RESEARCH ARTICLE

Molecular diversity and genetic relationships in *Secale*

E. SANTOS[1,3], M. MATOS[1,3]*, P. SILVA[1], A. M. FIGUEIRAS[2], C. BENITO[2] and O. PINTO-CARNIDE[1,4]

[1]*Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal*
[2]*Departamento de Genética, Facultad de Biologia, Universidad Complutense, C/ José Antonio Novais, 12, 28040 Madrid, Spain*
[3]*BioISI- Biosystems & Integrative Sciences Institute, University of Lisboa, Faculty of Sciences, Campo Grande, Lisboa, Portugal*
[4]*CITAB -Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

Abstract

The objective of this study was to quantify the molecular diversity and to determine the genetic relationships among *Secale* spp. and among cultivars of *Secale cereale* using RAPDs, ISSRs and sequence analysis of six exons of *ScMATE1* gene. Thirteen ryes (cultivated and wild) were genotyped using 21 RAPD and 16 ISSR primers. A total of 435 markers (242 RAPDs and 193 ISSRs) were obtained, with 293 being polymorphic (146 RAPDs and 147 ISSRs). Two RAPD and nine ISSR primers generated more than 80% of polymorphism. The ISSR markers were more polymorphic and informative than RAPDs. Further, 69% of the ISSR primers selected achieved at least 70% of DNA polymorphism. The study of six exons of the *ScMATE1* gene also demonstrated a high genetic variability that subsists in *Secale* genus. One difference observed in exon 1 sequences from *S. vavilovii* seems to be correlated with Al sensitivity in this species. The genetic relationships obtained using RAPDs, ISSRs and exons of *ScMATE1* gene were similar. *S. ancestrale*, *S. kuprijanovii* and *S. cereale* were grouped in the same cluster and *S. segetale* was in another cluster. *S. vavilovii* showed evidences of not being clearly an isolate species and having great intraspecific differences.

[Santos E., Matos M., Silva P., Figueiras A. M., Benito C. and Pinto-Carnide O. 2016 Molecular diversity and genetic relationships in *Secale*. *J. Genet*. **95**, 273–281]

Introduction

Rye (*Secale cereale* L.) is an important crop species with the ability to tolerate biotic and abiotic stresses. This species can get a good income in areas where there are no other crops grown, i.e. in areas that are not suitable for cultivation of other cereals (Bushuk 2001). Rye belongs to genus *Secale* which is small but a significant taxon in tribe Triticeae. *Secale* is a typical representative of the Mediterranean flora (Sencer and Hawkes 1980). It includes one cultivated and three wild species; perennial or annual, selfincompatible or selfcompatible (Vences *et al.* 1987). *Secale* are mostly allogamous promoting a great diversity of genotypes. All species in this genus have $2n = 14$ chromosomes (Frederiksen and Petersen 1998); however, there are also some tetraploid derivatives artificially obtained.

Over the years, there has been much controversy about the taxonomy of the *Secale* genus. It remains uncertain despite the large number of studies performed. At present, there are four recognized species of the genus *Secale*: the annual out breeder *S. cereale* L., the annual autogamous *S. sylvestre* Host and *S. vavilovii* Grossh, and the perennial out breeder *S. strictum* (Presl.) Presl. (syn. *S. montanum* Guss.) (De-Bustos and Jouve 2002; Germplasm Resources Informative Network (GRIN) 2015 (http://www.ars-grin.gov). There are eight subspecies included in *S. cereale*: *afghanicum* (Vavilov) K. Hammer, *ancestrale* Zhuk., *cereale* (the only cultivated), *dighoricum* Vavilov, *rigidum* Vavilov and Antropov (syn. *S. turkestanicum* Bensin), *segetale* Zhuk., *tetraploidum* Kobyl. and *tsitsinii* Kobyl. Within *S. strictum*, there are five subspecies: *africanum* (Stapf) K. Hammer (unlike the others, it is autogamous), *anatolicum* (Boiss.) K. Hammer, *ciliatoglume* (Boiss.) K. Hammer, *kuprijanovii* (Grossh.) K. Hammer and *strictum* (syn. *S. montanum* Guss.) (USDA, ARS, National

*For correspondence. E-mail: mmatos@utad.pt.

Keywords. random amplified polymorphic DNAs; intersimple sequence repeats; genetic diversity; *ScMATE1* gene; exons; *Secale*.

Genetic Resources Program. Germplasm Resources Informative Network (GRIN) 2015 (http://www.ars-grin.gov/cgi-bin/npgs/html/splist.pl?11022 (10 April 2015)).

Phylogenetic relationships between and among *Secale* species have been studied using different approaches, including morphological and ecological (e.g. Vavilov 1926; Stutz 1972), cytogenetic (Khush 1962; Cuadrado and Jouve 2002; Zhou *et al.* 2010), chemotaxonomic (Dedio *et al.* 1969), biochemical, like isozymes (Vences *et al*. 1987; Matos *et al*. 2001); and molecular methods such as amplified fragment length polymorphism (AFLP) (Chikmawati *et al.* 2005, 2012), ISSR (Matos *et al.* 2001; Ren *et al.* 2011), random amplified polymorphic DNA (RAPD) (Matos *et al.* 2001; Ma *et al.* 2004), restriction fragment length polymorphism (RFLP) (Loarce *et al.* 1996), simple sequence repeat (SSR) (Shang *et al*. 2006; Jenabi *et al.* 2011) and ribosomal DNA studies (De Bustos and Jouve 2002).

Several classifications were obtained resulting from the diversity of techniques used in phylogenetic analysis. Genetic diversity studies on rye and wild rye could contribute to the maintenance and rational use of germplasm resources in the improvement of rye and its related species (Ma *et al.* 2004). In the past two decades, use of molecular markers have become routine in plant biotechnology, such as genetic diversity studies. ISSR (Zietkiewicz *et al.* 1994) and RAPD (Williams *et al.* 1990) are polymerase chain reaction (PCR)-based markers popular for its advantages, cheap, quick and easy to assay. PCR amplification needs low quantities of DNA and, moreover, these markers have a high genomic abundance and good genome coverage, and does not require sequence information.

