BIOLOGIA PLANTARUM 37 (4): 481-489, 1995

PCR derived molecular markers and phylogenetic relationships in the *Secale* genus

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Abstract

DNA from 22 different species, accessions, cultivars and lines included in the *Secale* genus were analyzed by the polymerase chain reaction (PCR), using as primers five pairs of oligonucleotides derived from specific sequences. A total of 42 amplified bands were considered, and some of them appeared to be potentially useful as molecular markers for some of the analyzed groups. These amplified bands were used to generate molecular phenograms inside the *Secale* genus.

Key words: genetic markers, PCR band polymorphism, rye genetic diversity, rye phenograms.

Introduction

A great number of morphological and isozymic genetic markers have already been described in higher plants (Hart *et al.* 1993). However, the number of such markers available with an easy technical approach is still limited. In the case of the *Triticineae* a complete genetic map has not been reported and the taxonomic relationships among some species are a matter of controversy (Vences *et al.* 1987). Recently, there has been an increased interest in the use of techniques, such as restriction fragment length polymorphism (RFLPs), which can give rise to an almost unlimited number of possible molecular markers (Song *et al.* 1990).

An alternative way to easily obtain molecular markers is by the polymerase chain reaction (PCR) technique. This method has the advantage that it considerably reduces the experimental time required, and avoids also the radioactive isotope use, factors which facilitate the analysis of a high number of individuals (Benito *et al.* 1993). Polymorphism amplified fragments generated with short primers of arbitrary nucleotide sequences (RAPDs), have been used to establish taxonomic relationships in different plants (Demeke *et al.* 1992). Here we describe an analysis made using five pairs of primers, derived from specific sequences, among different members of the *Secale* genus in order to obtain good PCR molecular markers and to evaluate their use for studying phylogenetic relationships within the genus.

Received 8 February 1995, *accepted* 29 May 1995.

Materials and methods

The 22 different accessions of *Secale* genus used in this work were provided by several germoplasm banks (Table 1). The Spanish rye cultivars, as well as the inbred lines, were grown for a considerable number of generations in our laboratory. The cultivars Merced and Imperial came from the stocks of the Biology Department in the University of San Diego, La Jolla, USA. In all cases, voucher specimens are kept in the Herbarium of the Complutense University of Madrid.

Table 1. Characteristics of *Secale* species used

Plant species	Accession number or name	Number asigned in this work	Geographical origin
S. cereale subsp. *segetale*	USDA Cisc: 105S1	S1	Italy
	USDA PI 326284 cv. K5836	S2	Russia
	USDA PI 267107 cv. Perevaya	S3	Russia
S. cereale subsp. *ancestrale*	USDA PI 445976	S4	Spain
	USDA PI 445975	S5	Russia
	CPI 19359A	S6	Algeria
S. cereale subsp. *cereale*	line Transbaikal	S7	Russia
	cvs. Ailés, Albarracín	S8, S9	Spain
	line Gigantón	S10	Spain
	cvs. Elbon, Mercedes, Imperial	S11, S12, S13	USA
	cv. JNK	S14	Japan
	inbred lines Pool, Riodeva	S15, S16	Spain
S. montanum	NSGC PI 253956	S17	Iran
S. montanum subsp. *anatolicum*	USDA PI 206992	S18	Turkey
S. montanum subsp. *kuprijanovii*	USDA PI 209586	S19	Russia
S. vavilovii	NSGC cv. 1008	S20	Iran
	USDA PI 284842	S21	Hungary
S. silvestre	USDA	S22	USA

Five pairs of oligonucleotides, chosen from the described genes recorded in the Gene Bank, were used as primers in the PCR reactions (Table 2).

