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Research Article

Genetic diversity and phylogenetic relationships in the rye genus *Secale* L. (rye) based on *Secale cereale* microsatellite markers

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Abstract

The genetic diversity and phylogenetic relationships in the genus *Secale* L. (rye) was evaluated using 24 *Secale cereale* microsatellite (SCM) markers. The average polymorphism information content (PIC) value of each microsatellite locus in 30 *Secale* accessions evaluated was higher than that in 47 cultivated ryes (*Secale cereale* ssp. *cereale*). The mean genetic similarity (GS) index in *Secale* was lower than that in cultivated rye. The highest within-species GS index was observed for *S. sylvestre* and the lowest for *S. strictum*, whereas the highest between-species GS index was found between *S. cereale* and *S. vavilovii* and the lowest between *S. sylvestre* and *S. cereale*. There was no obvious difference in *GS* levels in the cultivated rye accessions from Asia, Europe, North America or South America. Cluster analysis indicated that all the *Secale* accessions could be distinguished by the 24 microsatellite loci. We also found that the *S. sylvestre* accessions were obviously divergent from the accessions of other species and that the *S. vavilovii* accessions were closely related to the *S. cereale* accessions. Our results also showed that *S. strictum* was heterogeneous and showed great within-species differences. The microsatellite-derived dendrogram faithfully reflected the phylogenetic relationships between *Secale* species but did not indicate a possible domestication process of the cultivated rye based on the geographical sources of the accessions.

Key words: cultivated rye, genetic diversity, microsatellite markers, phylogenetic relationships, *Secale* L.

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Introduction

The taxonomy of the genus *Secale* (rye) has for a long time been a matter of disagreement, despite the large number of studies performed. Historically, about 15 different species have been accepted (Roshevitz 1947, Delipavlov 1962), while Frederiksen and Petersen (1998) recognized only three *Secale* species, *i.e. S. sylvestre*, *S. strictum* (plus the subspecies ssp. *strictum* and ssp. *africanum* and the varieties var. *strictum* and var. *ciliatoglume*) and *S. cereale* (plus ssp. *cereale* and ssp. *ancestrale*). According to taxonomic systems adopted by the American Germplasm Resources Information Network (GRIN, http://www.ars-grin.gov), the genus *Secale* is presently recognized as containing four species, consisting of the annual outbreeder *S. cereale* L. , the annual autogamous *S. sylvestre* Host and *S. vavilovii* Grossh., and the perennial outbreeder *S. strictum* (Presl.) Presl. (syn. *S. montanum*) (Sencers and Hawkes 1980, De Bustos and Jouve 2002). There are 8 subspecies in *S. cereale* and 5 in *S. strictum*, and *S. cereale* ssp. *cereale* is the only cultivated rye.

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The different recent classifications of the genus *Secale* have involved phylogenetic analyses based on the use of various technologies, including some biochemical and molecular markers such as isozymes (Vences *et al*. 1987a,b), random amplified polymorphic (RAPDs) (Del Pozo *et al*. 1995), rDNA spacer-length (Reddy *et al*. 1990, Cuadrado and Jouve 2002) and internal transcribed spacers (ITS) (De Bustos and Jouve 2002) as well as traditional morphological and cytogenetical methods (De Bustos and Jouve 2002). Simple sequence repeats (SSRs or microsatellites) consisting of tandem repeats of 2 to 6 nucleotides are abundantly distributed throughout the nuclear genomes of all studied plant species (Tautz *et al*. 1986, 1989, Litt and Luty 1989). Because of their co-dominant inheritance, high polymorphism, good reproducibility and the convenience of the polymerase chain reaction (PCR) microsatellites have become the genetic markers of choice for studies involving plant species (Powell *et al*. 1996, Zhebentyayeva *et al*. 2003). Takezake and Nei (1996) concluded that microsatellite DNA seemed to be very useful for clarifying the

evolutionary relationships of closely related populations. However, the application of microsatellite markers to plant taxonomy is still very limited because the development of specific SSR primers is time-consuming and expensive plus the fact that genomic microsatellite markers are hard to transfer between the genomes of different species. Fortunately, at least 184 *S. cereale* microsatellite (SCM) markers have recently been developed (Saal and Wricke 1999, Hackauf and Wehling 2002).