Al-induced citrate transporter (*ScMATE1*) gene is of interest in rye since it is involved in aluminum tolerance in Poaceae family, and has been studied and characterized in *S. cereale* (Silva-Navas *et al.* 2012). In this work, ISSR and RAPD markers and six exons of *ScMATE1* gene were used to study the molecular diversity within the *Secale* genus and to estimate the genetic relationships among *Secale* spp. and between cultivars of *S. cereale* ssp. *cereale*. In this study, the *ScMATE1* gene was selected due to its importance in Al tolerance behaviour in different cultivars of rye. This is the first study of diversity of *ScMATE1* gene in different species of *Secale* genus.

Materials and methods

Plant materials and DNA extraction

Three species, *S. cereale* L., *S. strictum* (C. Persl) C. Persl and *S. vavilovii* Grossh. of the *Secale* genus were studied. Also, three subspecies of *S. cereale*: *S. cereale* ssp. *ancestrale* Zhuk., the cultivated *S. cereale* ssp. *cereale* and *S. cereale* ssp. *segetale* Zhuk.; and two of *S. strictum*: *S. strictum* ssp. *kuprijanovii* Grossh. and *S. strictum* ssp. *strictum* syn. *S. montanum* Guss. were also studied. These materials were kindly provided by Dr E. Larter, University of Winnipeg (Manitoba, Canada) (Dedio *et al.* 1969) and maintained in the germplasm bank of the Department of Genetics and Biotechnology at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Moreover, four rye varieties: Imperial (USA), JNK (Japan), Dankowskie Zlote (Poland) and Alvão (Portugal); three regional populations from the northern Portugal (two from Lamego and one from Montalegre), and the consanguineous line Riodeva (Spain) belonging to cultivated *S. cereale* were also studied.

For RAPD and ISSR analyses, each rye and wild rye were reduced to a pool of 15 plants and thereafter to 100 mg of young leaves from each plant. In the analyses of bulk samples, only frequently seen fragments in individual plants were scored (Loarce *et al.* 1996). Fragments seen at frequencies below 10% (Michelmore *et al.* 1991) or 14% (Loarce *et al.* 1996) were not amplified in the bulk DNA samples. The number of plants used to construct the bulk samples was similar to that used in the previous works. The fragments seen at frequencies below 13% were not amplified in our bulks. For *ScMATE1* study, individual plants were used. All the green tissues were frozen in liquid nitrogen and stored at −80°C until DNA extraction. For DNA extraction, a small-scale DNA isolation method was used (DNeasy Plant Mini kit, Qiagen Hilden, Germany) according to the procedures described by the manufacturer.

RAPD and ISSR markers

Polymerase chain reaction: For RAPDs, 21 10-mer oligonucleotides from sets A, B and C (Operon Technologies, Alameda, USA) were selected (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). For ISSRs, 16 primers from UBC primer set 100/9 (University of British Columbia, Canada) based on dinucleotides and pentanucleotides repeats were chosen (table 1 in electronic supplementary material). Primers were selected according to the degree of reproducibility, amplification and polymorphism.

RAPD and ISSR reactions were performed with minor modifications as described by Matos *et al.* (2001). These reactions were carried out in 25 and 20 μL volume, respectively using T-Professional Thermocycler (Biometra, Göttingen, Germany). PCR products were analysed on 1.8% agarose gel stained with ethidium bromide.

Data analysis: RAPD and ISSR amplifications were repeated at least thrice and only repetitive PCR products were scored. RAPD and ISSR markers were scored based on the presence (1) or absence (0) of homologous bands of all rye genotypes. Bands with same mobility were treated as identical fragment. Cluster analysis using the simple matching coefficient (SM) and the unweighed pairwise group method with arithmetic average (UPGMA) was achieved with the NTSYS-pc statistical package ver. 2.02 (Rohlf 1998). Further, cluster analyses were performed for each marker system based on Dice and Jaccard coefficient to reinforce the study (Nei and Li 1979).

Table 1. Genetic diversity parameters for 21 RAPDs and 16 ISSRs primers used for analysing 13 rye genotypes.

Primer	NB	PB	%P	UB	Rp	PIC	MI	DG
RAPDs								
A1	10	5	50.00	1	2.15	0.16	7.81	4
A4	8	5	62.50	0	5.38	0.22	13.50	8
A5	11	8	72.73	3	6.15	0.21	15.02	6
A17	13	9	69.23	3	6.62	0.14	9.92	5
B1	12	7	58.33	1	6.83	0.18	10.73	6
B5	12	7	58.33	1	7.69	0.17	10.00	8
B7	15	10	66.67	1	8.62	0.19	12.42	9
B10	13	5	38.46	0	3.23	0.17	6.45	7
C1	9	7	77.78	0	7.23	0.31	23.73	11
C2	7	2	28.57	1	0.62	0.07	2.03	1
C4	9	4	44.44	0	2.31	0.18	7.94	7
C5	13	8	61.54	1	8.62	0.20	12.32	11
C6	9	1	11.11	0	0.31	0.03	0.32	0
C7	11	10	90.91	2	8.31	0.27	24.25	6
C9	13	10	76.92	1	10.46	0.26	20.30	13
C11	9	4	44.44	0	3.23	0.19	8.41	6
C12	10	5	50.00	2	5.38	0.10	5.21	2
C13	21	20	95.24	4	18.00	0.29	27.79	13
C16	14	5	35.71	0	4.62	0.11	4.10	5
C19	15	10	66.67	1	9.69	0.21	14.31	10
C20	8	4	50.00	0	5.85	0.18	8.88	3
Total	242	146	60.33	22	6.25	0.18	11.69	6.71
ISSRs								
808	16	13	81.25	4	8.31	0.23	18.87	11
810	14	10	71.43	4	8.77	0.15	10.75	7
811	15	13	86.67	3	9.85	0.23	19.97	13
812	13	12	92.31	3	9.85	0.28	25.55	9
815	6	4	66.67	0	4.92	0.21	13.94	2
824	10	8	80.00	1	9.54	0.26	20.64	9
827	8	3	37.50	1	2.62	0.13	4.99	2
835	7	3	42.86	2	1.38	0.11	4.78	1
836	19	16	84.21	4	8.92	0.23	19.72	13
842	20	16	80.00	5	12.31	0.20	16.00	11
844	9	8	88.89	2	7.69	0.20	18.00	2
845	9	6	66.67	2	4.62	0.17	11.05	5
846	12	10	83.33	3	8.00	0.25	21.20	9
881	15	12	80.00	4	10.31	0.21	16.79	7
889	12	9	75.00	3	6.62	0.17	12.87	9
891	8	4	50.00	1	3.54	0.13	6.36	2
Total	193	147	76.17	42	7.33	0.20	15.09	7
RAPDs and ISSRs								
Total	435	293	67.36	64	6.79	0.19	13.39	6.86

