering routine, and TREE from program NTSYSpc version 2.1 (Rohlf 2000). The unweighted pair group method with arithmetic means (UPGMA), weighted pair group method with arithmetic means (WPGMA), complete-link, and single-link clustering methods were applied in all possible combinations with both the Dice and Jaccard similarity coefficients. Cophenetic correlation coefficients (r) were calculated and compared for each of the combinations using COPH and MXCOMP from NTSYSpc 2.1 procedures. Principal coordinate analysis (PCO) was performed using the procedures DCENTER, EIGEN, and MXPLOT from NTSYSpc 2.1*k*. The 2nd approach involved a phylogenetic analysis using a maximum parsimony method that was performed with the PAUP* (phylogenetic analysis using parsimony) program (Swofford 1998). Bootstrap tests were performed using 5000 replications for phenetic analysis and 1000 replications for phylogenetic analysis to assess confidence in producing tree topologies (Felsenstein 1985). The results from both phenetic and phylogenetic analyses were compared to determine the best phylogenetic relationship among the species. Fisher's exact test was used to test the correlation between life cycle

Table 2. Primer combination, total bands, polymorphic bands, level of polymorphisms, and average of *r* value with respect to the genus *Secale*.

Primer combination	Total bands	Polymorphic bands	Polymorphism (%)	Average of *r* value*
P-ACA/M-AAC	86	73	85	0.80
P-ACA/M-ACT	42	32	76	0.65
P-ACA/M-ATC	77	68	88	0.74
P-ACA/M-CCA	63	59	94	0.77
P-ACA/M-CCT	38	31	82	0.69
P-AGT/M-AAC	53	36	68	0.79
P-AGT/M-ACG	59	48	81	0.71
P-AGT/M-AGA	67	46	69	0.79
P-AGT/M-ATC	67	36	54	0.70
P-AGT/M-ATG	92	60	65	0.77
P-AGT/M-CCT	57	43	75	0.79
Average	64	48	76	—
E-ACA/M-ACG	54	33	61	0.72
E-AGC/M-ACG	64	39	61	0.73
E-CCT/M-CCA	75	48	64	0.80
E-CCT/M-CAC	72	40	56	0.75
E-CCT/M-CAG	51	33	65	0.67
E-CCT/M-CCT	63	34	56	0.77
E-CCT/M-CTG	50	30	60	0.60
Average	61	38	60	—
Total	1130	789	—	—

**r* value from Mantel's test.

character and AFLP data (Fisher 1970). Genetic structure of the *Secale* genus was analyzed using Wright's *F* test (Hartl and Clark 1997).

Results

Eighteen primer combinations were utilized in analyzing phylogenetic relationships among the various *Secale* species (Table 2). Amplified fragment length polymorphism analysis revealed a very large distinct number of fragments per primer pair in the members of the genus *Secale* (Fig. 1). The number of fragments present varied with each primer and ranged from 38 to 86. Each primer combination demonstrated approximately 54%–94% polymorphism. In total, 789 polymorphic markers of 1130 fragments were observed. Primer combinations P-/M- and E-/M- generally yielded similar numbers of fragments, 64 and 61 bands, respectively; however, P-/M- primer combinations produced a higher level of polymorphism (76%) than the E-/M- primer combinations (60%). The most efficient primer combination, which showed the largest number of polymorphic fragments, was the P-ACA/M-AAC (Table 2).

The Mantel test was performed to establish the correlation among primer combinations before constructing phenetic and phylogenetic trees. The correlation values ranged from 0.60 to 0.80 (Table 2) and were significant in all primer combinations. The 789 polymorphic markers resulting from the 18 primer combinations were then used to construct phenetic and phylogenetic trees.

Cophenetic correlation coefficients measured the goodness of fit of the cluster analysis to the similarity matrix, and the results showed that cophenetic correlation coefficients were >0.90 (Table 3) indicating that the goodness of fit for all combinations of the similarity matrix and cluster analysis were very good. Therefore, all those combinations were appropriate for use in analyzing the phenetic relationships. Among the cluster methods, UPGMA yielded the highest cophenetic correlation in all cases. The combinations of UPGMA with the Dice and Jaccard coefficients also yielded identical tree topologies. Therefore, they were considered the most suitable combinations for data analysis.