Genomic plant DNA was isolated from young leaves following the procedure described by Dellaporta *et al.* (1983). Amplification reactions were performed in a *Techne PCH-2* thermocycler (*Techne Corporation*, Cambridge, UK), programed for 1 cycle of 4 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 45 °C, 2 min at 72 °C and 1 cycle of 10 min at 72 °C, using the festest available transitions between each temperature. These conditions have proved to generate reproducible results. The reaction volume was 0.1 cm^3. It contained: deoxiribonucleotides triphosphate (dNTPs - 0.4 mM each), primers 2 μM each, 2.5 units of replitherm DNA polymerase (*Epicentre Technologies*, Madison, USA) and 20 - 40 ng of DNA template. The buffer solution was: 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.01 % (m/v) gelatine. A control tube without genomic DNA was also included in each set of reactions in order to detect possible contaminations. PCR

products were analyzed by electrophoresis in Tris, HCl, boric acid and EDTA (TBE) buffer on 1.4 % agarose gels, loading 0.05 cm^3 of each PCR product per lane. The gels were stained with ethidium bromide and the photographs were taken with a *Polaroid D534 Fotodyne* camera (*Polaroid*, Cambridge, MA, USA).

Table 2. Pairs of primers utilized in the PCR.

Designation	Strand + (-) sequence	Position	Source	Repetition grade
Tpi: triosephosphate isomerase	+ 5'GGACTGGAGCAACGTAG3'	1724	*Zea mays*	single/low copy
(Marchionni 1986)	- 5'TCGTCGTGTCGGACAGC3'	2184		c-DNA clone
Amy: amylase	+ 5'AGCCAGTCAGCCAATTCC3'	760	*Hordeum*	low copy
(Knox 1987)	- 5'CGGGGTGGGTGAGGATGT3'	1380	*vulgare*	c-DNA clone
Sec: secalins	+ 5'CAGCAGTCGAGCCCTGTGGC3'	181	*Secale*	low copy
(Kreis 1985)	- 5'GCCCGTGGTATTACACACTG3'	671	*cereale*	c-DNA clone
Het: heterochromatin	+ 5'CGGATACGCGGACAG3'	106	*Secale*	highly repetitive
(Appels 1981)	- 5'CTCCCGTTCTGCTGG3'	614	*cereale*	genomic clone
Ubi: ubiquitin	+ 5'CACCCTTCACCTGGTGGC3'	21	*Hordeum*	highly repetitive
(Gausing 1986)	- 5'CACCGACAACACAAGACC3'	323	*vulgare*	c-DNA clone

In order to facilitate the comparison among the different plant groups, amplified band patterns were made taking into account the data of at least five individuals from each of the 22 analyzed plant samples. A total of 42 amplified bands were recorded.

To establish the phylogenetic relationships the *Neighbour Joining* clustering method (*NTSYS-pc* program) was used (Rohf 1990). The genetic distance calculation was made by the Nei's index (Nei 1978). The general phenogram was elaborated using the frequency of one amplified band for each of the 22 plant groups analyzed. In the phenogram in which only *Secale* species are recorded, the mean frequency of each band for all the plant groups belonging to the same species was used. In this phenogram the branching errors have been calculated as described by Nei *et al.* (1985). This kind of data treatment has been extensively used with RAPD generated molecular markers in order to determine taxonomic identity (for review see Hardrys *et al.* 1992).

Results and discussion

Examples of the amplified DNA product electrophoresis are shown in Fig. 1. Most of the amplified bands appeared in all the analyzed individuals of a given taxon, and only few bands appeared at variable frequency (Fig. 2). On the other hand, it can also be observed that some amplified bands are present in all the *Secale* groups analyzed; however, few of them occurred only in particular groups.

The phylogenetic relationships obtained by frequency comparison of the 42 considered amplified bands among the 22 *Secale* groups are shown in the phenogram

of Fig. 3. Fig. 4 shows the phenogram constructed considering only the four analyzed *Secale* species.