NB, number of bands; PB, polymorphic bands; %P, percentage of polymorphism; UB, unique bands; Rp, resolving power values; PIC, polymorphism information content values; MI, marker index values; DG, distinguished genotypes.

To check the goodness of fit of ISSR and RAPD cluster analysis to the similarity matrix on which it was used, the cophenetic correlation coefficient (r) was calculated and Mantel test was performed with 1000 permutations (Mantel 1967) using COPH and MXCOMP tools from NTSYS-pc package. The coefficients values obtained for both markers were compared. Further, the correlation of the combined similarity data matrices (RAPD and ISSR) and the cophenetic matrices of the three coefficients used (SM, Dice and Jaccard) were estimated and compared to verify their reliability.

To determine the confidence limits of UPGMA-based dendrograms, bootstrap tests were performed using 10,000 replications for phylogenetic analysis using the Winboot program (Yap and Nelson 1996).

Discriminatory power: Primer resolving power (Rp) was calculated according to Prevost and Wilkinson (1999) formula $Rp = \Sigma Ib$, where Ib (band informativeness) takes the value of $1-[2\times(0.5-p)]$, p being the proportion of the 13 rye genotypes analysed containing the band. Polymorphic information content (PIC) was calculated as $PIC = 2fi(1-fi)$, as proposed by Roldán-Ruiz *et al.* (2000), where fi is the frequency of the marker bands present and $(1-fi)$ is the frequency of absent marker bands. PIC was averaged over the bands for each primer. To estimate the effectiveness of each marker system, marker index (MI) was calculated according to Sorkheh *et al.* (2007) that defined it as the product of the polymorphism percentage and PIC value.

ScMATE1 gene

Exon amplifications: *ScMATE1* complete gene (genomic sequence) was previously obtained from Silva-Navas *et al.* (2012). Different pair of primers which were designed from the sequences of *ScMATE1* gene were used to amplify the exons 1, 3, 4, 8, 9 and 10 from rye genomic DNA (gDNA) by PCR (table 2 in electronic supplementary material).

Sequence and phylogenetic analyses: Sequences were analysed with Chromas Lite 1.0 (Technelysium, South Brisbane, Australia). A BLASTN search (http://www.ncbi.nlm.nih.gov/) was performed to confirm the DNA sequences predicted from the analysis. Alignments between different

Table 2. Diversity parameter, for six *ScMATE* exons, obtained comparing different sequences of gDNA from different cultivated and wild ryes using the DnaSP v5.0 software.

	Ex1	Ex3	Ex4	Ex8	Ex9	Ex10
Exon size (bp)	200	207	194	164	72	117
SWAG	6	0	0	0	0	0
IMS	177	202	182	150	70	111
VPS	17	5	12	14	2	6
SVS	1	1	2	0	0	1
PIS	16	4	10	14	2	5
h	19	7	14	12	4	6
Hd	0.918	0.648	0.75	0.623	0.453	0.526
ND (Pi)	0.02066	0.00410	0.01149	0.01776	0.00677	0.00767
k	3.9	0.848	2.228	2.913	0.487	0.897
SC	0.912	0.976	0.938	0.915	0.972	0.949

Ex, exon; SWAG, sites with alignment gap; IMS, invariable (monomorphic) sites; VPS, variable (polymorphic) sites; SVS, singleton variable sites; PIS, parsimony informative sites; Hd, haplotype (gene) diversity; ND (Pi), nucleotide diversity; k, average number of nucleotide differences; SC, sequence conservation.

ScMATE1 sequences were made using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/clustalw). The sequences obtained in this study were compared and several diversity parameters were utilized to study the difference among the DNA sequences of *ScMATE1* using DnaSP v5.0 (Librado and Rozas 2009). Genetic relationships among exons of *ScMATE1* gene in different *Secale* genotypes were studied using MEGA 5.1 (Tamura *et al.* 2007) with the evolutionary distance (Kimura 2-parameter) and the UPGMA clustering method. Bootstraps with 10,000 replicates were performed to test the robustness of the dendrograms. The sequence of *HvMATE1* gene from *Hordeum vulgare* was used as outgroup.

Results

RAPD analysis

A total of 242 fragments were produced from all the primers ranging from 250 to 2800 bp with 60.33% polymorphic (table 1 and figure 1 in electronic supplementary material). The average band per primer and the average polymorphic band per primer were 11.52 and 6.95, respectively. Moreover, a total of 22 exclusive bands were observed with 13 RAPDs primers, OPC13 being the most significant. More number of unique bands were found in the cultivated species, especially in the consanguineous line Riodeva. The Rp of 21 RAPD primer was 6.25. Primer OPC13 reached the highest Rp value (10.46), being able to distinguish all the 13 rye genotypes as OPC9 primer. The average PIC and MI values were 0.18 and 11.69, respectively (table 1).

