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Rye (*Secale cereale* L.) and Wheat (*Triticum aestivum* L.) Simple Sequence Repeat Variation within *Secale* spp. (Poaceae)

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Transferability of 25 rye and 20 wheat simple sequence repeat (SSR) markers were examined across *Secale* spp. to elucidate their genetic relationships. One hundred percent of rye and 75% of wheat SSR markers could be amplified in weedy and wild forms of *Secale*. However, only 84% of rye SSR markers were amplified in *S. sylvestre* indicating that this species had the furthest relation to the cultivated rye. The PCR products resulting from rye SSR tended to have similar size as the original size, while wheat SSRs tended to have a shorter size than that of the original size. Polymorphism in the allelic number and size was found in 68% of the rye and 40% of the wheat SSR markers. Several alleles were unique for the annual and perennial rye taxa, which were counted for 22 and 15%, respectively. Some SSR markers also produced specific alleles that could be used to identify certain taxa. The genetic relationships within the genus *Secale* based on SSR markers were discussed.

Keywords: rye SSRs, *Secale cereale*, SSR variation, wheat SSRs

INTRODUCTION

Secale is a genus of grasses in the Triticeae tribe and is closely related to barley (*Hordeum*) and wheat (*Triticum*). *Secale* includes cultivated (*Secale cereale* L.), weedy, and wild rye species. The most known member of *Secale* is rye (*S. cereale* L.) that is grown extensively as a grain and as a forage crop. Rye grain has been used for flour, rye bread, rye beer, and several types of whiskey, and vodka. It can also be consumed as a whole, either as a boiled rye berries, or a rolled rye, similar to rolled oats. Rye is a hardy grain which is more tolerant to frost, aluminum and drought stress than wheat. It is the most winter hardy of all cereals, and is frequently grown under conditions where other cereals fail (Small 1999). Meanwhile, the wild and weedy rye species demonstrated a huge reservoir of genetic diversity that is readily accessible for the improvement of cultivated rye (Chikmawati *et al*. 2012).

Knowledge about relationships among genotypes within germplasm pools is required to support plant breeders in making crop improvement decisions (Archak *et al*. 2003). Correct identity of plants is also very important before developing new plant genotypes. In addition, reliable and definitive cultivar identification becomes critical to maintain varietal purity and to protect breeder and consumer rights (Kubik *et al*. 2009). However, it has been difficult to identify *Secale* species due to lack of diagnostic characters. Many classification efforts using phenetic approaches have been reported. Up to now, systematic classifications have recognized three to 14 species of *Secale* genus depending on the identification criteria used.

Simple sequence repeat (SSR) is a molecular marker that generated based on tandem repeats of short (2-6 bp) DNA sequences. These repeats are highly polymorphic, even among closely related cultivars, because of the high frequency of mutations of the repeats causing variation in the number of repeating units. Polymorphisms at individual loci are easily detected using specific primers developed from the flanking regions of such loci followed by subsequent PCR amplification (Litt & Luty 1989). The high levels of polymorphism, combined with a high interspersion rate, make SSRs an abundant source of genetic markers (Fahima *et al*. 1998). Simple sequence repeats have been proven to discriminate not only among species, but also among accessions within a species, such as olives (*Olea europaea*), and Korean grapevine cultivars (*Vitis vinifera*) (Ercisli *et al*. 2011; Cho *et al*. 2011). Simple sequence repeat analysis was also able to identify the putative parents of two Turkish

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cultivars of European hazelnut (*Corylus avellana* L.) (Gu¨rcan *et al*. 2010). In wild-collected black raspberry germplasm, SSR markers could become useful tool for identifying whether wild plants with good horticultural attributes are truly wild or are closely related to cultivated material (Dossett 2012). Since the cultivated rye genome is composed of highly repetitive sequences (± 85%), SSRs are able to distinguish the taxa in the genus *Secale* either among species or within species.

The amplification of SSR loci by means of PCR assay requires knowledge of DNA sequence, which is expensive and time consuming to obtain. One way to increase the utility of SSR loci, once they have been isolated and sequenced in a source species, is to transfer these markers to related species. Success in the cross-species amplification of any DNA sequence is inversely related to the evolutionary distance between the two species and the degree of synteny (Steinkellner *et al*. 1997). Peakall *et al*. (1998) demonstrated successful cross-species amplification of several SSR loci within and among legume genera. Useful transferability, however, is mostly restricted to the same genera. Within forage species, primers have been shown to work even across different genera. A vast majority of the SSR markers amplified across all forage and turf grass species rather than across cereal species (Saha *et al*. 2006). The present study reported the ability of SSR primers developed from cultivated rye and wheat to amplify weedy and wild rye DNA, and the use of SSR allelic data to distinguish species identity among *Secale* species and to evaluate the phylogenetic relationships within the genus *Secale*.