The phenogram and the cladogram, from combinations of UPGMA and Dice similarity, had similar tree topologies (Figs. 2 and 3). In both cluster analyses, *S. sylvestre* was first separated (100% support), followed by *Secale ciliatoglume* (70% and 62% support in dendrogram and cladogram, respectively). The rest of the accessions were clustered into 2 major groups. Group III, *S. montanum, Secale anatolicum*, *S. kuprijanovii, Secale chaldicum,* and *S. africanum*, consisted of all perennial taxa (42% and 72% support in dendrogram and cladogram, respectively). Group II, *S. ancestrale,* S. *afghanicum*, *S. cereale, S. dighoricum, Secale turkestanicum*, S. *segetale*, and *Secale vavilovii*, consisted of annual taxa (91% and 37% support in dendrogram and cladogram, respectively).

The results of PCO analysis showed that the first 2 axes accounted for 16.38% and 7.24% of the data variance (Fig. 4). Cumulatively, the PCO results represented 24% of the data, which were sufficient to resolve all the analyzed accessions into 3 distinct groups. Group 1 contained *S. sylvestre* and the 2 outgroups, group II contained the annual taxa, and group III contained the perennial taxa. The

Fig. 1. An example of the AFLP polymorphism among *Secale* species, detected with the *Pst*I-ACA and *Mse*I-AAC primer combination.

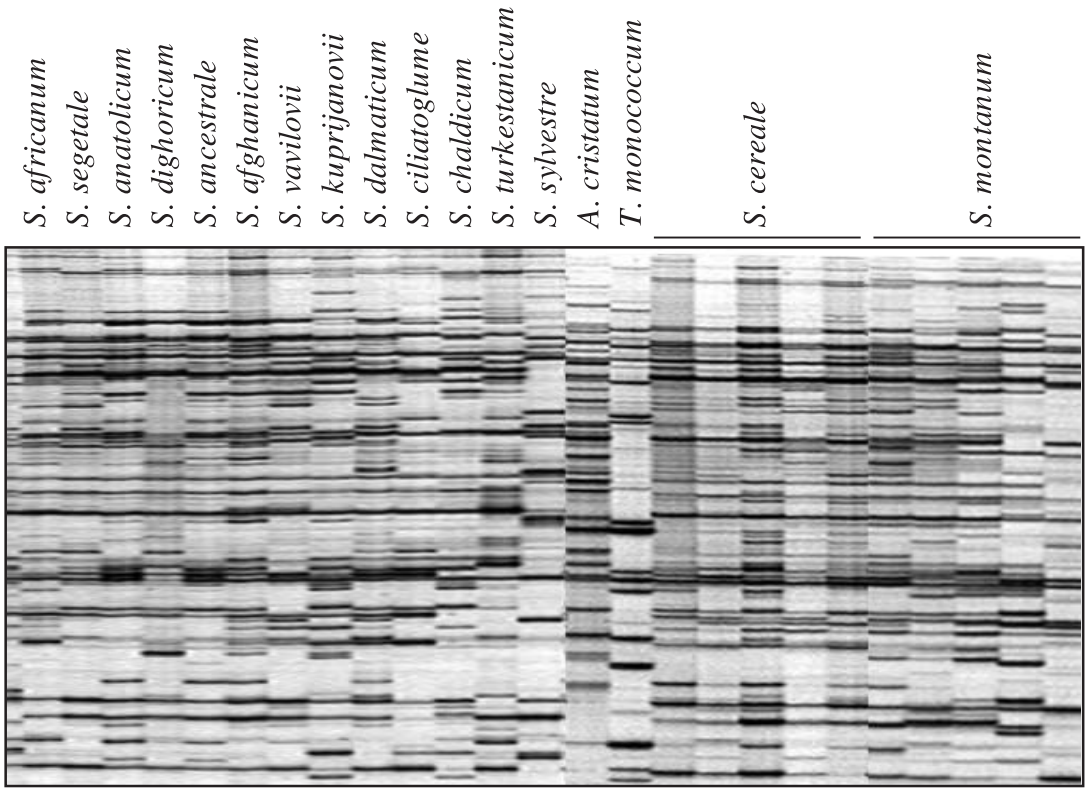


Table 3. Cophenetic correlation coefficients for amplified fragment length polymorphism data of all *Secale* species used. UPGMA, unweighted pair group method with arithmetic means; WPGMA, weighted pair group method with arithmetic means.