ISSR analysis

The average degree of polymorphism obtained with ISSR markers was higher than that of RAPD markers, reaching 76.17% (table 1). The total number of amplified products was 193 ranging from 320 to 3000 bp, with 147 being polymorphic. The average band per primer and the average polymorphic band per primer were 12.06 and 9.19, respectively. The polymorphism obtained with ISSR markers was significant because 69% of selected primers achieved at least 70% of polymorphism. The number of exclusive bands was 42 (twice as much as RAPD markers). Five unique bands were obtained with only *UBC842* primer. As in RAPDs, ISSR markers revealed the highest exclusive band number in the cultivated species, with special relevance to the consanguineous line Riodeva and JNK varieties, with 14 and 15 exclusive bands, respectively, average of Rp was 7.33. Primers with higher Rp were able to distinguish a greater number of genotypes, although it was not so linear. Primers UBC811 and UBC836 distinguished all rye genotypes (figure 1 in electronic supplementary material). The average PIC and MI were 0.2 and 15.09, respectively (table 1).

Genetic relationships using RAPDs and ISSRs data

Dendrograms using only RAPD markers, only ISSR markers and both the markers combined were obtained. These dendrograms were generated using three coefficients (SM, Dice and Jaccard (Rohlf 1998; Nei and Li 1979) with the UPGMA grouping method. The three coefficients used produced identical dendrograms with slight variations in the similarity degree obtained (data not shown). The best results were obtained by the combined data (ISSR and RAPD). Two different kinds of dendrograms were created for this study: one with seven distinct cultivars of *S. cereale* (figure 1a) and another with six different species/subspecies of the *Secale* genus (figure 2a), where the cultivar Imperial was the representative of the *S. cereale* ssp. *cereale*. The results obtained with the combined data and with ISSRs data were the same, whereas the dendrograms obtained with RAPD data showed minor modifications.

The dendrogram that resulted from the cultivated rye data (figure 1a) had three main clusters: one with five cultivars split into two subclusters (cluster I) and two divergent samples, the consanguineous line Riodeva (cluster II) and the JNK variety (cluster III). In cluster I, rye varieties Alvão, Imperial and D. Zlote were included in the same subcluster (A) and the regional Portuguese populations from Montalegre and Lamego were grouped together (subcluster B). The dendrogram obtained with one representative of each *Secale* species/subspecies, which originated three main groups are shown in figure 2a. Cluster I was divided into two subclusters, one with the subspecies *S. ancestrale* and *S. kuprijanovii* (subcluster A) and another with the variety Imperial (*S. cereale*) and the species *S. vavilovii* (subcluster B). In the clusters II and III, the subspecies *S. segetale* and the species *S. strictum*, respectively, were isolated. Mantel test revealed high correlation values between the dendrograms and the original matrices for both approaches (*S. cereale*: $r = 0.960$ and *Secale* species/subspecies: $r = 0.776$). The correlation values were also significant using Jaccard and Dice coefficients (data not shown).

ScMATE1: sequences and genetic relationships

At least one sequence from each rye genotype per exon was obtained. Exon 1 was the most variable with haplotype diversity (Hd), almost reaching the value 1 (0.918) and with the highest average number of nucleotide differences (k) and nucleotide diversity (Pi) with 3.9 and 0.02066, respectively (table 2). Two different sequences of exon 1 were detected in *S. vavilovii*, one showed two indels with 3 bp each. Exon 3 had the largest size (207 bp), whereas exon 9 had the smallest (72 bp) and both exhibited the highest sequence conservation (SC) with 0.976 and 0.972, respectively. While, exon 3 revealed the lowest Pi value (0.0041), exon 9 showed the smallest Hd (0.453) and k (0.487) values. The sequences comparison revealed intracultivar, intercultivar, intraspecific and interspecific variabilities.

All exon sequences were joined to construct two dendrograms, the first was made with one sequence of every cultivar of *S. cereale* ssp. *cereale* (figure 1b) and the second was made with one sequence of each *Secale* species/subspecies