MATERIALS AND METHODS

Plant Materials and DNA Isolation. Plant materials used in this study were similar as plant materials used by Chikmawati *et al.* (2005). Total of 29 accessions of *Secale* spp. consisting of 13 wild, 9 weedy, and 7 cultivated rye were used in this research. Two outgroup species, *Agropyron cristatum* (L.) Gaertn and *Triticum monococcum* (L.), were also used (Monte *et al*. 1995). DNA of 5-10 plants of each accession was isolated from young freeze-dried tissue collected using a cetyltrimethyl ammonium bromide (CTAB) method (Saghai-Maroof *et al*. 1994).

Simple Sequence Repeat Analyses. Forty-five SSR primer sets were examined. Of those, 25 primer pairs were developed by Saal and Wricke (1999) from cultivated rye (SCM), and 20 primer pairs were wheat SSR primers developed by Roder *et al*. (1995) (Table 1). The primers were between 18 and 26 bp in length with annealing temperatures ranged from 50 to 63 ^{o}C.

Polymerase chain reactions were carried out in a volume of 20 ul (non-radioactive) using a Tetrad Thermocycler (M.J. Research, Waltham-MA). The reaction mixtures contained 50 ng of template DNA, 250 nM of each primer, 200 uM of each dNTP, 0.5 U Taq DNA polymerase (New England Biolabs, Beverly-MA) in 1 x reaction buffer (10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3). A typical PCR profile consisted of one cycle at 95 ^{o}C for 5 min, 40 cycles for denaturation at 95 ^{o}C for 1 min, annealing temperature at 50-63 ^{o}C for 1 min, extension step at 72 ^{o}C for 1 min, and followed by one cycle of a final elongation step at 72 ^{o}C for 10 min. Amplification products were mixed with 1:5 volume of loading buffer (50% glycerol, 0.5% bromophenol blue, 1 mM EDTA) and separated on 4% super fine agarose gel in 1 x TBE (Tris-Borate EDTA) buffer for 4-6 h at 5.5 V/cm gel length.

Data Analysis. The genetic relationships within *Secale* were analyzed using allelic data that was scored as binary data, e.g., presence and absence. Two or more alleles with the same position were treated as the same allele. Accessions with the same allele position were scored as present (code 1), and accessions without the allele in the same position were score as absent (code 0). All allele scores from all loci were combined, and analyzed as binary data. A similarity matrix was calculated using the Nei-Li index (Nei & Li 1979). The data was subjected to the genetic diversity and relationship analysis using neighbor-joining method that was performed using the PAUP program (Swofford 1998). Robustness of the phylogenetic tree was tested by bootstrap analysis with 1000 replications.

RESULTS

Cross-Species Amplification of Rye SSRs within the Genus *Secale*. Simple sequence repeat markers that were developed for cultivated rye clearly demonstrated their ability to make amplicons in weedy and wild rye (Figure 1). Twenty-five SSR markers of rye were successfully amplified in all *Secale* accessions of all taxa except in *S. sylvestre*. *Secale sylvestre* amplified 21 rye SSR markers (84%), while *T. monococcum* and *A. cristatum* (outgroups) amplified only 18 (72%) and 16 (64%) rye SSR markers, respectively. Sixty-eight percent of rye SSR markers were polymorphic. In total, 98 alleles were produced from 25 rye SSR markers. The resulting alleles of each polymorphic marker

Table 1. List of SSR loci, forward and reverse primers, repeat motifs, annealing temperature used, expected PCR product, number of alleles, and PCR product in *Secale* spp.