Although the primers used in this experiment came from known sequences, some of them (Tpi, Amy and Ubi) came from other *Gramineae* (heterologous primers). This fact, together with the few restrictive annealing temperature used, does not allow us to assure that the amplified bands obtained would correspond to the amplification of the same sequences from which the primers used were chosen. In this respect, our technique could be considered as intermediate between RAPDs and

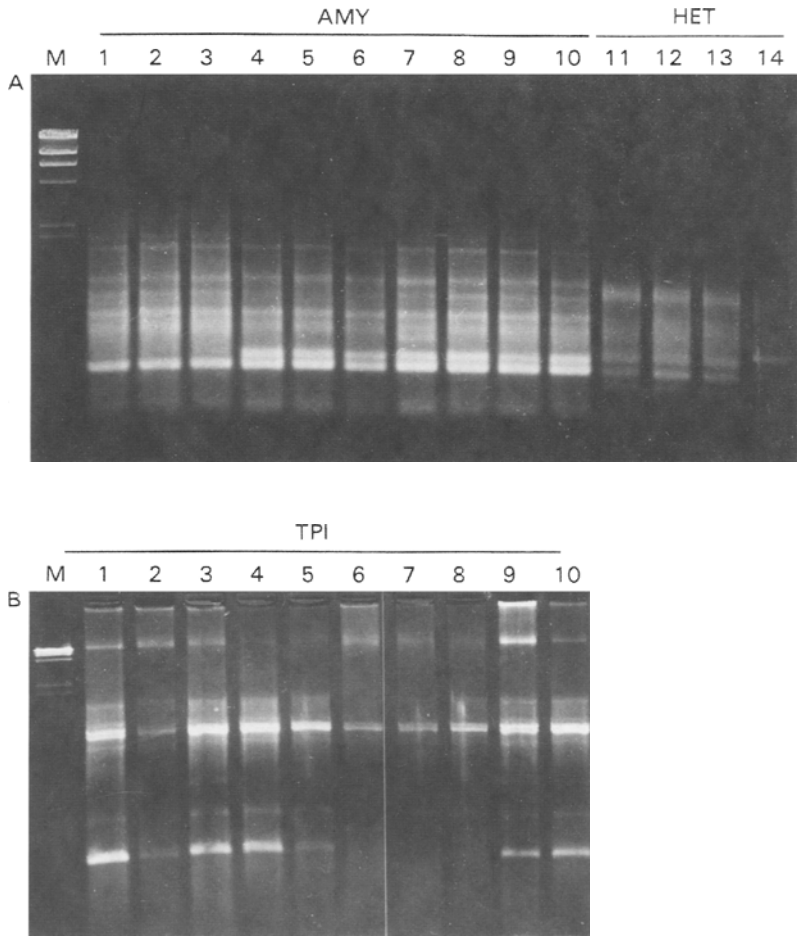
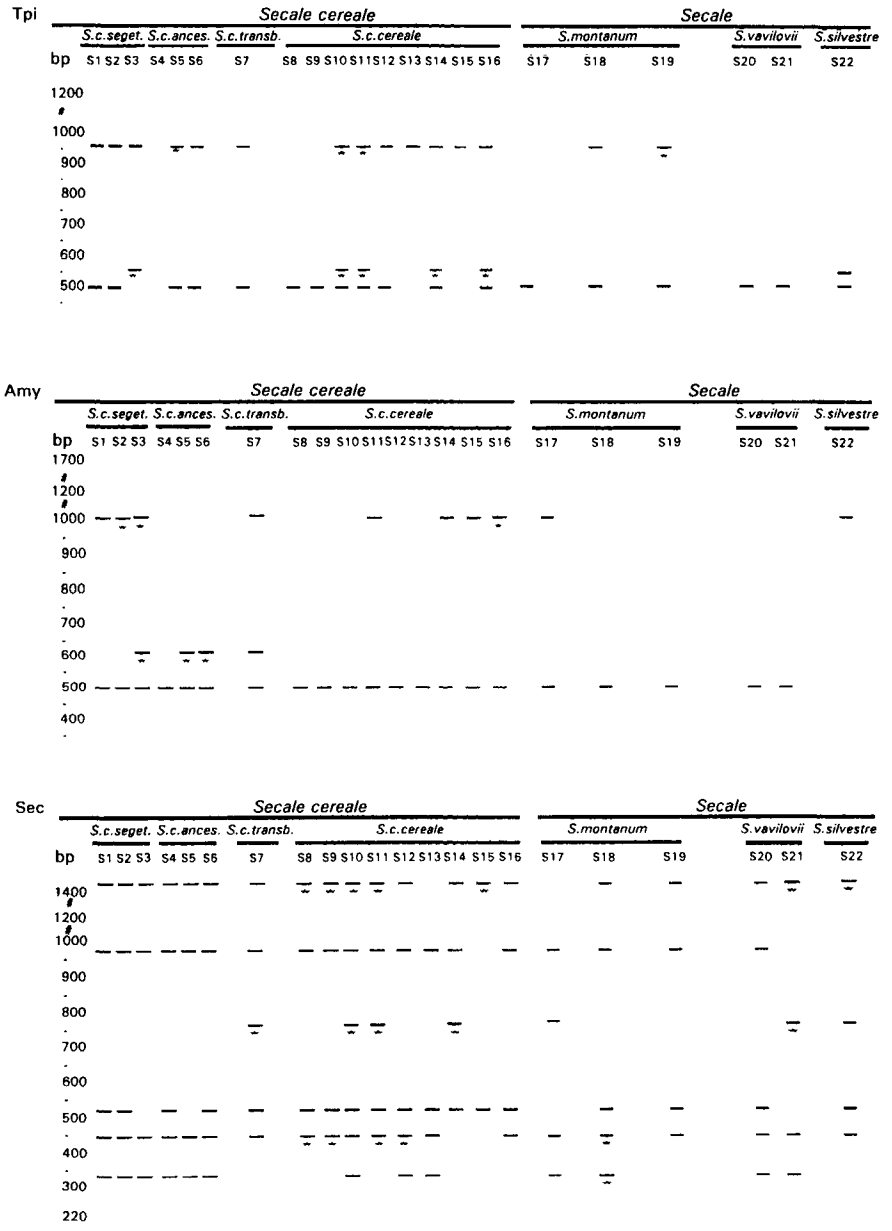