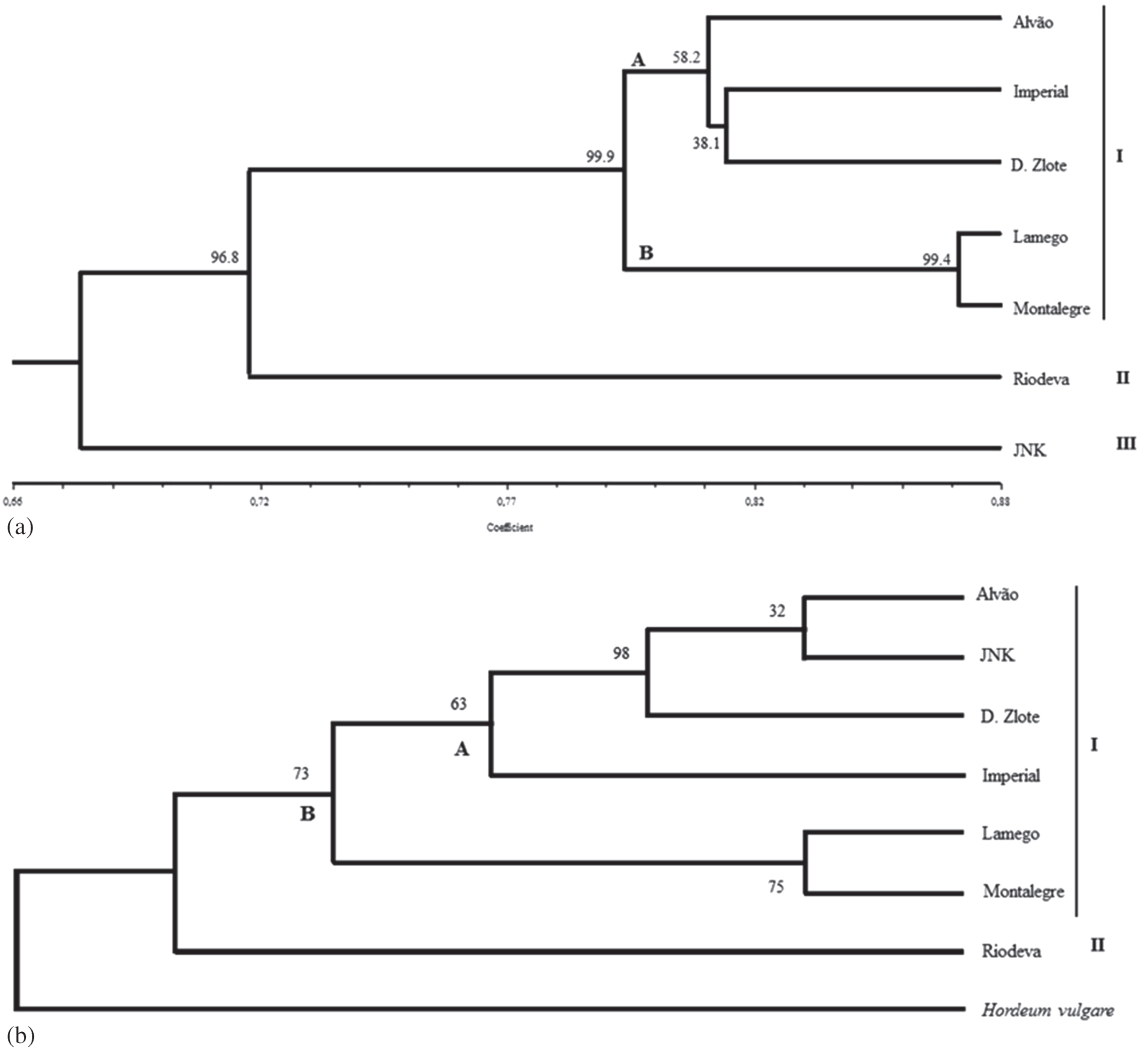

Figure 1. Dendrogram showing the genetic similarity within cultivated ryes belonging to *S. cereale* ssp. *cereale* using (a) RAPD and ISSR markers data and (b) data of six exons of *ScMATE1* gene. I, II, III indicate cluster denomination and (A) and (B) subclusters denomination. Dendrogram (a) was generated using UPGMA method and SM coefficient, whereas dendrogram (b) was generated using the evolutionary distance Kimura 2-parameter and the clustering method UPGMA. The numbers at the nodes are the bootstrap probability values (%).

(figure 2b) with cultivar Imperial as the representative of the *S. cereale*. This first dendrogram (figure 1b) was similar with the dendrogram which was obtained with the dominant markers (RAPDs and ISSRs), with only a change in the variety, JNK. The most divergent were the Riodeva inbred line. The second dendrogram (figure 2b) originated two main clusters with the subspecies *ancestrale* and *kuprijanovii*, and the cultivar Imperial in cluster I and the species *S. strictum* and *S. vavilovii*, and the subspecies *segetale* in cluster II. This was also very similar to the dendrogram obtained with RAPDs and ISSRs with an alteration in the species *S. vavilovii* (figure 2a).

Discussion

Molecular markers have various advantages, such as low cost, easy and quick assay, that make them increasingly required as ubiquity among others. Molecular data have shown more effectiveness than the morphological and physiological data. Matos *et al.* (2001) and Fernández *et al.* (2002) concluded that for genetic relationships studies, RAPD and ISSR markers were more useful than isozymes. Two RAPD and nine ISSR primers generated more than 80% of polymorphism. The bulk method is very convenient to get information about the interspecific genetic variability. Several authors used bulks in their studies and confirmed their effectiveness and usefulness (Loarce *et al*. 1996; Matos *et al*. 2001; Fernández *et al*. 2002; Tanyolac 2003).

Estimation of genetic variability in Secale taxa

Polymorphism quantification is crucial in genetic studies because it indicates the variance degree among plants of