SSR loci	Forward primer (5`-3`)	Reverse primer (5`-3`)	Repeat motifs	T	Expected PCR product (bp)	Number of alleles	PCR product (bp)
SCM2	GATGACTATGACTACCAGGATGAA	GGAGTGAGAAGGCCGAGAAC	(GT)10	62	113	5	110-150
SCM5	TCGCGATACATCAAGATCGTG	CTAGCATCGACGTAACCCTTT	(GA)16	50	285	6	200-325
SCM9C	TGACAACCCCCTTTCCCTCGT	TCATCGACGCTAAGGAGGACCC	(GT)8	62	220	2	200-250
SCM28	CTGGTCCTGGTCTGGTGGGTC	CGCATCGGGTGTGTCGCATAC	(GT)26	63	159	7	130-190
SCM39 C	GACCTCAGTGGAGCCTCTAGGT	GGACATCTGCCGTGACAATACC	(GT)8(GC)6..(GT)53	60	230	5	200-250
SCM40 B	CCCTTCAGCGGTCATTGTTG	CACATCTTGGGCCTGACACC	(GT)18	63	159	7	130-280
SCM43 AB	CTAGGGGATTACAGGGAGGGCA	GTTCCCTTGTCCTACTCGTTACCG	(GT)11	63	100	8	<100-115
SCM65	CCCGTCCTTCAGTTATGTATGG	TTTGATCGATGAAGAGAGCCA	(CA)7..(CA)8	60	295	1	350-400
SCM75 C	TTTTCTATCTCAGCGATTCATGC	TCCTGAGATCAAGTGCGTGTG	(CA)7(CT)15..(CA)10	63	191	2	165-190
SCM86	CAGATAGATGGGTGTTGTGCG	CTCTTCTCGACATCCACACTCC	(GT)20	60	117	4	<100-125
SCM101B	GCCAGCCGCCACCTTAATTG	AGCCCAACTCTTTCGTGCATG	(CT)18	60	170	7	140-168
SCM102	AACAAGTGCAGAACTCGCGT	CAGAAAGTCTTGGGGCCAG	(AG)27	60	208	1	180
SCM104	GATAGTGGGACATTTGGGTACG	GCCATTCCCCATTAGTACGG	(TG)9	60	173	1	165
SCM109	AACCCCCTTTCGTACCTTGT	TAAAGCAAACCACCAGAGCC	(GT)9	60	98	8	100-175
SCM111	TGCAGATTCACTTATCAACACACAC	TCTGATATCTTTCCAACGGCGT	((GT)9…(GT)26	63	233	1	700
SCM120	CATTGTTGCGAGTGTTGAAGC	TGTGCTGTCGTCGATGTTGTC	(AC)10	62	127	2	125-150
SCM138 AB	ATAGCCGCAGATGGTTGAGGAC	GAGAAGTCTACAAATCAAGGGGGC	(AC)23	63	188	5	150-200
SCM180	GTTTCGTCCCCGTTGCCATC	ACGTGTCGCTTTCCATTGCCC	(GT)6(GA)7	63	140	3	165
SCM206	TCATCGAAAGACGGGACACCT	ACGATTGCTGCTAATAGCCATGTG	(TA)28GA(CA)9	63	171	2	120
SCM243	AGTTGCCGCAGTATTTCCTCA	AACTTAACAATGGTTTCGAGGG	(CA)11	63	110	1	<100-230
SCM256	CTAATTGCCTCACAGAACTATCA	CCAGAGAACAACACAGATCAGC	(GT)9	63	136	1	460
SCM268	GCGCACCCCACACAACACG	GCGGTGGCGGTTGAGGAC	(CA)9	63	153	3	115-160
SCM273	GAGTGGTCGCCATATAACCGTG	TTCGTGAATACCCTGTTTAGCAAG	(GT)28	60	214	1	160-200
SCM304	CATCGGATCACATTCACTTAGTTCG	TAACGCCACCACCAAGCCTTC	(CA)36	60	267	4	225
SCM307	GTAATCAATCTACTACTCTTTCA	CCTTCTGCTGTCATTGTTGG	(AC)9	62	132	11	150-600
WMS 102 D	TCTCCCATCCAACGCCTC	TGTTGGTGGCTTGACTATTG	(CT)15	60	153	-	-

