Phylogenetic relationships among Portuguese rye based on isozyme, RAPD and ISSR markers

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The phylogenetic relationships of 10 rye landraces and cultivars from the north of Portugal and from Brazil were analysed using 20 isozyme loci, and a total of 511 PCR markers (342 ISSRs and 169 RAPDs).

The isozymes were analysed in at least 100 plants of each population/cultivar and, therefore, we have data about intra and inter population/cultivar genetic variability. However, the analyses with ISSRs and RAPDs were obtained using a mix of 25 plants of each population. Therefore, each population/cultivar was reduced to one tube and we have no data about intra genetic variability.

As expected in a cross pollinated crop we found genetic diversity and a larger variation within than among the populations using isozymes. Somewhat unexpectedly, however, we found that the breeding cultivars have the same level of heterozygosity as the landraces.

The phylogenetic relationships obtained using isozymes among the landraces, synthetic cultivar and the cultivars from breeding programs do not reflect their origin. Moreover, the cultivar from Brazil is not separated from the remaining populations/cultivars studied.

However, the data observed using RAPDs and ISSRs are in agreement with their known origin. The populations maintained by the farmers in the north of Portugal are grouped in a cluster in the phenogram and the C902591 (from Brazil), the Alvão (synthetic variety) and Larouco (a hybrid between Montalegre and Brazil) are in a different cluster.

The ISSRs and RAPDs provide a rapid method for the production of polymorphic markers, which appear to correspond to known pedigree information.

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Rye culture is of huge importance in the north of Portugal (Trás-os-Montes). The rye populations maintained by the farmers in this area of Portugal are highly varied and some of them show high levels of aluminium tolerance (PINTO-CARNIDE and GUEDES-PINTO 1999), their culture being more extensive than the culture of modern cultivars. The genus *Secale* has the most efficient group of genes for aluminium tolerance among cultivated species of *Triticeae* (ANIOL and KACZKOWSKI 1979). 80 % of Portuguese soils are acid (ALMEIDA 1955). Moreover, rye populations are very well adapted to different adverse conditions such as cold and other harsh conditions and show resistance to different diseases. Therefore, to maintain and exploit rye germplasm resources efficiently, an understanding of the genetic variability and its variations among populations is required.

Isozyme markers have been used traditionally in the studies of genetic variability and in the establishment of phylogenetic relationships in many different animal and plant species. Several studies have analysed the variability and the relationships among different species of the genus *Secale* or among cultivars of *Secale cereale* L. using isozyme markers (PÉREZ DE LA VEGA and ALLARD 1984; RAMIREZ et al. 1985; ADAM et al. 1987; FIGUEIRAS et al. 1988; VAQUERO et al. 1990; ALONSO-BLANCO et al. 1993; CARNIDE et al. 1997). However, these studies have concentrated on variation among present-day cultivars from Argentina, Europe, Japan and USA. Until today only one isozyme study of rye landraces has been carried out by PERSSON and BOTHMER (2000).

The new technological developments have expanded the range on DNA polymorphism assays for genetic mapping, marker assisted plant breeding, genome fingerprinting and for investigating genetic relations (POWEL et al. 1996). Random-Amplified Polymorphic DNA (RAPD) (WILLIAMS et al. 1990) and Inter Simple Sequence Repeat PCR (ISSR) (ZIETKIEWICZ et al. 1994) are two new molecular technologies used in the above mentioned studies. The RAPD markers have been previously used to study the genetic variability among different *Secale cereale* L. cultivars or species of the genus *Secale* (BENITO et al. 1993; POZO et al. 1995; LOARCE et al. 1996). However, there are currently no studies of the genetic variability among populations of *Secale cereale* L. using ISSR markers.