The objectives of the study described in the present paper were to investigate the phylogenetic relationship of four *Secale* species using microsatellite markers and to evaluate the genetic diversity and genetic relationships of cultivated rye (*S. cereale* ssp. *cereale*) from different countries.

Materials and Methods

Plant materials

For this study we selected 30 *Secale* accessions, consisting of 5 cultivated rye and 25 non-cultivated rye, to represent the 4 species and 10 subspecies of the rye genus (Table 1). Meantime we used 47 cultivated rye (*S. cereale* ssp. *cereale*) accessions from different countries (Table 1). Accession CN31389 was kindly provided by Dr. D. Kessler of Plant Gene Resources of Canada (PGRC). Accession NGB5073 was kindly provided by Dr. L. Bondo at Nordic Gene Bank, Sweden. Accessions R953/90 and R955/90 were kindly provided by Dr. A. Graner at the Genebank Gatersleben, Institute of Plant Genetic and Crop Plant Research (IPK). Accessions As3045 and As3033 came from the Triticeae Research Institute, Sichuan Agriculture University (TRISAU). Most of the accessions with accession numbers starting with PI were kindly provided by Dr. H. Bockelman of the American Germplasm Resources Information Network (GRIN).

DNA isolation and PCR amplification

Genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) procedure of Doyle and Doyle (1987). The samples for each accession consisted of DNA bulked from 25-30 individual plants.

We used 24 microsatellite markers (Table 2), consisting of 13 markers described by Saal and Wricke (1999) and 11 markers described by Hackauf and Wehling (2002). All the primers were synthesized by Shenergy Biocolor, Biological Science and Technology Co., China. The final reaction volume was 25 μL, containing approximately 50-100 ng template DNA, 1 unit of Takara rTaq polymerase (Takara Bio, Inc., Kyoto, Japan), 0.2 μM of each primer, 200 μM of each deoxynucleotide triphosphates (dNTP) (Takara Bio, Inc., Japan), 1.5 mM $MgCl_2$, and 1xPCR buffer. The PCR amplifications were carried out in a PTC-240 thermocycler (Genetic Technologies, MJ Research, USA) under the conditions described by Saal and Wricke (1999) and Hackauf and Wehling (2002) with minor modification. The PCR amplification products were separated on a 6% (w/v) denatured polyacrylamide gel and visualized by silver staining.

Data scoring and analysis

Polymorphism information content (PIC) values were calculated for each microsatellite locus according to the formula:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where p_{ij} is the frequency of the jth allele for the ith marker summed over n alleles (Anderson *et al*. 1993). For each genotype x marker combination, the presence (1) or absence (0) of a microsatellite allele was treated as an independent character. The data matrix was then used to calculate the genetic similarity (GS) index (Nei and Li 1979) as $GS = 2N_{ij}/(N_i + N_j)$, where N_{ij} is the number of microsatellite alleles common to genotypes i and j, while N_i and N_j are the total numbers of microsatellite alleles observed for genotypes i and j, respectively. Genetic relationships among *Secale* accessions were estimated using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis of the *GS* matrix (Rohlf, 2000)

Results

Five cultivated rye (*S. cereale* ssp. *cereale*) accessions and 25 accessions of other *Secale* species or subspecies were selected to detect the genetic variations and relationships within and between *Secale* species. For the 30 accessions selected a total of 113 microsatellite alleles were amplified, of which 107 (94.7%) were polymorphic with an average of 4.7 alleles per locus and a range of from 2 to 8. The average PIC value of the 13 markers from Saal and Wricke (1999) was 0.589 while that of the 11 markers from Hackauf and Wehling (2002) was 0.620. The average PIC value of the 24 microsatellite loci was 0.604 and ranged from 0.315 to 0.799. In these accessions the highest PIC value was for the SCM101 marker. These results indicated a high level of the microsatellite polymorphism in the 30 accessions