Table 1. Continue

SSR loci	Forward primer (5`-3`)	Reverse primer (5`-3`)	Repeat motifs	T	Expected PCR product (bp)	Number of alleles	PCR product (bp)
WMS 107	ATTAATACCTGAGGGAGGTGG	GGTCTCAGGAGCAAGAAGAACAC	(CT)21	60	188	1	190
WMS 125	GCAGGCGTGTTACTCCAAGT	CCGAGGTGGATAGGAGGAAA	(CA)29	52		2	100-125
WMS 131	AATCCCCACCGATTCTTCTC	AGTTCGTGGGTCTCTGATGG	(CT)22	60	165	2	110-125
WMS 153	GATCTCGTCACCCGGAATTC	TGGTAGAGAAGGACGGAGAG	(GA)18	60	183	3	290-500
WMS 155 D	CAATCATTTCCCCCTCCC	AATCATTGGAAATCCATATGCC	(CT)19	60	143	-	-
WMS 160 D	TTCAATTCAGTCTTGGCTTGG	CTGCAGGAAAAAAAGTACACCC	(GA)21	60	184	-	-
WMS 165	TGCAGTGGTCAGAGTTTTCC	CTTTTCTTTCAGATTGCGCC	(GA)20	60	257	6	175-225
WMS 169	ACCACTGCAGAGAACACATACG	GTGCTCTGCTCTAAGTGTGGG	(GA)23	60	220	1	<100
WMS 182D	TGATGTAGTGAGCCCATAGGC	TTGCACACAGCCAAATAAGG	(CT)18	60	163	0	-
WMS 194	GATCTGCTCTACTCTCCTCC	CGACGCAGAACTTAAACAAG	(CT)32	50	136	2	330-500
WMS 210	TGCATCAAGAATAGTGTGGAAG	TGCAGTTAACTTGTTGAAAGGA	(GA)20	60	303	1	<100
WMS 212D	AAGCAACATTTGCTGCAATG	TGAGAGGAAGGCTCACACCT	(CT)20	60	102	0	-
WMS 257	AGAGTGCATGGTGGGACG	CCAAGACGATGCTGAAGTCA	(GT)30	60	190	3	180-250
WMS 271	CAAGATCGTGGAGCCAGC	AGCTGCTAGCTTTTGGGACA	(CT)4imp(GA)10	60	179	6	160
WMS 295	GTGAAGCAGACCCACAACAC	GACGGCTGCGACGTAGAG	(GA)25	60	254	1	225
WMS 608	ACATTGTGTGTGCGGCC	GATCCCTCTCCGCTAGAAGC	(GA)16	60	166	2	115-175
GDM34	GATCGGACTTTGTGGATGCT	ATGGCTGTAGGACCAAC	(GA)24	52		5	250-1100
GDM40	GCAGGCATGGTACCCTTG	GCAGGCATGGTACCCTTG	(CT)15(CA)7	52		5	250-350
GDM129	GAGCAGGCAGCAGCTAGC	TGCATCATCATCGGTCAAGT	(CA)15	52		3	300-900
						141	

T = annealing temperature, A = not amplified in *S. sylvestre* and two outgroups, B = not amplified in two outgroups, C = not amplified in *A. cristatum*, D = not amplified in *Secale* spp., - = no PCR product.

varied in number and size. The number of alleles ranged from 1 to 11 with 1 to 2 common alleles for each marker. The size of the SSR alleles ranged from 100 to 600 bp and the most common size was between 100-200 bp (48%) (Figure 2). Most of the alleles produced by cultivated rye have a similar number and size as those from weedy annual rye, and were more homogenous when compared to wild perennial rye. *Secale sylvestre* produced the same or fewer alleles than the other accessions.

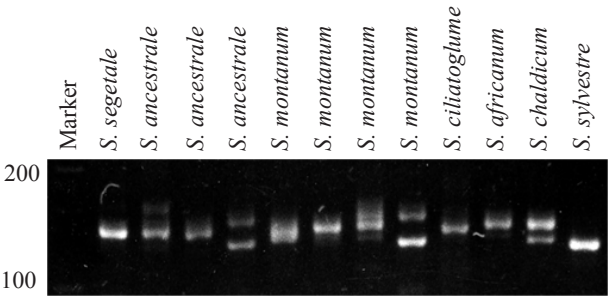

Figure 1. Electrophoregram of rye SSR SCM 101 PCR products among species in the genus *Secale*. The PCR products were electrophoresed on 4% superfine agarose gel with 1x TBE buffer for 3 h.

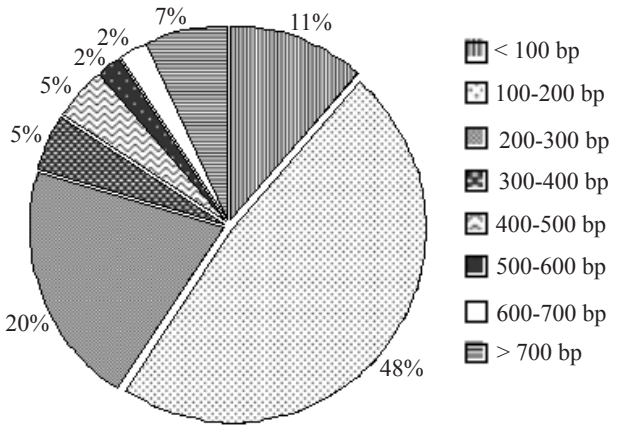

Figure 2. Pie diagram of the distribution of band size categories among SSR loci.