Fig. 1. Amplifed DNA product electrophoresis using Tpi, Amy and Het as pairs of primers: *A*) Amy primers: lane 1, S22; lane 2, S22; lane 3, S22; lane 4, S1; lane 5, S9; lane 6, S10; lane 7, S7; lane 8, S14; lane 9, S15; lane 10, 517. Het primers: lane 11, S22; lane 12, S22; lane 13, S22; lane 14, S4. *B*) Tpi primers: lane 1, S1; lane 2, S10; lane 3, S18; lane 4, S12; lane 5, S19; lane 6, S3; lane 7, S13; lane 8, S7; lane 9, S5. For abbreviations see Tables 1 and 2.

PCR amplification of known sequences, being the experimental temperature and, therefore, the annealing conditions unspecific; however, a pair of quite long primers (20 bp) were used in all the reactions. In our laboratory, other researchers belonging to different groups have shown that two RAPDs primers used simultaneously generated a poor band pattern (few bands and badly defined) (Gallego *et al.*, unpublished). We and Dr. Vazquez's group have found that single longer RAPD



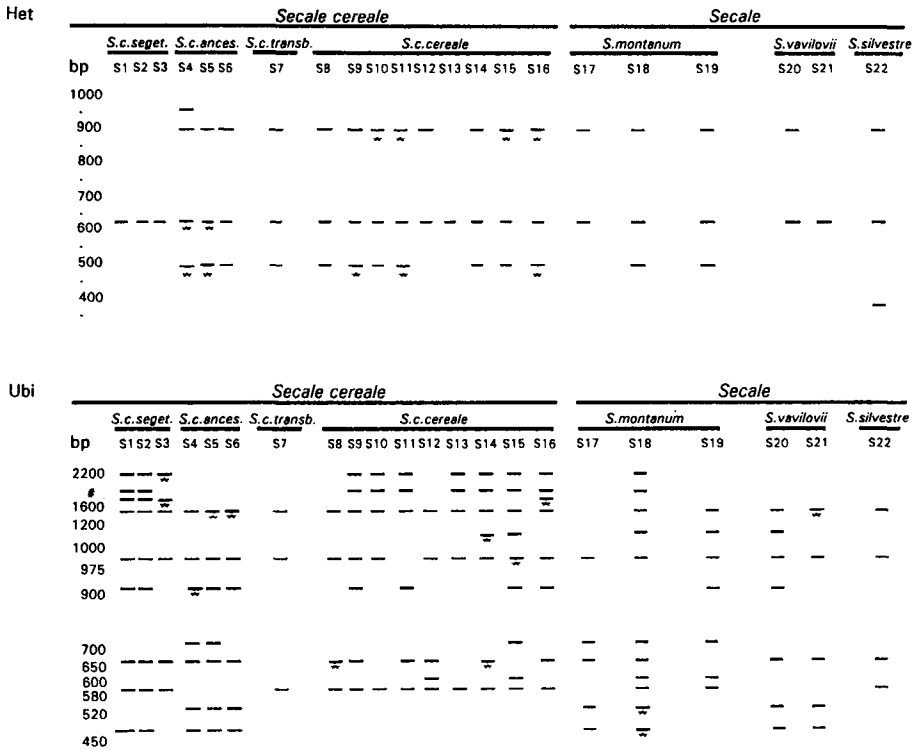


Fig. 2. Amplied bands patterns for the five pairs of primers utilized. For abbreviations see Tables 1 and 2.

primers (20 bp) at lower annealing temperatures (45 - 37 °C) generated more repetitive results than the standard short RAPD primers. It is our experience that the results improved when a pair of specific long primers are used at low temperatures. As far as the experiments shown to be repetitive, the amplified band polymorphism generated by this means provides very good molecular markers. Those polymorphic bands within a given taxonomic group would constitute an excellent material for future mapping studies by the analysis of their linkage relationships in programmed crosses.