The genetic similarity and range of variation within and between species were calculated based on the *GS* matrix (Table 3). The GS index for the 30 accessions ranged from 0.326 to 0.932 with a mean of 0.633. The highest genetic similarity occurred between *S. sylvestre* accessions R953/90 and R955/90 while the lowest was between *S. segetale* accession PI61867 and *S. sylvestre* accession CN31389. The highest within-species *GS* index (0.884) was for *S. sylvestre* and the lowest (0.649) was for *S. strictum*. The lowest between-species *GS* index (0.444) was for *S. sylvestre* and *S. cereale*, and the *GS* indices be-

Table 1 - The 72 *Secale* accessions used in this study.

Secale accessions (n = 72)					
Cultivated rye (n = 47)			Non-cultivated rye (n = 25)		
Taxon and accession number	Country, State	Continent	Taxon and accession number	Country, State	Continent
S. cereale ssp. *cereale*			*S. cereale* ssp. *afghanicum*		
CIse 20	Sweden, Malmohus	Europe	PI618662	Armenia	Asia
CIse 26	Canada, Saskatchewan	North America			
CIse 35	United States	North America	*S. cereale* ssp. *ancestrale*		
CIse 79	Australia	Oceania	PI618663	Turkey	Asia
PI168199**	Turkey, Isparta	Asia	PI618666	Turkey	Asia
PI221478	Afghanistan, Bamian	Asia			
PI240676	Argentina, Pico	South America	*S. cereale* ssp. *dighoricum*		
PI254806	Austria, Lower Austria	Europe	PI618667	Sweden	Europe
PI260055	Ukraine, Kharkiv	Europe	PI618668	Russian Federation	Europe
PI272333	Hungary, Heves	Europe			
PI280839	Russian Federation, Kirov	Europe	*S. cereale* ssp. *rigidum*		
PI289827	Pakistan	Asia	PI618669	Turkey	Asia
PI290420	Hungary, Pest	Europe			
PI290423	Slovakia, West Slovakia	Europe	*S. cereale* ssp. *segetale*		
PI306488	Romania	Europe	PI618671	Turkey	Asia
PI323449	Poland, Lublin	Europe	PI618673	Turkey	Asia
PI330422	Poland	Europe			
PI344983	Yugoslavia, Montenegro	Europe	*S. cereale* ssp. *tetraploidum*		
PI345001	Macedonia	Europe	PI573647	Ukraine, Kharkiv	Europe
PI346416**	Australia	Oceania			
PI362399	Yugoslavia, Montenegro	Europe	*S. vavilovii*		
PI372116	Belarus, Minsk	Europe	PI573649	Afghanistan	Asia
PI372117	Ukraine, Chernihiv	Europe	PI618678	Armenia	Asia
PI372118	Russian Federation, Leningrad	Europe	PI618680	Turkey	Asia
PI372119	Belarus	Europe			
PI410534	Pakistan, Azad Kashmir	Asia	*S. sylvestre*		
PI410800	Poland	Europe	PI592294	Ukraine, Mykolayiv	Europe
PI412949**	South Africa, Cape	Africa	NGB5073	Denmark	Europe
PI414080	United Kingdom, England	Europe	R953/90	Denmark	Europe
PI430003	India, Himachal Pradesh	Asia	R955/90	Denmark	Europe
PI430004	India, Himachal Pradesh	Asia	CN31389	Germany	Europe
PI436188	Chile, Los Lagos	South America	AS3045		
PI436192	Chile, Los Lagos	South America			
PI446020	Japan	Asia	*S. strictum*		
PI446025	Mexico	North America	PI401400	Iran, Kordestan	Asia
PI446366	Bulgaria	Europe	PI531829	Armenia	Asia
PI446379	Ukraine, Lviv	Europe	PI568257	Russian Federation	Europe
PI446433	Bulgaria	Europe			
PI447337**	China, Xinjiang	Asia	*S. strictum ssp. africanum*		
PI452132	China, Guizhou	Asia	As3033	South Africa, Calvinia	Africa
PI534981	Japan	Asia			
PI535003	Finland	Europe	*S. strictum* ssp. *anatolicum*		
PI535005	Finland	Europe	PI445973	United States	North America
PI535018	Portugal	Europe	PI445974	Canada, Manitoba	North America
PI542468	Mexico, Sonora	North America			
PI573634	Armenia, Syunik	Asia	*S. strictum* ssp. k*uprijanovii*		
PI602997**	United States, Georgia	North America	PI326282	Russian Federation, Krasnodar	Europe