Clustering/Similarity	Jaccard	Dice
UPGMA	0.98	0.98
WPGMA	0.96	0.96
Complete-link	0.95	0.96
Single-link	0.97	0.97

PCO results were congruent with the results of cluster analysis.

Genetic differentiation among annual and perennial taxa was analyzed using the Wright's *F* test (Table 4). Since *S. sylvestre* showed really distinct characters, this taxon was excluded from the analysis. The genetic differentiation level of annual taxa was smaller than that of perennial taxa; however, the genetic diversity within the annual taxa was much higher than that of perennial taxa.

Discussion

Phylogenetic relationships among *Secale* species

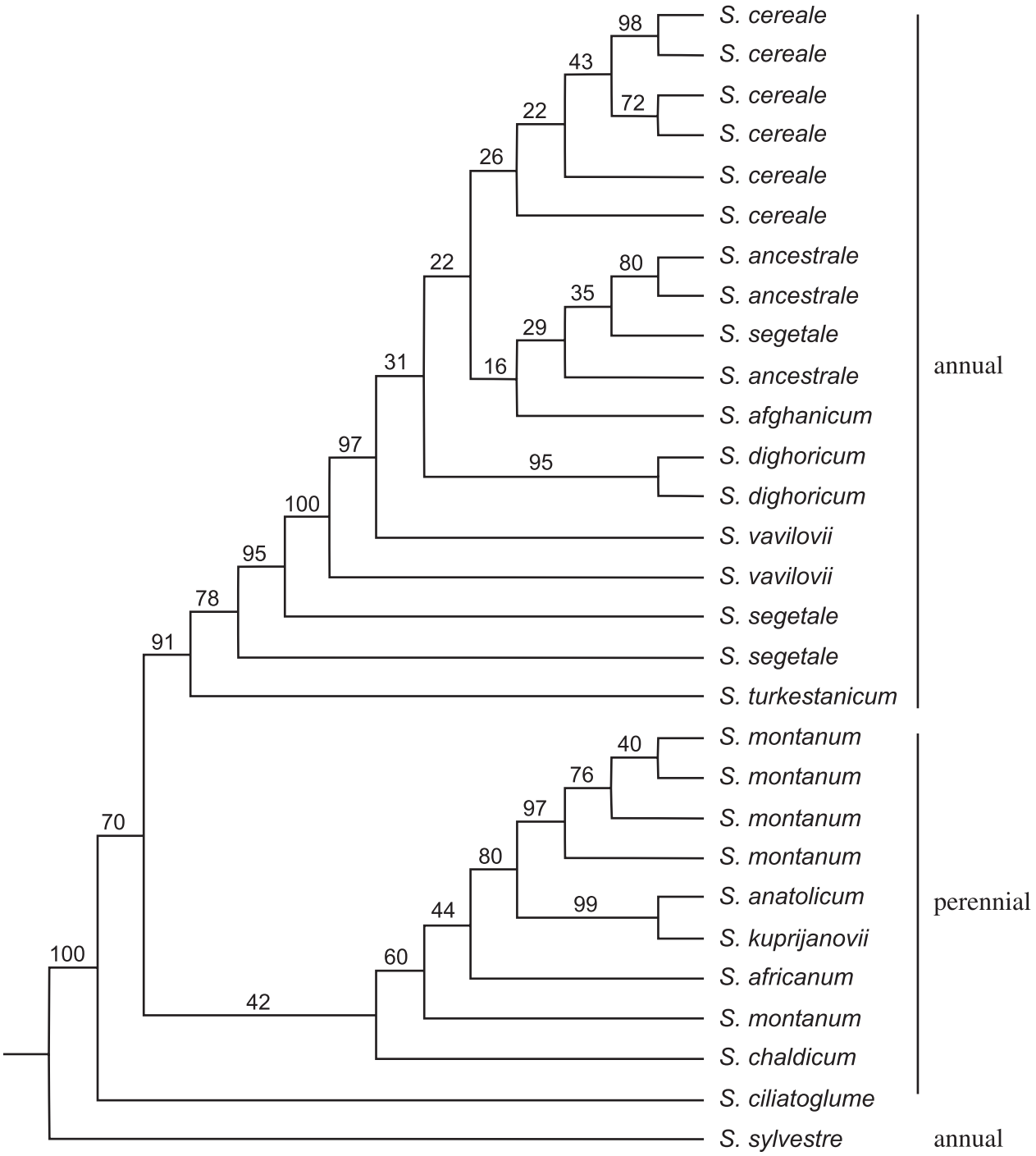
The AFLP technique produced a wide range of variability among *Secale* taxa that was sufficient to clearly resolve all analyzed accessions into 3 major groups: group 1 contained of *S. sylvestre*, group II contained all of the perennial taxa, and group III contained all of the annual taxa. This indicated that the perennial versus annual life cycle probably played an important role in determining the relationships among the *Secale* species. Further analysis using Fisher's exact test supported this by showing that 24% of the AFLPs detected were associated with the character life cycle. Our results supported the validity of 3 major series within *Secale* as recognized by Roshevitz (1947). *Secale montanum* and all perennial forms constituted 1 major group, the series *Kuprijanovia* Roshev.; *S. cereale* and all weedy annual relatives constituted the series *Cerealia* Roshev.; and *S. sylvestre* stood alone as an annual and constituted the series *Silvestria* Roshev.