Table 1. *Summary of isozyme systems studied and primers (ISSR and RAPD) used*

Enzymes	Oligonucleotides with sequences of microsatellites – ISSRs (UBC)	10-mer oligonucleotides RAPDs (Operon)
GOT	808 5′ AGAGAGAGAGAGAGAGC 3′	B8 5′ GTCCACACGG 3′
PGM	818 5′ CACACACACACACACAG 3′	R2 5′ CACAGCTGCC 3′
PGI	834 5′ AGAGAGAGAGAGAGAGYT 3′	R20 5′ ACGGCAAGGA 3′
MDH	835 5′ AGAGAGAGAGAGAGAGYC 3′	S17 5′ TGGGGACCAC 3′
NDH	844 5′ CTCTCTCTCTCTCTCTRC 3′	
6PGD	857 5′ ACACACACACACACACYG 3′	
AC	873 5′ GACAGACAGACAGACA 3′	
IPO	879 5′ CTTCACTTCACTTCA 3′	
ACPH	889 5′ DBDACACACACACACAC 3′	
PER	B = C,G,T; D = A,G,T; R = A,G; Y = C,T	

In the investigation reported here, isozyme, ISSR and RAPD techniques were used to estimate the genetic relationships among 10 rye landraces and cultivars with known pedigree. The aim of this work was to compare the groups of landraces and cultivars, based on genetic distances, generated by each type of marker.

MATERIALS AND METHODS

Plant materials

The investigation comprised six rye (*Secale cereale* L.) landraces (Montalegre, Gimonde, Malhadas, Vila Pouca, Lamego and Padrela obtained from the farmers of northern Portugal) and four cultivars (Res. Vila Pouca – obtained by University of Trás-os-Montes and Alto Douro (UTAD) from a Vila Pouca population selection, Larouco – obtained by UTAD from the hybrid Montalegre × C902591, Alvão – obtained by UTAD from a synthetic variety and C902591 – kindly supplied by Augusto Baier from Embrapa, Brasil).

Isozyme analyses

The enzymes assayed were phosphoglucose isomerase (Pgi), phosphoglucose mutase (Pgm), acid phosphatase (Acph), malate dehydrogenase (Mdh), 6-phosphogluconate dehydrogenase (6-Pgd), aconitase (Aco), NADH-dehydrogenase (Ndh), glutamate-oxaloacetate-transaminase (Got), indophenol oxidase (Ipo) and peroxidase (Per). The analyses were carried out with 15-day-old leaves from approximately 100 plants from each population/cultivar and by following the extraction, electrophoresis and staining methods previously described by PÉREZ DE LA VEGA and ALLARD (1984) and FIGUEIRAS et al. (1985, 1988).

Genomic DNA extraction

Each landrace/cultivar was reduced to a pool of 25 plants, and 100 mg of green tissue (15-day-old seedling leaves) from each plant were used to create the pool. The pieces of leaves collected for each pool were frozen in liquid nitrogen. The extraction was carried out using a small-scale DNA isolation method (Dneasy Plant Mini Kit, from Qiagen).

Polymerase chain reaction

The ISSR technique has been previously described by ZIETKIEWICZ et al. (1994). A total of nine primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were used (Table 1). The oligonucleotide primers were obtained from UBC primer Set 100/9 (University of British, Columbia).

The RAPD technique has been previously described by WILLIAMS et al. (1990). A total of four 10-mer oligonucleotides from set B, R and S (Operon Technologies, Alameda, CA) were employed (Table 2).

AmpliTaq DNA polymerase (Stoffel fragment), together 10 × concentrated PCR buffer and $MgCl_2$, was supplied by Perkin-Elmer. The PCR was performed in a Perkin-Elmer 480.

Amplification reaction conditions

The amplification conditions were rigorously tested in order to optimise the generation of RAPDs and ISSRs in rye. The ISSR reactions were carried out in a 25 µl volume containing 1.25 units AmpliTaq DNA polymerase, 30 ng of genomic DNA template, 10 pmoles of primer, 2.5 mM of each dATP, dCTP, dGTP and dTTP; 4 mM $MgCl_2$ and 2,5 µl 10 × AmpliTaq DNA polymerase reaction buffer. The RAPD reactions were carried out in a 25 µl volume containing 1.25 units Stoffel Fragment, 60 ng of genomic DNA template, 15pmoles of primer, 2.5 mM of each dATP, dCTP, dGTP and dTTP; 4 mM $MgCl_2$ and 2.5 µl 10 × AmpliTaq DNA polymerase reaction buffer.