**Means the representative accessions selected for the subspecies *S. cereale* ssp. *cereale*.

Table 2 - Repeat motif, number and size range of alleles, polymorphic information content (PIC) value and chromosome location of the *Secale* microsatellite markers used in this study. The markers came from the studies of Saal and Wricke (1999) and Hackauf and Wehling (2002).

Marker*	Repeat motif	Observed alleles	Size range (bp)	PIC in cultivated rye	PIC in *Secale*	Chromosome location
Saal and Wricke (1999)						
SCM2	$(GT)_{10}$	6	110-147	0.146	0.507	6RL
SCM5	$(GA)_{16}$	7	190-331	0.797	0.789	3RL
SCM9	$(GT)_{8}$	5	190-242	0.506	0.606	1RS
SCM39	$(GT)_{8}(GC)_{6}(GT)_{53}$	3	190-331	0.427	0.599	1R
SCM43	$(GT)_{11}$	4	67-147	0.156	0.616	2R
SCM86	$(GT)_{20}$	3	110-147	0.449	0.638	7R
SCM101	$(CT)_{18}$	8	147-190	0.647	0.799	6R
SCM120	$(AC)_{10}$	5	110-147	0.580	0.556	5RL
SCM138	$(AC)_{23}$	6	110-190	0.434	0.681	5RS
SCM180	$(GT)_{6}(GA)_{7}$	2	110-190	0.000	0.358	6RL
SCM304	$(GA)_{36}$	3	190-331	0.000	0.315	6RS
WMS6	$(GA)_{40}$	4	110-190	0.358	0.700	5RL
WMS44	$(GA)_{28}$	2	67-147	0.473	0.498	6R
Hackauf and Wehling (2002)						
SCM63	$(CCG)_{5}$	6	110-190	0.521	0.521	Unknown
SCM66	$(CCG)_{7}$	5	147-190	0.506	0.680	Unknown
SCM92	$(GCG)_{6}$	4	147-190	0.698	0.633	Unknown
SCM98	$(CTG)_{5}$	6	110-147	0.688	0.727	Unknown
SCM116	$(TAT)_{7}$	3	110-190	0.489	0.464	Unknown
SCM126	$(AACC)_{4}$	5	110-190	0.780	0.744	Unknown
SCM152	$(AG)_{7}$	8	190-331	0.558	0.558	Unknown
SCM153	$(AT)_{9}$	6	147-190	0.764	0.779	Unknown
SCM164	$(CCT)_{5}$	7	67-190	0.496	0.700	Unknown
SCM166	$(CCT)_{5}$	6	190-331	0.611	0.649	Unknown
SCM169	$(CGG)_{7}$	4	147-190	0.230	0.368	Unknown

*Grouped by citing author (see reference list).

Table 3 - Genetic similarities (GS) and range of variations within and between *Secale* species.

Species	*S. cereale*	*S. vavilovii*	*S. sylvestre*	*S. strictum*
S. cereale	0.779 (0.696 to 0.885)			
S. vavilovii	0.783 (0.705 to 0.867)	0.812 (0.764 to 0.817)		
S. sylvestre	0.444 (0.326 to 0.543)	0.487 (0.419 to 0.554)	0.884 (0.822 to 0.932)	
S. strictum	0.642 (0.471 to 0.833)	0.662 (0.548 to 0.747)	0.463 (0.375 to 0.533)	0.649 (0.500 to 0.880)

tween *S. sylvestre* and *S. vavilovii* and between *S. sylvestre* and *S. strictum* are also lower. The results indicated that the *S. sylvestre* accessions studied were more divergent from the accessions of the other species. The *GS* index between *S. vavilovii* and *S. cereale* was the highest at 0.783, indicating that *S. vavilovii* was closely related to *S. cereale*.