Among the annual taxa, *S. cereale* had a closer relationship to *S. ancestrale, S. afghanicum, S. dighoricum,* and *S. segetale* than to *S. vavilovii*. Even though both *S. turkestanicum* and *S. cereale* are cultivated plants, they had a distant relationship with each other. The breeding system differences between the 2 taxa (*S. turkestanicum* is self-pollinated and *S. cereale* is cross-pollinated) may offer a possible explanation.

Among perennial taxa, *S. ciliatoglume* showed the most distant relationship from the others. *Secale ciliatoglume* is an isolated weedy population with pubescent culms that appear to be endemic to orchards and vineyards near Mardin, Turkey. It is possible that the very limited distribution of this taxon allowed it to maintain a distinct identity from the others. Within group II, *S. africanum* had the furthest relationship from *S. montanum*, whereas *S. anatolicum* and *S. kuprijanovii* had the closest relationship to *S. montanum*. Somewhat surprisingly, *S. sylvestre* was closer to the perennial taxa than to the annual taxa. Since *S. sylvestre* had the closest relationship to the outgroups, it can be considered as the most ancient among *Secale* species, and *S. cereale* can be considered as the youngest of the *Secale* species.

Based on cytological, ecological, and morphological

Fig. 2. A dendrogram constructed from the AFLP data, using Nei–Li's distance and UPGMA clustering. Numbers on the branches are bootstrap and range from 16% to 100%.