The reaction mixture was overlayed with one drop of mineral oil and subjected to PCR. The program

Table 2. *Genetic diversity in the total population and in subpopulations*

Loci	Hs	Js	Ht	Jt	Dst	Gst
Got1	0.0000	1.0000	0.0000	1.0000	0.0000	******
Got2	0.0000	1.0000	0.0000	1.0000	0.0000	******
Got3	0.3568	0.6432	0.3711	0.6289	0.0143	0.0385
Pgm1	0.2302	0.7698	0.2346	0.7654	0.0044	0.0187
Pgi1	0.5462	0.4538	0.5946	0.4054	0.0484	0.0814
Mdh1	0.0000	1.0000	0.0000	1.0000	0.0000	******
Mdh2	0.2854	0.7146	0.3126	0.6874	0.0273	0.0872
Ndh1	0.2189	0.7811	0.2245	0.7755	0.0055	0.0247
6Pgd1	0.0000	1.0000	0.0000	1.0000	0.0000	******
6Pgd2	0.3867	0.6133	0.3944	0.6056	0.0077	0.0195
Aco1	0.6047	0.3953	0.6166	0.3834	0.0119	0.0194
Aco2	0.5004	0.4996	0.5052	0.4948	0.0048	0.0095
Ipo1	0.0000	1.0000	0.0000	1.0000	0.0000	******
Acph1	0.0020	0.9980	0.0020	0.9980	0.0000	0.0000
PerA	0.2175	0.7825	0.2241	0.7759	0.0066	0.0296
PerB	0.4494	0.5506	0.4946	0.5054	0.0452	0.0913
PerC	0.4382	0.5618	0.4752	0.5248	0.0370	0.0778
PerD	0.4286	0.5714	0.4978	0.5022	0.0692	0.1390
PerE	0.3570	0.6430	0.3870	0.6130	0.0300	0.0774
PerF	0.1562	0.8438	0.1714	0.8286	0.0152	0.0890
Average	0.2589	0.7411	0.2753	0.7247	0.0164	0.0595

Hs = Gene diversity in subpopulations, Js = Gene identity in subpopulations, Ht = Gene diversity in the total population, Jt = Gene identity in the total population, Dst = Gene diversity among subpopulations, Gst = Coefficient of gene (or population) differentiation.

used in both cases (ISSR and RAPD) was preliminary step of 5 min at 94°C, 45 cycles of 30 sec at 94°C, 45 sec at 52°C and 2 min at 72°C and a final step of 5 min at 72°C. PCR reactions were stored at 4°C until their resolution by electrophoresis.

Samples of 10 µl PCR products were analysed on 8 % acrylamide gels in TBE buffer running at 100 V for 12.30 hours. The gels were stained using silver nitrate.

Data analysis

Cluster analyses with isozymes, using the unweighted pairwise group methods with arithmetical average (UPGMA), were performed with the GENESTAT 5 and the NTSYSpc statistical packages. The results were used to generate a dendrogram displaying the hierarchical associations among all populations/cultivars.

ISSR and RAPD markers were scored on the basis of the presence (1) or absence (0) of each band for all populations/cultivars. Pairwise comparisons were made between populations using monomorphics and polymorphics bands. Cluster analyses, using SM coefficient and unweighted pairwise group method with arithmetical average (UPGMA), were performed with the NTSYSpc statistical package. The results were used to generate a dendrogram displaying the hierarchical associations among all populations/cultivars.