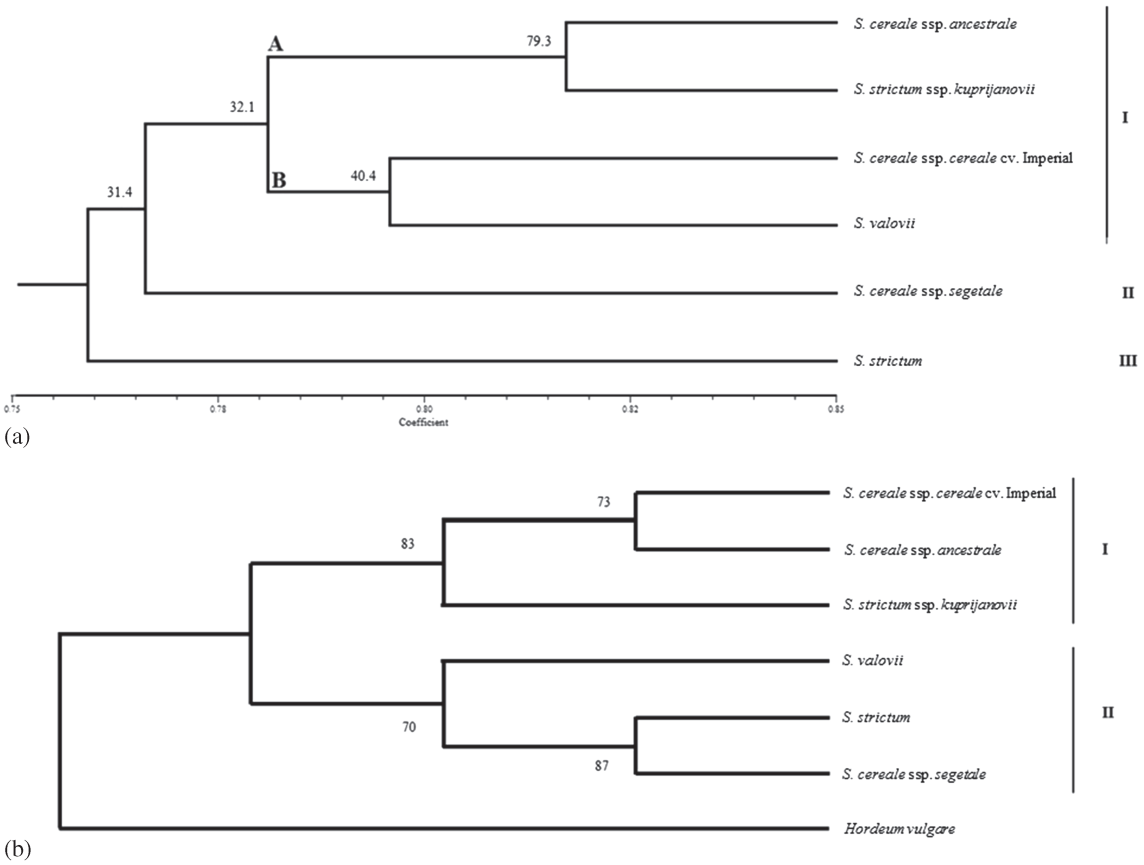

Figure 2. Dendrogram showing the genetic similarity among six different species/subspecies of the *Secale* genus using (a) RAPD and ISSR markers data and (b) data of six exons of *ScMATE1* gene. I, II, III indicate cluster denomination and (A) and (B) subclusters denomination. Dendrogram (a) was generated using UPGMA method and SM coefficient, whereas dendrogram (b) was generated using the evolutionary distance Kimura 2-parameter and the clustering method UPGMA. The numbers at the nodes are the bootstrap probability values (%).

the same or different species. We found high polymorphism values with both markers. The cultivars of *S. cereale* and wild species of the genus *Secale* showed a high variability which is important to reduce vulnerability to biotic and abiotic stresses. Raina *et al.* (2001) reached lower values of polymorphism with the same markers in cultivars and wild species of peanuts. ISSRs were more polymorphic and informative than RAPDs, being the more efficient marker system. Similar results were found with Portuguese rye cultivars (Matos *et al.* 2001), barley cultivars (Fernández *et al.* 2002), wild barley populations (Tanyolac 2003), cultivars and wild species of peanut (Raina *et al.* 2001) and white mulberry (Srivastava *et al.* 2004). RAPDs and ISSRs have dominant inheritance. This disadvantage could be partially overcome while analysing large number of amplification products to enlarge the genome sampling, and to obtain a better assessment of variability and phylogenetic relationships. The 435 fragments (242 RAPDs and 193 ISSRs) analysed are probably sufficient to obtain accurate genetic relationships.

The *ScMATE1* gene has been involved in Al tolerance in some rye cultivars (Silva-Navas *et al.* 2012). The variability detected in exon 1 is consistent with previous results in *S. cereale* (Silva-Navas *et al.* 2012) and other *Poaceae* species. Two sequences of exon 1 from *S. vavilovii* suggest that this gene have at least two copies in this species. The same results were obtained by Silva-Navas *et al.* (2012) in the inbred-line Riodeva. These results indicate a potential relation with Al sensitivity/tolerance, since both genotypes were previously classified as sensitive to Al-stress in acidic soil. The diversity parameters analysed (table 2) were high indicating the existence of a great genetic variability within the *Secale* genus. This variability has a high selection value that can be exploited through breeding programmes to improve the yield/performance of related crops, and even within the *Secale* genus, relative to the presence of aluminum in acidic soil, since this cereal is one of the most tolerant to this stress.

Genetic relationships among Secale taxa

The taxonomy and origin of the *Secale* genus remain highly controversial despite the large number of studies performed over the years. Rye, as barley and wheat, are crops with economic importance that are distributed worldwide. The studies of genetic diversity in the *Secale* genus are important to get a better characterization of its genome for breeding purposes.

Polymorphisms have a great importance in the determination of genetic relationships. The bulk method is a good tool for this approach due to the high degree of polymorphism observed in this study. Use of different markers are essential to obtain more reliable genetic relationships (Bolibok *et al*. 2005). The relationships obtained simultaneously using RAPD and ISSR markers are more accurate and the bootstrap values are higher than using one kind of marker. Loarce *et al.* (1996) and Matos *et al.* (2001) reached the same conclusion.

Both Portuguese landraces (Lamego and Montalegre) grouped together (figure 1a) probably due to a possible adaptation to local environmental conditions. Cultivars with the same geographical location have been grouped together previously (Tanyolac 2003; Ma *et al*. 2004; Srivastava *et al*. 2004). Alvão, Imperial and D. Zlote, all rye varieties were together in the subcluster A. Matos *et al.* (2001) obtained similar results with Lamego, Montalegre and Alvão. Using DArT (diversity arrays technology) markers, Bolibok-Brągoszewska *et al.* (2009) grouped Imperial and D. Zlote, whereas the inbred lines analysed were separated in other cluster. In our case, the inbred-line Riodeva was also separated. The consanguineous ryes are characterized by high homozygosity and low genetic variation. The JNK variety was the most divergent (cluster III). This rye exhibited a wide genetic diversity possibly due to the presence of supernumerary chromosomes (Jones *et al.* 2008).