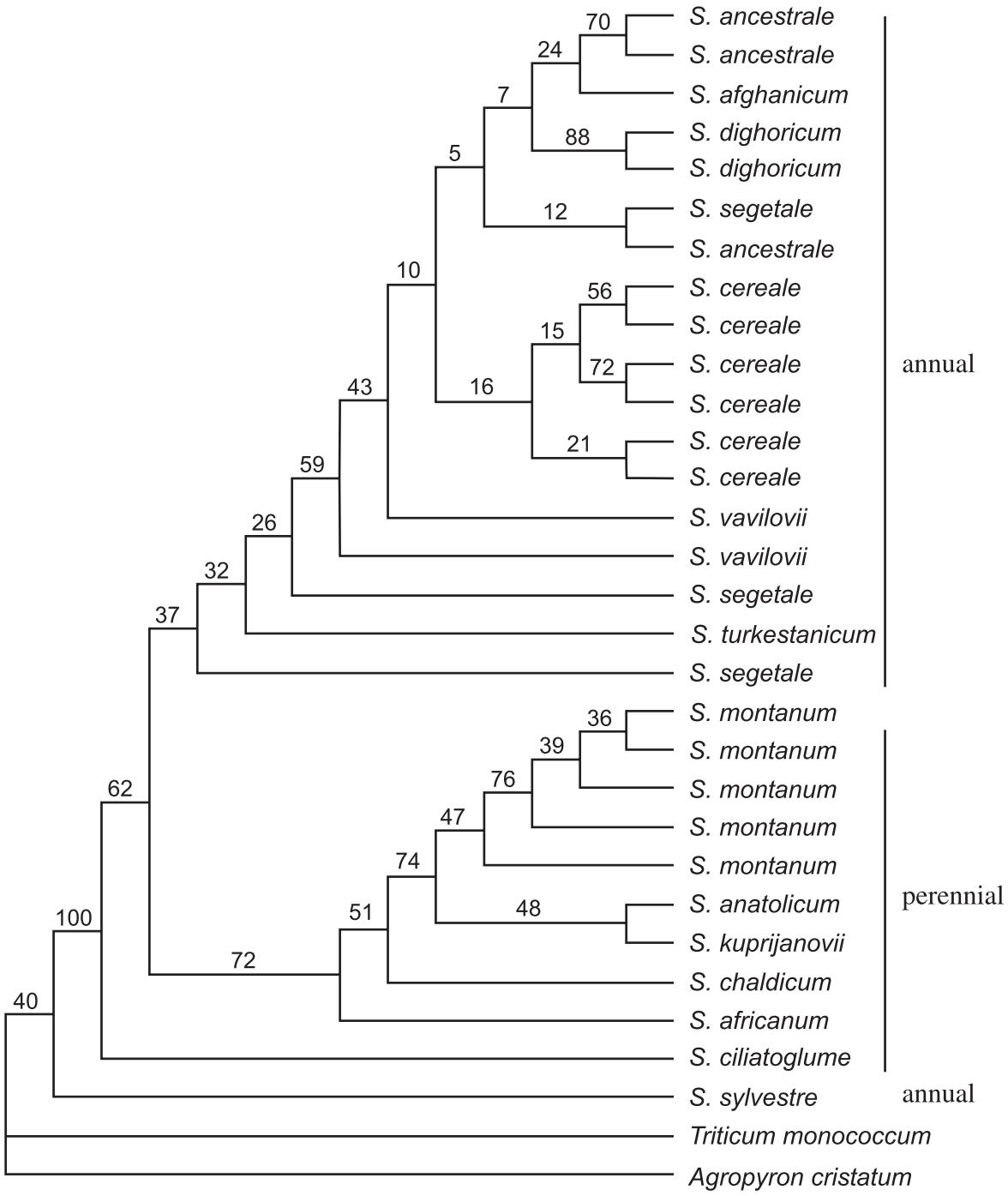


studies, Stutz (1972) demonstrated that cultivated rye (*S. cereale* L.) originated from the weedy progeny derived from introgression of *S. montanum* (syn. *S. strictum*) into *S. vavilovii*, *S. africanum, Secale dalmaticum, S. ciliatoglume*, and *S. kuprijanovii* as they had close relationships with each other and appeared to be only slightly modified isolated populations of *S. montanum*. Populations of *S. anatolicum* were thought to be weedy forms of *S. montanum*, genetically and chromosomally distinct from the weedy annual forms. In general, the species relationships within genus *Secale* as based on AFLP data were consistent with Stutz (1972). However, Stutz (1972) also suggested that *S. montanum* was the common ancestor of all the *Secale* species, which conflicts with the present AFLP data. The present data clearly demonstrated that *S. sylvestre* was the most ancient species that split off first from the common ancestor, whereas *S. montanum* split off after the separation of *S. sylvestre*.

Molecular taxonomy of *Secale* based on AFLP

Secale sylvestre is a low growing plant with a fragile

Fig. 3. A cladogram resulting from the maximum parsimony method that was conducted using heuristic search methods with TBR branch swapping, collapse of zero-options, and weighting of all characters equally. Numbers on branches are bootstrap values and range from 5% to 100%. Consistency index (CI) = 0.18, retention index (RI) = 0.35, rescale consistency index (RCI) = 0.06.



rachis, widely distributed from central Hungary eastward throughout the sandy steppes of southern Russia. This taxon can be easily distinguished from other taxa by its long awned glumes (Stutz 1972). Khush and Stebbins (1961) showed that *S. sylvestre* was cytogenetically very distant from *S. cereale*, and was geographically, ecologically, and reproductively isolated from *S. montanum* (Sencer and Hawkes 1980). In addition, *S. sylvestre* also has other unique characteristics, such as distinct chloroplast DNA (Petersen and Doebley 1993), a spacer length variant of the ribosomal DNA (Reddy et al. 1990), and an internal transcribed spacer of the 18S–5.8S–26S rDNA (ITS-1) region, as compared with the other *Secale* species. Given the strong distinction of *S. sylvestre* from other taxa, it is easy to consider *S. sylvestre* as a distinct species. Amplified fragment length polymorphism analysis showed that *S. sylvestre* also demonstrated a

Fig. 4. Principal coordinate analysis of *Secale* species based on AFLPs. I = *S. sylvestre* and 2 outgroups; II = annual taxa; and III = perennial taxa.