On the other hand, some of the amplified bands would be used to quickly identify species, subspecies, accessions, lines or cultivars, because they only appear in particular *Secale* groups (Fig. 2). An example of this situation would be, in the case of Tpi primers, the 500 bp band, which is missing in *S. cereale* subsp. *segetale* (Zhuk) cv. Perevaya (S3), *S. cereale* subsp. *ancestrale* (Zhuk) accession S4, cv. Imperial (S13) and the inbreed line Pool (S15) of *S. cereale* L. Another example would be the 600 bp band, which appear only in *S. cereale* cv. Transbaikal (S7), *S. cereale* subsp. *segetale* (S3) and in *S. cereale* subsp. *ancestrale* accession S5 and S6 when the Amy primers are used. With the same pairs of primers, the absence of the 500 bp band seems to be a good marker for *S. silvestre* (Host) (S22). With the Sec primers, a 750 bp bond appear in all the analyzed species, but only in some groups just inside them. Using the same Sec primers, a 300 bp band would be useful to characterize *S. cereale* subsp. *segetale* (S1, S2, S3), *S. cereale* subsp. *ancestrale*

(S4, S5, S6), the *S. cereale* line Gigantón (S10), the *S. cereale* cvs. Merced (S12) and Imperial (S13), and also the sample from Iran of *S. montanum* (Grosseheim) (S17), S. *montanum* subsp. *anatolicum* (S18) together with *S. vavilovii* (S20, S21). With the Het primers, a 380 bp band appears to be exclusive for *S. silvestre* (S22), and using the same pair of primers, a 950 bp band seems to be a good marker for the Spanish accession of *S. cereale* subsp. *ancestrale* (S4).

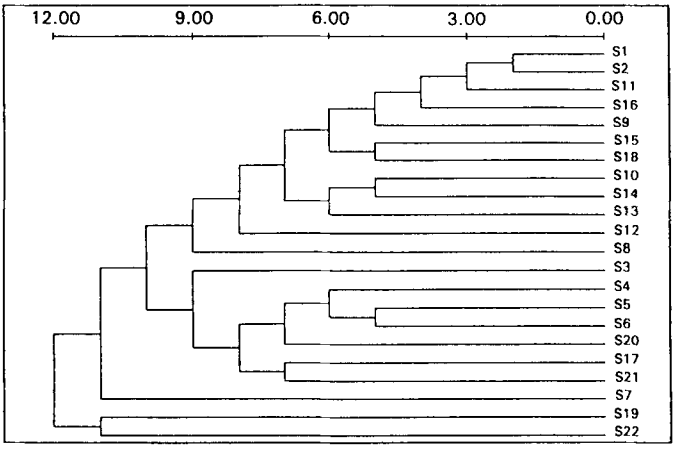


Fig. 3. *Secale* phenogram obtained comparing the PCR amplificated band polymorphism of the 22 plant groups analyzed. The *Neibourg Joining* method was used. For abbreviations see Table 1.