The subspecies *cereale* and *ancestrale* of *S. cereale* were together (figure 2a), whereas the subspecies *segetale* was separated, however, this maintained a close relationship with this group. The subspecies *ancestrale* and *segetale* have been grouped using other molecular markers (De Bustos and Jouve 2002; Chikmawati *et al*. 2005; Shang *et al*. 2006; Fu *et al*. 2010; Ren *et al*. 2011). The subspecies *segetale* was generally the farthest in the group (Chikmawati *et al.* 2005; Shang *et al.* 2006; Achrem *et al.* 2014). *S. segetale* was the representative of *S. cereale* species closer to the wild rye *S. strictum*. It appears to be an immediate form among cultivated and wild ryes. This conclusion was also reached by Chikmawati *et al*. (2012). We had a unique representative of each subspecies, but high genetic similarity among different *S. cereale* subspecies was detected. *S. vavilovii* was closely related to the cultivated rye (figure 2a). This species showed strong link to *S. cereale* subspecies which is in agreement with various other works (Cuadrado and Jouve 2002; De-Bustos and Jouve 2002; Chikmawati *et al*. 2005; Shang *et al*. 2006; Fu *et al*. 2010; Zhou *et al*. 2010; Ren *et al*. 2011; Achrem *et al*. 2014). Moreover, some authors suggested that the species *S. strictum* is the common ancestor of *S. vavilovii* and *S. cereale* species (Cuadrado and Jouve 2002; De Bustos and Jouve 2002; Zhou *et al.* 2010; Achrem *et al.* 2014). *S. strictum* separated first, being the most divergent (figure 2a) and, possibly the most ancient rye in this study. The subspecies *kuprijanovii*, a perennial rye as *S. strictum*, was grouped with annual rye (figure 2a). Chikmawati *et al.* (2005) separated the perennial from the annual rye using AFLP markers. We only had two perennial ryes and they were separated. However, Shang *et al.* (2006) and De Bustos and Jouve (2002) also had not grouped *S. strictum* and *S. strictum* ssp. *kuprijanovii* being last, close to the annual rye. Further, the *S. strictum* complex proved to be a heterogeneous group with variations between and within different taxa, mainly in the subspecies *kuprijanovii* (Cuadrado and Jouve 2002; De Bustos and Jouve 2002; Shang *et al*. 2006; Achrem *et al*. 2014). De Bustos and Jouve (2002) concluded that it was not clear if this perennial rye can be considered a subspecies of *S. strictum*.

The dendrograms obtained using six exons of *ScMATE1* gene (figures 1b and 2b) were almost similar to the dendrograms obtained using RAPDs and ISSRs markers (figures 1a and 2a). The main differences were in the variety JNK (figure 1b) and the species *S. vavilovii* (figure 2b). With both dominant markers (RAPD and ISSR), the whole genome was covered, which included the B chromosomes of JNK and as we discussed above, this could be the reason for its remoteness (figure 1a). In this case (figure 1b), a particular genome location was covered that should not include the supernumerary chromosomes, and JNK grouped with the others rye varieties. *S. vavilovii* was closely related to the cultivated species above (figure 2a) and here it was more related to the wild species *S. strictum* (figure 2b). This species have great within species differences (Fu *et al.* 2010). This change may be due to the use of different kinds of molecular markers. Therefore, we obtained good genetic relationships with different molecular markers between different species of the *Secale* genus and within the cultivated species.

Conclusion

The bulk method using RAPD and ISSR markers proved to be effective method to detect different levels of polymorphism in rye where ISSRs were more polymorphic and informative than RAPDs. The bulk analysis is an effective strategy for genotype identification in *Secale*. Regarding the genetic relationships, the regional populations of *S. cereale* clustered according to the geographic localization. *S. cereale* cultivars showed high genetic diversity which is favourable for breeding programme. Two different species would be expected to have obvious molecular differences but this was not found between *S. cereale* and *S. vavilovii*. The subspecies *segetale* could be an immediate form among cultivated and wild ryes. However, the heterogeneity of the perennial accessions verified. The *ScMATE1* gene showed a high genetic diversity in the *Secale* genus. Rye show a high tolerance to Al in acidic soils and the variability detected in this study could be related with the abiotic stress response. Moreover, the availability of plant genetic resources, with different tolerance behaviours and with different sequences, is an important basis for future crop breeding programmes. The genetic relationships obtained reinforce the dendrograms

with the dominant markers and showed the inconsistency prevailing in *S. vavilovii*. The conclusions were in agreement with the last revision of the genus *Secale* made by Frederiksen and Petersen (1998) who did not recognize *S. vavilovii* as a species and distinguished *S. cereale* and *S. strictum.*

Acknowledgements

This work was supported by Acción Integrada España-Portugal (PT2009-0096 and E-171/10) and PhD grant from Fundação para a Ciência e Tecnologia de Portugal (SFRH/BD/65040/2009).

References

Achrem M., Kalinka A. and Rogalska S. M. 2014 Assessment of genetic relationships among *Secale* taxa by using ISSR and IRAP markers and the chromosomal distribution of the ACC microsatellite sequence. *Turk. J. Bot.* **38**, 213–225.

Bolibok H., Rakoczy-Trojanowska M., Hromada A. and Pietrzykowsky R. 2005 Efficiency of different PCR-based marker systems in assessing genetic diversity among winter rye (*Secale cereale* L.) inbred lines. *Euphytica* **146**, 109–116.

Bolibok-Brągoszewska H., Heller-Uszyńska K., Wenzl P, Uszyński G., Kilian A. and Rakoczy-Trojanowska M. 2009 DArT markers for the rye genome—genetic diversity and mapping. *BMC Genomics* **10**, 578.

Bushuk W. 2001 Rye production and uses worldwide. *Cereal Foods World* **46**, 70–73.

Chikmawati T., Skovmand B. and Gustafson J. P. 2005 Phylogenetic relationships among *Secale* species revealed by amplified fragment length polymorphisms. *Genome* **48**, 792–801.

Chikmawati T., Miftahudin M., Skovmand B. and Gustafson J. P. 2012 Amplified fragment length polymorphism-based genetic diversity among cultivated and weedy rye (*Secale cereale* L.) accessions. *Genet. Resour. Crop Evol.* **59**, 1743–1752.

Cuadrado A. and Jouve N. 2002 Evolutionary trends of different repetitive DNA sequences during speciation in the genus *Secale*. *J. Hered.* **93**, 339–345.

De Bustos A. and Jouve N. 2002 Phylogenetic relationship of genus *Secale* based on the characterization of rDNA ITS sequences. *Plant Syst. Evol.* **235**, 147–154.

Dedio W., Kaltsikes P. J. and Larter E. N. 1969 Numerical chemotaxonomy in the genus *Secale*. *Can. J. Bot.* **47**, 1175–1180.

Fernández M. E., Figueiras A. M. and Benito C. 2002 The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among barley cultivars with known origin. *Theor. Appl. Genet.* **104**, 845–851.