The genetic relationships in *Secale* as estimated using UPGMA cluster analysis of the genetic distance 1-*GS* matrix are shown in Figure 1. All 30 accessions could be distinguished by 24 microsatellite markers. The six *S. sylvestre* accessions were divergent from the other accessions and all closely clustered into group V. The *S. strictum* ssp. *africanum* accession As3033 and *S. strictum* accession PI401400 were divergent from the other *S. strictum* accessions and clustered into group IV, while *S. strictum* accessions (PI531829 and PI568257) were clustered into group III. The *S. strictum* ssp. *kuprijanovii* accession PI326282 and two *S. strictum* ssp. *anatolicum* accessions (PI445973 and PI445974) were clustered into group II. The three *S. vavilovii* accessions and all *S. cereale* subspecies were genetically related and clustered into group I.

For the 47 *S. cereale* ssp. *cereale* (cultivated rye) accessions (Table 2) a total of 82 microsatellite alleles were amplified, of which 69 (84%) were polymorphic with an

average of 3.3 alleles per locus and a range of from 1 to 7. We also found that 22 out of the 24 (91.67%) microsatellite markers used were polymorphic in the 47 cultivated rye accessions studied, the remaining two microsatellite markers (SCM 180 and SCM304) being monomorphic. The average PIC value of the 13 markers from Saal and Wricke (1999) was 0.383 while that of the 11 markers from Hackauf and Wehling (2002) was 0.576. In cultivated rye the average PIC value of the 24 microsatellite loci was 0.471 and ranged from zero for SCM180 and SCM304 to 0.797 for SCM5. These results indicate that the microsatellite polymorphism in the cultivated rye was lower.

The mean GS index for the 47 cultivated rye accessions was 0.773 and ranged from 0.622 to 0.921. The highest GS index (0.921) was observed between the Australian accession PI346416 and accession PI372118 from the Russian Federation, while the lowest (0.622) was between accessions PI542468 from Mexico and PI345001 from Macedonia. The genetic similarity and range of variation between cultivated rye accessions from different continents were calculated based on the GS matrix (Table 4). The *GS* index for the European accessions was the highest (0.794) while that for the North and South American accessions was the lowest (0.731). The highest *GS* value (0.777) was observed between Asia and Europe, and the lowest (0.742) between Europe and North American. Our microsatellite-derived data showed no obvious differences in *GS* index between the cultivated ryes from different continents.

The genetic relationships in cultivated rye (Figure 2) were estimated by UPGMA cluster analysis of the genetic distance (1-*GS*) matrix. Accessions PI323449 and PI330422 from Poland, PI542468 from Mexico and PI240676 from Argentina clustered in group J and were the most divergent from other accessions, although accessions PI345001 from Macedonia, CIse79 from Australia and PI452132 from China each formed a separate cluster (groups I, H and F respectively) and were also divergent from the other groups. Accessions PI430003 from India, PI221478 from Afganistan, PI446025 from Mexico, CIse35 from United States, and PI436192 from Chile were genetically related and clustered into group G, while accession PI168199 from Turkey and PI290420 from Hungary formed group E. Accessions PI446020 and PI534981 from Japan were related to accession PI280839 from the Russian Federation and clustered into group D, while accession PI447337 from China, PI290423 from Slovakia and PI410534 from Pakistan formed group C. Accessions CIse20, PI446366, PI535003 and PI535018 from Europe and accession PI573634 from Asia clustered into group A. And all the remaining accessions were clustered into one group B.