RESULTS AND DISCUSSION

Isozyme analyses

The loci Got1, Got2, Mdh1, 6Pgd1, and Ipo1 were monomorphic in the ten landraces/cultivars analysed (Table 2). The observed heterozygosity (Ho), the expected (unmodified) heterozygosity (H) and the expected heterozygosity unbiased for simple size (Hu) are very similar (Table 3), indicating that the landraces/cultivars studied are in equilibrium for the loci analysed. However, the populations (landraces) usually showed the same higher heterozygosity (Ho, H and Hu) as mean number of alleles per locus (A, Ap) than the breeding cultivars. These results suggest that the landraces and the breeding cultivars conserved a good level of variability. The average heterozygosity obtained was 0.26, being higher than the heterozygosity detected for different rye cultivars in previous papers, 0.12 for cultivars without translocations and 0.16 for cultivars with translocations (FIGUEIRAS et al. 1988). It was also higher than the heterozygosity detected in different species (ranging from 0.08 to 0.24) of the genus *Secale* (VENCES et al. 1987). These data indicate that the Portuguese rye populations are very polymorphic. However, the average heterozygosity, the mean number of alleles per locus and the percentage of polymorphic loci obtained for Portuguese rye landraces and breeding cultivars are very similar

Table 3. *Polymorphism indices obtained with the 20 isozyme loci analysed*

	N	A	Ap	P	Ho	H	Hu	U	p(1)
Malhadas	119.40	2.25	2.92	0.65	0.27	0.26	0.26	1	0.026
Lamego	99.80	1.85	2.31	0.65	0.24	0.25	0.25	0	******
Padrela	97.05	1.95	2.36	0.70	0.26	0.27	0.27	0	******
Gimonde	129.15	1.90	2.38	0.65	0.26	0.26	0.26	0	******
Montalegre	193.10	2.15	2.64	0.70	0.27	0.27	0.27	0	******
Vila Pouca	141.50	2.10	2.57	0.70	0.27	0.27	0.27	0	******
Res. Vila Pouca	99.65	1.95	2.36	0.70	0.25	0.25	0.25	0	******
C902591	226.10	2.10	2.47	0.75	0.27	0.28	0.28	0	******
Larouco	116.75	1.80	2.23	0.65	0.22	0.22	0.22	0	******
Alvão	168.05	1.90	2.29	0.70	0.24	0.24	0.24	0	******
Average	139.05	1.99	2.45	0.68	0.26	0.26	0.26	0.1	0.026

N = The average number of individuals sampled (mean across all loci), A = The mean number of alleles per locus, Ap = The mean number of alleles per polymorphic locus, P = The proportion of polymorphic loci, Ho = The observed heterozygosity, H = The (unmodified) expected heterozygosity, Hu = The expected heterozygosity unbiased for sample size, U = The number of alleles unique to that population, p(1) = the average frequency of private alleles.

to that observed by PERSSON and BOTHMER (2000) for Swedish landraces and cultivars (0.24, 2.0 and 64.9 %, respectively)

One exclusive allele (allele 3 of Pgm) has been detected in the Malhadas population (Table 3) although at a low frequency (0.026).

As expected in a cross-pollinated crop the gene diversity was higher within populations (Hs = 0.2589) than between populations (Dst = 0.0164) and the coefficient of gene (or population) differentiation was Gst = 0.0595. Therefore, there was higher genetic variability within than between populations. Only a small percentage of total variability (5.9 %) was detected between different populations. Similar results have been found for Swedish landraces and cultivars (PERSSON and BOTHMER 2000).

The genetic distances and the dendrogram (Fig. 1a) obtained using Nei's indice and UPGMA with the NTSYSpc statistical package do not agree with the known pedigree of the landraces/cultivars analysed. The C902591 (from Brazil) was not grouped with Larouco (hybrid from Montalegre × C902591). A similar problem was observed for Vila Pouca and Res. Vila Pouca (selection from Vila Pouca). Moreover, C902591 was in the same cluster as the populations maintained by the farmers. Alvão is a synthetic cultivar and was also grouped in the same cluster as the farmer populations. Therefore, the results obtained with the 20 isozyme loci analysed do not reflect the known pedigree of these populations/cultivars. However, the dendrogram obtained by PERSSON and BOTHMER (2000) using isozymes showed three main clusters and separate commercial varieties from landraces, although their cluster II included both landraces and modern cultivars.