The amplified band variability observed in our PCR experiments, can also be used for the elaboration of phenograms (Fig. 3) in a similar way as it has been prepared with other kind of markers (Vences *et al.* 1987, Song *et al.* 1990). The phenogram has been done using the *Neighbour Joining* method. We have used two other clustering criteria *UPGMA* and *Complete Link* (data not shown) which have also proved to be useful in systematic and phylogenetic studies (Taneto *et al.* 1982, Nei *et al.* 1983, Hart *et al.* 1993). The tree phenograms look very similar. The major discrepancy are formed in *S. silvestre* (S22). This taxon appears alone in an early separated branch with the *UPGMA* and *Complete Link* methods, together with *S. montanum* subsp. *kuprijanovii* (S19) although also in an early branch.

In general, the phenogram obtained is in agreement with the *Secale* groups phylogeny proposed by Khush (1962); however, according to our data *S. cereale* subsp. *ancestrale* would not be included in *S. cereale* as it was according to morphological criteria (Zhukovsky 1926), cytogenetic data and hybrid viability (Khush and Stebbins 1961) and isozymic markers (Vences *et al.* 1987).

Among the *Secale* genus, the classification of *S. montanum* subsp. *anatolicum* (Boiss) (S18) and *S. montanum* subsp. *kuprijanovii* (Grossheim) (S19) was the subject for a controversy (Khush 1962). Our data favours the idea of the *S. cereale* subsp. *anatolicum* being closer to *S. cereale* than to *S. montanum*, and *S. montanum* subsp. *kuprijanovii* would be closer to *S. silvestre* although with the other data

clustering criteria (*UPGMA* and *Complete Link*) remains closer to *S. cereale*. This fact together with the species phenogram would fit also with the possible existence of a common ancestor for *S. silvestre* and *S. montanum*, instead of *S. montanum* being the ancestor for *S. silvestre*, *S. cereale* and *S. vavilovii*, a possibility also mentioned by Khush and Stebbins (1961).

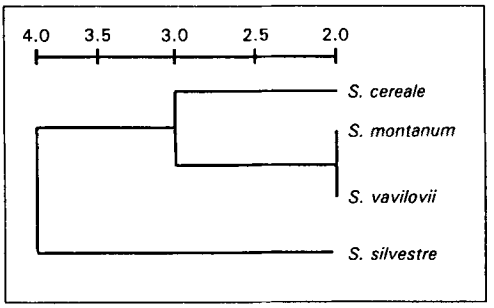


Fig. 4. Phenogram obtained for PCR amplificated band polymorphism only at the species level.

Our data favour the exclusion of *S. cereale* subsp. *ancestrale* (S4, S5 and S6) from the *S. cereale* species, and remains closer to *S. cereale* subsp. *segetale* cv. Perevaya (S3). This cultivar differs from the other *S. cereale* subsp. *segetale* analyzed and seems also not to be included in the *S. cereale* species.

On the other hand, our method does not seems to be strong enough to fit accurately with the known pedigrees of some lines and cultivars inside *S. cereale*. Thus, the relation found between the cv. Imperial and the synthetic tetraploid Gigantón (developed by Dr. E. Sanchez Monge by crossing several nonregistered Spanish *S. cereale* cultivars), does not makes too much sense. However, our data would indicate, at least, similarities for the amplified sequences.

Finally, our data indicate that the PCR experiment using primers of known sequence represents a useful tool which determine genetic diversity, avoiding, in great proportion, the experimental problems derived from the RAPDs use (Fritch *et al.* 1993) which are extremely dependent upon very specific primer characteristics and experimental conditions to obtain repetitive results.

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