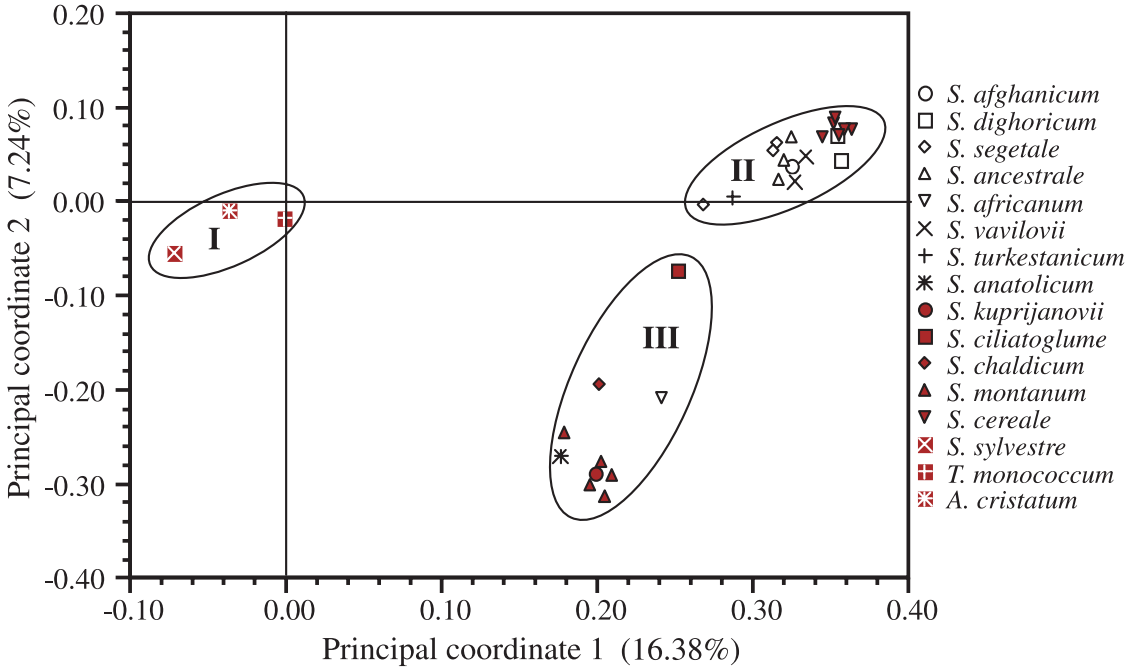


Table 4. Total genetic diversity, genetic diversity within population, genetic differentiation and genetic identity of *Secale* accessions. Ht, total genetic diversity; Hs, genetic diversity within population; Gst, genetic differentiation; GI, genetic identity.

Groups	Ht	Hs	Gst	GI
Annual taxa	0.25±0.03	0.09±0.0062	0.63	0.68–0.89
Perennial taxa	0.30±0.03	0.02±0.0009	0.90	0.24–0.48

very distinct profile in all primer combinations, and it was well separated from others in all analyses. Thus, AFLP analyses confirm *S. sylvestre* as a distinct species.

The presence of a high degree of similarity among wild, weedy annual forms and cultivated rye has been demonstrated. Khush (1963) showed that there was no evidence of structural differences between the genome of cultivated rye and several weedy ryes (*S. cereale, S. vavilovii, S. ancestrale, S. afghanicum, S. dighoricum,* and *S. segetale*), which had previously been recognized as varieties, subspecies, or even species. They all readily crossed and produced vigorous F_1s, which had similar chromosome arrangements, breeding habit, periodicity, and crossability, and they also demonstrated geographical continuity. Therefore, Khush (1963) proposed all annual forms are subspecies of *S. cereale*. Further study based on morphometrical analyses concluded that it was impossible to recognize each annual taxon based on their morphology (Frederiksen and Petersen 1997). They proposed 2 intraspecific taxa within a single species (*S. cereale*), which are *S. cereale* subsp. *cereale* for cultivated rye and *S. cereale* subsp. *ancestrale* for weedy and wild annual rye taxa. The most recent study based on ITS-1 region also found no differences between the weedy forms and cultivated rye (Bustos and Jouve 2002). Thus, all previous studies demonstrated that morphologically and genetically all annual taxa were similar to each other and that it was impossible to discriminate them clearly.