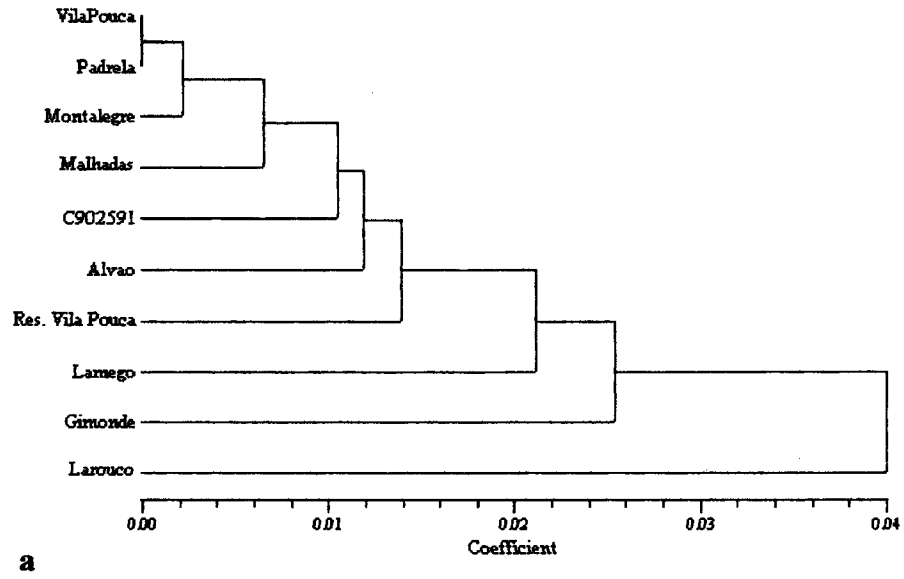
RAPD amplification

DNA from bulks of the ten landraces/cultivars was amplified using four oligonucleotides as primers to generate RAPD markers. These primers (B8, R2, R20 and S17) were selected from among 120 different 10-mer oligonucleotides from the KIT A, B, C, F, R and S (Operon) as they produce many amplification products using a high annealing template temperature (52°C). The results obtained using this annealing temperature show a high reproducibility. For each primer-DNA combination, amplification was repeated at least twice and only fragments present in both replicates were scored.

The four primers produced a total number of 169 fragments. On average, 42.25 bands were amplified per RAPD primer. The total number of polymorphic bands among the ten populations/cultivars were 61 (36 %) (Table 4). Therefore, only with four primers is it possible to obtain many amplification products. LOARCE et al. (1996) obtained 149 fragments with 33 different primers, of which 45 % were polymorphic. Eight different exclusive bands were observed in five different populations.

ISSR amplification

DNA from bulks of the ten landraces/cultivars was amplified using nine oligonucleotides as primer to generate ISSR markers. These primers (808, 818, 834, 835, 844, 857, 873, 879 and 889) were selected among 100 different oligonucleotides from the Set 100/9 UBC, as they generate many amplification fragments. For each primer-DNA combination, amplification was repeated at least twice and only fragments present in both replicates were scored. The results indicate that microsatellites in rye frequently contain