Cross-Species Amplification of Wheat SSRs within the Genus *Secale*. Seventy-five percent of 20 wheat SSR markers were amplified in the *Secale* species, but the polymorphism level of wheat SSR markers (40%) was markedly lower than that of rye SSR markers (68%). The number of allele ranged from 1 to 6 for each marker. In total, 43 alleles were produced from 15 wheat markers. Three loci, GDM34, WMS257, and WMS608, produced the same alleles in all accessions except for *S. sylvestre*. The WMS608 produced single fragment in all *Secale* accessions, but there was an additional fragment with larger size in *S. sylvestre*. In contrast, GDM34 produced five fragments in every *Secale* accession except *S. sylvestre*, which produced only two fragments. The WMS165 marker produced more variation than all other wheat markers. Its exhibited both common and additional fragments in all annual rye taxa except for *S. sylvestre*. Perennial rye taxa exhibited more polymorphism than annual rye taxa.

Specific Alleles. Several alleles were unique for the annual and perennial rye taxa, which counted for 22 and 15%, respectively. In addition, some SSR markers produced species-specific alleles that could be used to identify certain taxa (Table 2). *S. sylvesre* could be identified using SCM 2, SCM 101, SCM 180, SCM 268, SCM 307, WMS 608, and GDM 34. While *S. afghanicum*, *S. africanum*, and *S. vavilovii* could be identified using SCM 2, SCM180, and SCM 307, respectively.

Genetic Relationship Analysis. A dendrogram was constructed using 129 alleles from 23 SSR markers within the genus *Secale* (Figure 3). The cluster clearly separated *S. sylvestre* from the rest of the taxa. Bootstrap values showed that the separation of *S. sylvestre* was the only separation that was well supported (88%).

Table 2. List of SSR loci, common alleles, and specific alleles in *Secale* spp.

SSR loci	Number of common alleles	Specific alleles
SCM 2	1-2	2 additional bands in *S. afghanicum* 1 additional band in *S. sylvestre*
SCM 101	1-3	1 small-sized band in *S. sylvestre*
SCM180	1	1 additional smaller-sized band in *S. africanum* 1 additional larger-sized band in *S. sylvestre*
SCM 268	1-2	1 larger-sized band in *S. montanum* from Iran 1 small band in *S. sylvestre*
SCM 307	2-6	1 band in *S. vavilovii*
WMS 608	1	1 additional, larger-sized band in *S. sylvestre*
GDM 34	5	2 in *S. sylvestre*