Frederiksen S. and Petersen G. 1998 A taxonomic revision of the genus *Secale* (Triticeae; Poaceae). *Nord. J. Bot.* **18**, 399–420.

Fu S., Tang Z., Ren Z., Zhang H. and Yan B. 2010 Isolation of rye-specific DNA fragment and genetic diversity analysis of rye *Secale* L. using wheat SSR markers. *J. Genet.* **89**, 489–492.

Jenabi T., Saeidi H. and Rahiminejad R. M. 2011 Biodiversity of *Secale strictum* in Iran measured using microsatellites. *Genet. Resour. Crop Evol.* **58**, 497–505.

Jones R. N., González-Sánchez M., González-García M., Vega J. M. and Puertas M. J. 2008 Chromosomes with a life of their own. *Cytogenet. Genome Res.* **120**, 265–280.

Khush G. S. 1962 Cytogenetic and evolutionary studies in *Secale* II. Interrelationships in the wild species. *Evolution* **16**, 484–496.

Librado P. and Rozas J. 2009 DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–1452.

Loarce Y., Gallego R. and Ferrer E. 1996 A comparative analysis of genetic relationships between rye cultivars using RFLP and RAPD markers. *Euphytica* **88**, 107–115.

Ma J. F., Nagao K., Sato K., Ito H., Furukawa J. and Takeda K. 2004 Molecular mapping of a gene responsible for Al-activated secretion of citrate in barley. *J. Exp. Bot.* **55**, 1335–1341.

Mantel N. 1967 The detection of disease clustering and a generalized regression approach. *Cancer Res.* **27**, 209–220.

Matos M., Pinto-Carnide O. and Benito C. 2001 Phylogenetic relationships among Portuguese rye based on isozyme, RAPD and ISSR markers. *Hereditas* **134**, 229–236.

Michelmore R. W., Paran I. and Kesseli R. V. 1991 Identification of markers linked to disease resistance genes by bulk segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. *Proc. Natl. Acad. Sci.USA* **88**, 9828–9832.

Nei M. and Li W. H. 1979 Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* **76**, 5269–5273.

Prevost A. and Wilkinson M. J. 1999 A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl. Genet.* **98**, 107–112.

Raina S. N., Rani V., Kojima T., Ogihara Y., Singh K. P. and Devarumath R. M. 2001 RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. *Genome* **44**, 763–772.

Ren T. H., Chen F., Zou Y. T., Jia Y. H., Zhang H. Q., Yan B. J. and Ren Z. L. 2011 Evolutionary trends of microsatellites during the speciation process and phylogenetic relationships within the genus *Secale*. *Genome* **54**, 316–326.

Rohlf M. 1998 NTSYS-pc. Numerical taxonomy and multivariate analysis system, version 2.02i. Department of Ecology and Evolution. State University of New York, Setauket, USA.

Roldán-Ruiz I., Dendauw J., Van Bockstaele E., Depicker A. and De Loose M. 2000 AFLP markers reveal high polymorphic rates in ryegrass (*Lolium* spp.) *Mol. Breed.* **6**, 125–134.

Sencer H. A. and Hawkes J. G. 1980 On the origin of cultivated rye. *Biol. J. Linn. Soc.* **13**, 299–313.

Shang H. Y., Wei Y. M., Wang X. R. and Zheng Y. L. 2006 Genetic diversity and phylogenetic relationships in the rye genus *Secale* L. (rye) based on *Secale cereale* microsatellite markers. *Genet. Mol. Biol.* **29**, 685–691.

Silva-Navas J., Benito C., Téllez-Robledo B., El-Moneim D. A. and Gallego F. J. 2012 The *ScAACT1* gene at the Q_{alt5} locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.) *Mol. Breed.* **30**, 845–856.

Sorkheh K., Shiran B., Gradziel T. M., Epperson B. K., Martíne-Gómez P. and Asadi E. 2007 Amplified fragment length polymorphism as a tool for molecular characterization of almond germplasm: genetic diversity among cultivated genotypes and related wild species of almond, and its relationships with agronomic traits. *Euphytica* **156**, 327–344.

Srivastava P. P., Vijayan K., Awasthi A. K. and Saratchandra B. 2004 Genetic analysis of *Morus alba* through RAPD and ISSR markers. *Indian J. Biotechnol.* **3**, 527–532.

Stutz H. C. 1972 On the origin of cultivated rye. *Am. J. Bot.* **59**, 59–70.

Tamura K., Dudley J., Nei M. and Kumar S. 2007 MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596–1599.

Tanyolac B. 2003 Inter-simple sequence repeat (ISSR) and RAPD variation among wild barley (*Hordeum. vulgare* subsp. *spontaneum*) populations from west Turkey. *Genet. Resour. Crop Evol.* **50**, 611–614.

Vavilov N. 1926 Studies of the origin of cultivated plants. *Bull. Appl. Bot. Genet. Plant Breed.* **16**, 1–248.

Vences F. J., Vaquero F. and Pérez de la Vega M. 1987 Phylogenetic relationships in *Secale* (Poaceae): an isozymatic study. *Plant Syst.* **157**, 33–47.

Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A. and Tingey S. V. 1990 DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531–6535.

Yap I. and Nelson R. J. 1996 Winboot: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series No. 14. International Rice Research Institute, P.O. Box 933, Manila, Philippines.

Zhou J., Yang Z., Li G., Liu C., Tang Z., Zhang Y. and Ren Z. 2010 Diversified chromosomal distribution of tandemly repeated sequences revealed evolutionary trends in *Secale* (Poaceae). *Plant Syst. Evol.* **287**, 49–56.

Zietkiewicz E., Rafalski A. and Labuda D. 1994 Genome fingerprinting by simple sequence repeat (SSR-An shored) polymerase shine reaction amplification. *Genomics* **20**, 176–183.

Received 20 February 2015, in final revised form 10 July 2015; accepted 3 September 2015
Unedited version published online: 9 September 2015
Final version published online: 18 April 2016