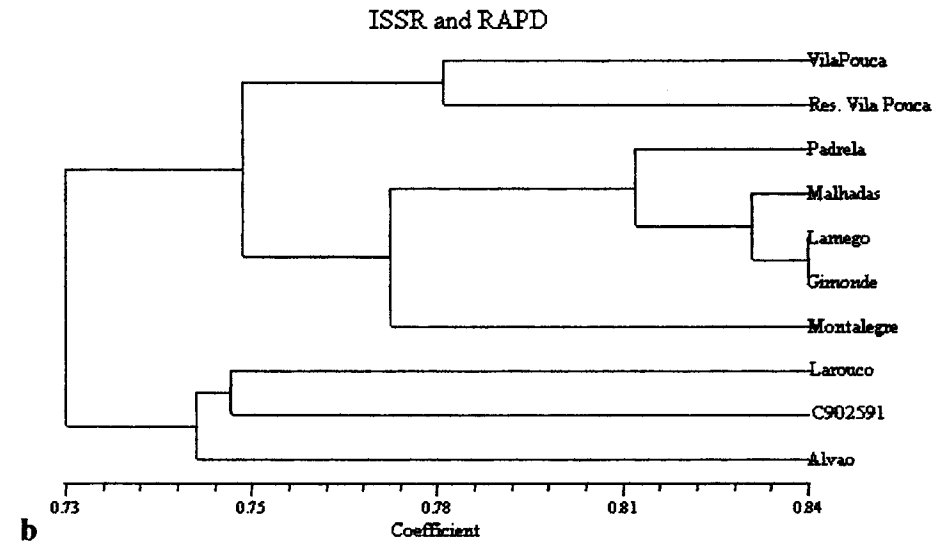


Fig. 1. Dendrograms showing the genetic relationships among rye populations/cultivar using isozymes (**a**), RAPDs together with ISSRs (**b**). The isozyme dendrogram was drawn based on UPGMA cluster analysis and the Nei's indice, whereas the RPADs + ISSRs dendrograms were drawn based on UPGMA cluster analysis and SM coefficient.

the repeated dinucleotides AG, AC and TC. A high reproducibility was observed in all cases. A sample of the ISSR pattern is shown in Fig. 2.

The nine primers produced a total number of 342 fragments. On average, 38 bands were amplified per ISSR primer. The total number of polymorphic bands among the ten populations/cultivars was 280 (82 %) (Table 4). Twenty four different exclusive bands were observed in eight different populations/cultivars with only six primers.

RAPD and ISSR amplifications

Each landrace/cultivar was reduced to one tube that contained the same quantity of green tissue (15-day-old seedlings leaves) for each of the 25 different plants. Therefore, all analyses were conducted using one bulk for each landrace/cultivar. Since bulk samples of DNA were used to produce RAPDs and ISSRs, a mixture of sequences with different degrees of homology with the primer could be amplified. The

Table 4. *Polymorphism obtained with RAPDs and ISSRs*

RAPDs			
Primer	Number of bands	Polymorphic bands	Percentage polymorphism
B8	27	18	67
R2	61	7	11
R20	46	13	28
S17	35	23	66
Total	169	61	36
ISSRs			
Primer	Number of bands	Polymorphic bands	Percentage polymorphism
808	48	41	85
818	33	33	100
834	37	30	81
835	40	39	97
844	36	31	86
857	32	24	75
873	29	19	65
879	51	41	80
889	36	22	61
Total	342	280	82
ISSRs and RAPDs			
Primer	Number of bands	Polymorphic bands	Percentage polymorphism
Total	511	341	67

final quantity of amplifications depended upon sequence frequencies in the bulk sample. Competition among them means that only a reduced number of sequences of all those possible were effectively resolved as defined bands on the gel. In the analyses of bulk DNA samples, only frequently seen fragments in the individual plants were observed (LOARCE et al. 1996). Fragments seen at frequencies below 10 % (MICHELMORE et al. 1991) or 14 % (LOARCE et al. 1996) were not amplified in bulk sample DNA.

The reduction in the number of scored fragments when bulk DNA samples were amplified could introduce a bias in the estimate of genetic distances among cultivars. The fragments pattern of a cultivar would be composed by fragments amplified from sequences highly represented in the sample (in many individual plants). On the other hand, the rare sequences presented in a bulk DNA sample would have higher probability of being amplified when DNA from individual plants was analysed. Therefore, two cultivars whose differences related to poorly represented sequences in both cultivars would show a stronger similarity when bulk DNA samples were used, compared with those obtained if individual plants were analysed. The opposite would occur for two distant related cultivars: two cultivars whose similarities were reduced to poorly represented sequences would show a lower genetic distance when using bulk DNA samples than when using DNA from individual plants (LOARCE et al. 1996). Bulk analyses are economic and rapid but it is not possible to obtain information about the genetic variability inside the populations, it is only possible to get genetic variability between different populations. However, the analyses carried out with isozyme loci in this work indicate the existence of an important genetic variability within populations.