The AFLP results showed that 6 accessions of *S. cereale*, which originated from different locations, composed a monophyletic group. The *S. dighoricum* accessions also clustered together, which was not too surprising since they both originated from the same location. Thus, it is highly possible that those accessions are duplicated accessions, whereas other annual taxa that were represented by more than 1 accession did not cluster together. Principal coordinate analyses intermingled them with each other. Nei–Li's distances yielded very small genetic-distance values among them indicating they had a high degree of AFLP similarity. This was also shown from the intermediate level of genetic differentiation (Gst = 0.63). Except for cultivated rye, it was still difficult to discriminate between wild and weedy rye using AFLPs, therefore, AFLP analysis supported Frederiksen and Petersen (1997).

Among perennial species, only *S. ciliatoglume* did not cluster together with the others. *Secale ciliatoglume* stood alone between annual and perennial taxa in cluster analyses, but the separation was intermediately supported (62% in phylogenetic and 70% in phenetic analysis). The PCO analysis placed this accession in the same quadrant with the other perennial taxa. The information about *S. ciliatoglume* from previous studies was very limited, and only dealt with morphological data. This taxon has been shown to be morphologically similar to *S. montanum*, and only deviated by having a dense cover of hairs over the internodes, leaf sheaths, and blades (Frederiksen and Petersen 1998). Frederiksen and Petersen (1997) suggested that *S. ciliatoglume* should be given an intraspecific rank.

Stutz (1972) demonstrated that several perennial forms (*S. anatolicum*, *S. africanum*, *S. dalmaticum*, and *S. mon-*

tanum) readily crossed to each other, and that crossing among them yielded normal chromosome configurations indicating no reproductive barrier. Khush (1962) proposed all perennial taxa as subspecies of *S. montanum*. Furthermore, Sencer and Hawkes (1980) showed that all the wild perennial forms had a fairly common morphological resemblance. Our analysis, based on AFLPs, clustered the 5 accessions of *S. montanum* together with low bootstrap support (47% in cladogram). The separation of the other perennial taxa was with intermediate bootstrap support (47%–74% in cladogram). However, the genetic differentiation level among perennial taxa was very high (Gst = 0.90). This result suggests that AFLP marker polymorphism levels within the perennial taxa were sufficient to discriminate and place them in an intraspecific rank, instead of in an interspecific rank.

In summary, this is the first phylogenetic study of the genus *Secale* based on AFLP analyses. Analyzing AFLPs among 29 accessions representing 14 of the most recognized ranked species or subspecies in the genus *Secale* demonstrated that AFLP marker technology is a better tool for analyzing phylogenetic relationships among *Secale* species, because it produced high levels of polymorphism that were sufficient to resolve all accessions into 3 distinct groups. Group I contained *S. sylvestre*, group II contained all perennial taxa, and group III contained all annual taxa. (*Secale ciliatoglume* stood alone between annual and perennial taxa in cluster analyses.) The phylogenetic relationships among *Secale* taxa based on AFLPs strongly supported Stutz (1972). The AFLP data were clearly able to distinguish 3 *Secale* species: *S. sylvestre*, *S. cereale, and S. montanum*. In addition, AFLPs were also able to recognize 2 intraspecific taxa of *S. cereale*, namely *S. cereale* subsp. *cereale* for cultivated rye and *S. cereale* subsp. *ancestrale* for wild and weedy rye annual taxa, and 6 intraspecific taxa of *S. montanum*, namely *S. montanum* subsp. *montanum*; *S. montanum* subsp. *africanum*; *S. montanum* subsp. *anatolicum*; *S. montanum* subsp *chaldicum*; *S. montanum* subsp. *ciliatoglume*; and *S. montanum* subsp. *kuprijanovii*. These results were consistent with the last revision of the genus *Secale* by Frederiksen and Petersen (1998) who recognized only 3 species within the genus *Secale*: *S. sylvestre, S. strictum* (syn. S. *montanum*), and *S. cereale*.

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