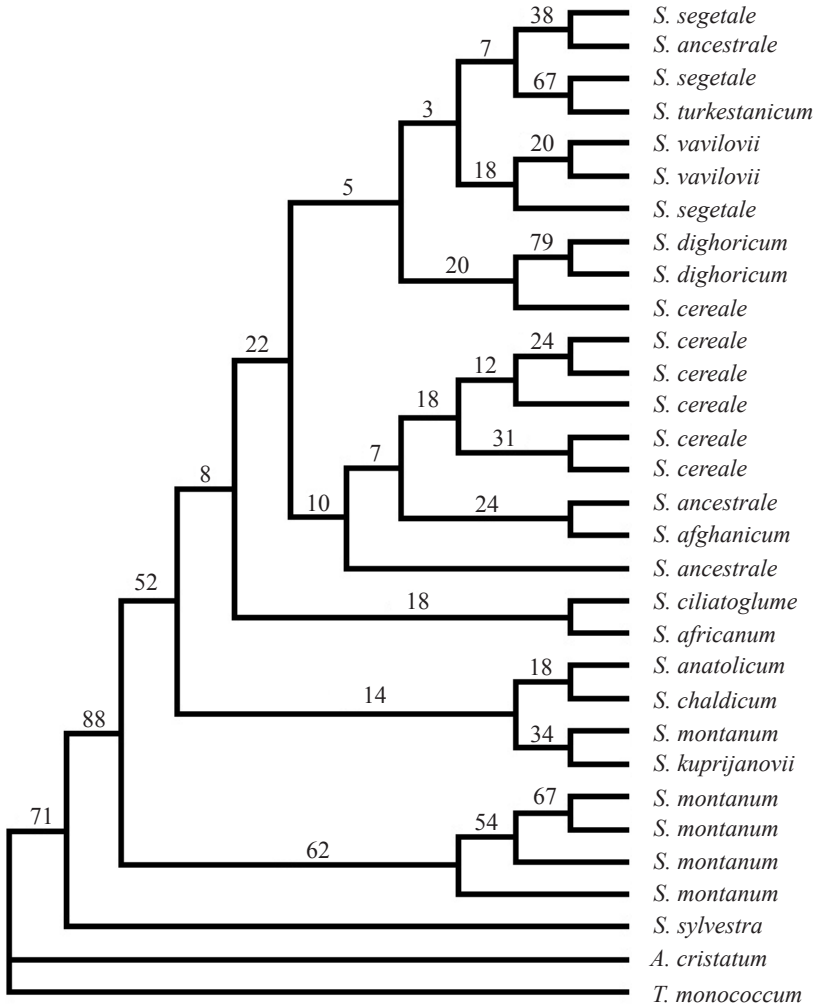

Figure 3. Dendrogram constructed from SSR allelic data, using Nei and Li's distance and neighbor-joining clustering method. Numbers on the branches are bootstrap values.

DISCUSSION

Previous studies showed that the probability of successful cross-species amplification of simple sequence repeat primers depends on the relatedness between species (Fields & Scribner 1997). A decline in amplification success was observed with the increase of genetic distance in some plant species (Roa *et al*. 2000). For instance, between *A. thaliana* and *A. rabis* (van Treuren *et al*. 1997), for which the divergence time between those two species was less than 10 million years, the amplification success was quite high, whereas, Whitton *et al*. (1997) found a much lower rate of success over evolutionary distances of 15-29 million years. Roa *et al*. (2000) found that there were no DNA amplification products detected in the most distant species from the source, and the same SSR loci could not be found in species with a low degree of relationship. Our study clearly demonstrated all SSR markers developed from *S. cereale* were successfully amplified in all *Secale* taxa except for *S. sylvestre*, which amplified 84% of the markers. Many studies using different approaches, including our previous study using AFLPs (Chikmawati *et al*. 2005, 2012), showed that *S. sylvestre* had the most distant relationship to *S. cereale*. This fact may explain the lower rate of this species to have rye SSR sequences compared to that of other *Secale* taxa. In addition, the amplification of rye SSR markers in *T. monococcum* and *A. cristatum* was lower than that in all *Secale* taxa. This result suggested that the success rate for amplification of rye SSRs was higher within the *Secale* genus than between *Secale* and other genera. Our results were similar to those of Peakall *et al*. (1998) who showed that cross-species amplification in plants appears to be moderate to complete (50-100% primer conservation) within genera, however, successful cross-species amplification between closely-related genera appears to be much lower. According to Smulders *et al*. (1997), the lack of allele amplification in certain accessions can be the result of divergence of the area flanking the microsatellite, thus creating a null allele.

A majority of the PCR product sizes resulting from rye SSRs were similar to the size when they were amplified in the original source, indicating that we amplified the correct SSR. However, PCR products resulting from wheat SSRs tended to be shorter than that of the original source. This result is in agreement with Roder *et al*. (1995) who showed that when wheat SSR markers were amplified in rye and barley, shorter fragment size or no PCR product was produced. Reciprocal studies demonstrated that this phenomenon occurs between pairs of related species in both directions (Ellegren *et al*. 1995).

Cluster analysis based on the presence of the SSR alleles separated *S. sylvestre* first from the rest of the taxa indicating *S. sylvestre* had the most distant relationship to the others. Although weakly supported by bootstrap analysis, all annual taxa formed a monophyletic group suggesting that they had close relationships with each other. Perennial taxa were separated after the separation of *S. sylvestre*. This result is in accordance to most of the previous studies based on various approaches such as rDNA spacer-length variation (Reddy *et al*. 1990), rDNA ITS sequences (De Bustos & Jouve 2002), and AFLP approaches (Chikmawati *et al*. 2005).

Based on cluster analysis and the successful of the rye and wheat SSRs amplification, we could draw conclusions on the genetic relationships among *Secale* species. *Secale sylvestre* was the most distant from the others. Since it had the closest relationship to the other outgroups, *S. sylvestre* could be considered as the most ancient of the *Secale* species. *S. montanum* seemed to be the direct progeny of *S. sylvestre*, while the other perennial taxa were originated from *S. montanum*. Annual taxa were clustered together indicating closer relationships among them, and suggested that they might originated from a common progenitor. Perennial taxa were the possible progenitor of the annual taxa.

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