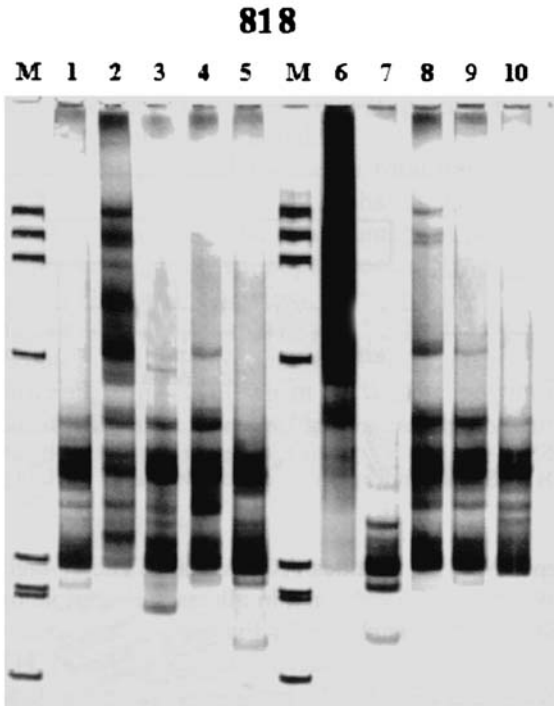


Fig. 2. Electrophoresis of ISSR amplification reactions using 818 UBC primer. M = DNA molecular weight marker (φX174/HaeIII); 1 = Alvao; 2 = C902591; 3 = Larouco; 4 = Gimonde; 5 = Lamego; 6 = Malhadas; 7 = Montalegre; 8 = Padrela; 9 = Res. Vila Pouca; 10 = Vila Pouca.

The 13 (RAPD and ISSR) primers produced a total of 511 bands, of which 67 % were polymorphic (341 bands). The dendrogram generated by the RAPD + ISSR (Fig. 1b) matrix agrees very well with the genealogy of the rye landraces/cultivars studied, better than the dendrogram generated by isozymes (Fig. 1a). C902591 is present with Larouco (hybrid from Montalegre × C902591) and is also in the same cluster with Alvão. Vila Pouca and Res. Vila Pouca (selection from Vila Pouca landrace) are also together. The regional populations (landraces) are grouped in another cluster. This seems to reinforce the information concerning genetic proximity by one of the markers obtained from the other. Therefore, both kinds of markers may be considered appropriate tools for disclosing genetic relationships between rye cultivars.

The mean number of amplification products obtained with RAPDs and ISSRs is similar (42.25 and 38.00, respectively); however, the percentage of polymorphic bands is much higher with ISSR (82 %) than with RAPD (36 %). Therefore, the ISSR markers are more polymorphic than RAPD markers.

In general, the isozyme study reflected greater similarities between rye cultivars than those shown by DNA markers. This is explained by the conservative nature of these isozyme loci compared to RAPDs and ISSRs which might detect non-coding, and therefore, more polymorphic DNA. Also the similarities detected with RAPDs are greater than the similarities observed with ISSRs. In general, there exist relationships between the polymorphism of each kind of marker and the similarities detected. Markers with a low level of polymorphism showed greater similarities between cultivars than markers with a high level of polymorphism. Differences were found among the dendrogram generated by isozymes and DNA markers. The discrepancies observed between dendrograms obtained with isozymes and RAPDs + ISSRs could also be explained by the low number of isozyme loci analysed (20 loci), reinforcing again the importance of the number of loci and how they represent the overall genome, in obtaining reliable estimates of genetic relationships among rye cultivars. Similar results have been observed by LOARCE et al. (1996).

With this study we can conclude that the bulked analyses of RAPD and ISSR markers were useful for studying the genetic relationships between Portuguese rye populations and the isozyme loci were useful for studying the genetic variability within population.

A combination of RAPD and ISSR markers produces a better estimate of genetic relationships (in accordance with the known pedigree), indicating that both types of markers, if present in sufficient numbers, can produce accurate dendrograms.

Finally, the Portuguese rye populations maintained by the farmers have a large genetic variability detected using isozyme, RAPD and ISSR markers. These populations also show a high aluminium tolerance and therefore are good examples to study the relationships between this important trait and these genetic markers.

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