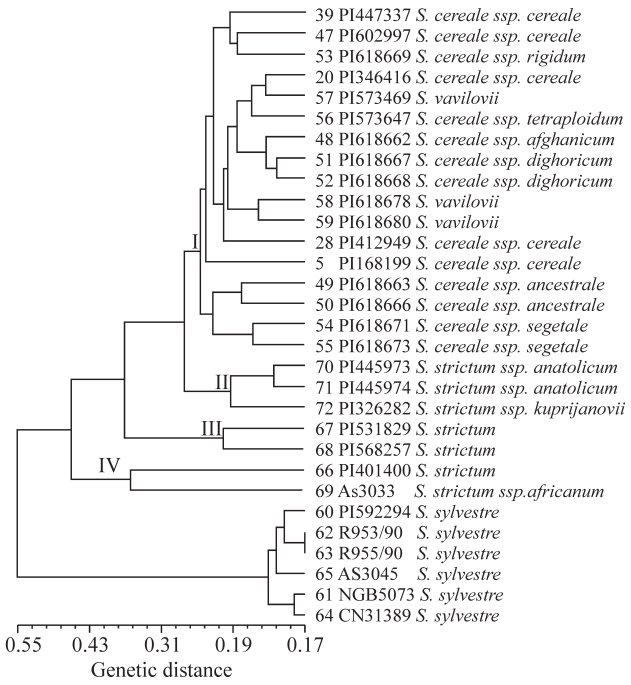

Figure 1 - The genetic relationships in *Secale* species based on microsatellite markers.

Discussion

The average microsatellite marker PIC index was 0.604 for the 30 *Secale* accessions of different species or subspecies, whereas for the cultivated rye accessions the average PIC index was 0.471. The mean *GS* index for the 30 *Secale* accessions of different species or subspecies was 0.633 while for the 47 cultivated rye accessions the mean *GS* index was 0.773. These results suggest that the genetic diversity in the *Secale* as a whole is more extensive than that in cultivated ryes, supporting our previous findings based on RAMP (Random amplified microsatellite polymorphic DNA) markers (Shang *et al*. 2004). For the 13 markers from Saal and Wricke (1999) the average PIC value was 0.589 for 30 *Secale* representatives and 0.383 for cultivated rye, whereas for the 11 markers from Hackauf and Wehling (2002) the average PIC value was 0.620 for 30

Table 4 - Genetic similarities (GS) and range of variations between cultivated rye accessions from different continents.

Continent	Asia	Europe	North America	South America
Asia	0.769 (0.667 to 0.857)			
Europe	0.777 (0.634 to 0.902)	0.794 (0.658 to 0.920)		
North America	0.744 (0.636 to 0.833)	0.742 (0.622 to 0.867)	0.731 (0.683 to 0.775)	
South America	0.759 (0.675 to 0.847)	0.761 (0.629 to 0.871)	0.750 (0.681 to 0.838)	0.731 (0.689 to 0.763)

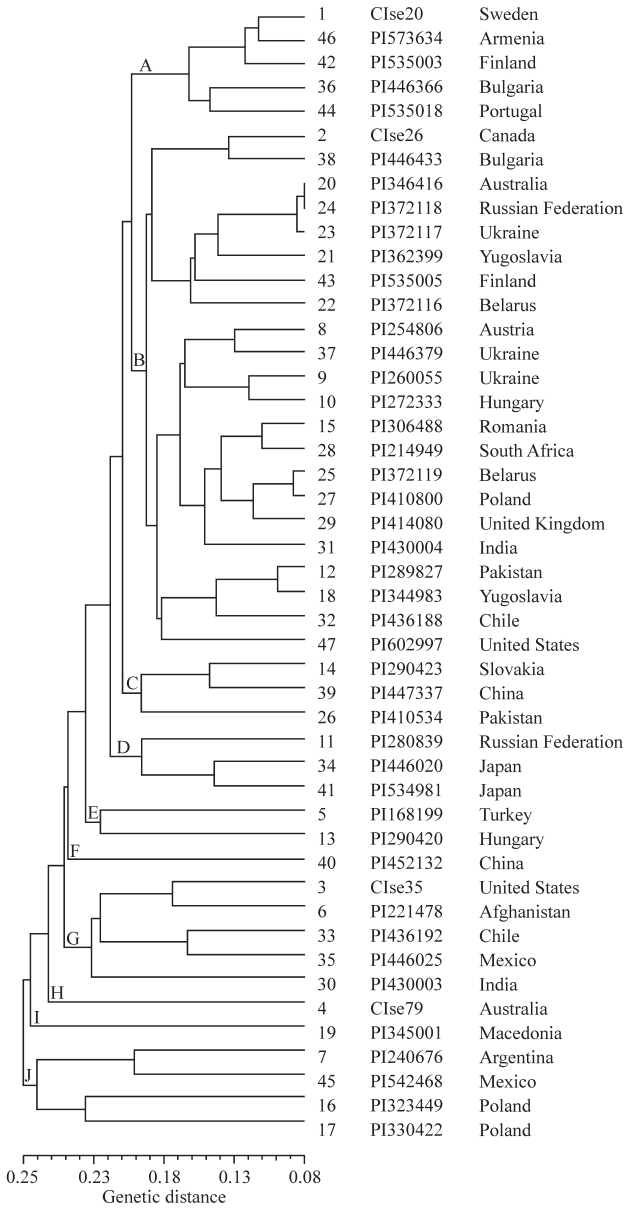

Figure 2 - The genetic relationships of cultivated rye accessions based on microsatellite markers.

Secale representatives and 0.576 for 47 cultivated ryes, respectively. Saal and Wricke (1999) developed the microsatellite markers from a genomic library, whereas the markers developed by Hackauf and Wehling (2002) were derived from expressed sequence tags (EST) sequences. Our results suggest that the EST-derived microsatellite markers were superior to the microsatellite markers from genomic library, and could reveal a relatively higher level of polymorphism in the genus *Secale*, especially in cultivated rye.

The phylogenetic relationships of *Secale* at the species level has been investigated using diverse techniques (Venses *et al*. 1987a, Reddy *et al*. 1990, Del Pozo *et al*. 1995, De Bustos and Jouve 2002). Our study showed that the *S. sylvestre* accessions were very divergent from the accessions of the other species investigated, agreeing with the majority of previous studies (Reddy *et al*. 1990, Del Pozo *et al*. 1995, De Bustos and Jouve 2002). Our *S. strictum* accessions were heterogeneous, with large differences between subspecies. For example, we found that *S. strictum* ssp. *africanum*, once considered to be a separate species (Khush 1962), was indeed clustered in a separate group, while *S. strictum* ssp. *kuprijonovii*, the hypothetical ancestor of the other taxa (Hammer, 1990), was clearly similar to *S. strictum* ssp. *Anotolicum* but divergent from the other *S. strictum* subspecies, supporting the results published by Hammer (1990) and De Bustos and Jouve (2002). Traditionally, *S. vavilovii* has been considered to be a separate species (Khush 1962, Del Pozo *et al*. 1995, Cuadrado and Jouve 1997, De Bustos and Jouve 2002) but we found that it was closely related to *S. cereale*. No obvious differences were found between the *S. cereale* subspecies, supporting the results of *Secale* rDNA ITS analysis reported by De Bustos and Jouve (2002).

It is thought that cultivated rye originated in the Mount Ararat and Lake Van area of eastern Turkey, linguistic evidence suggesting that the introduction of cultivated rye to southern and western Europe and Central Asia were independent of each other (Sencer and Hawkes 1980). Khush (1962) concluded that cultivated rye probably entered Europe by two routes, one being through the northern Caucuses and the other through central Asia. Bushuk (1976) proposed that cultivated rye was probably distributed from south-western Asia to Russia, and thence into Poland and Germany from where it gradually spread throughout most of Europe and eventually to North America and western South America. Rye was introduced into China from Turkey and later the species was into Japan. Ma *et al*. (2004) found that American cultivars were more closely related to Chinese cultivars than to European cultivars and that temporal isolation had influenced the genetic diversity of rye more than geographical isolation.

In their book concerning the origin of isolating mechanisms in flowing plants, Max *et al*. (1978) observed that geographical, ecological and reproductive isolation should be taken into account when studying plant evolution. In our study we analyzed the genetic similarities of cultivated rye accessions from Asia, Europe, North America and South America, but could not make any deductions regarding the domestication process of cultivated rye, indicating that further studies are needed to detect the phylogenetic relationships and evolution process of cultivated rye.

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