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VARIABILITY OF THE SWEET CHERRY *S*-LOCUS IN THE GENE CENTRE

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1. INTRODUCTION

Develpoments in the fruit growing industry requires new cultivars that fit well with the existing ecological/economical conditions. Molecular genetics may help to promote and speed up traditional breeding activity and hence molecular analysis of the perspective genotypes (landraces, wild species, hybrids etc.) has become more intensive in all producer countries.

The sweet cherry (*Prunus avium* L.) is thought to have originated between the Caspian Sea and Western Anatolia (De Candolle, 1894). The English word of cherry and the Latin name of *Cerasus* possibly come from the name of the city Kerasun, currently known as Giresun in the Black Sea region of Turkey (Faust and Surányi, 1997). Among several cherry species, the ground cherry (*Prunus fruticosa*) and sour cherry (*Prunus cerasus*) are also abundant in the Anatolian region (Ercisli, 2004). Since sour cherry was shown to have arisen from the cross between *P. fruticosa* and *P. avium* (Iezzoni and Hancock, 1996; Tavaud et al., 2004), their overlapping habitats may indicate the centre of origin of sour cherries.

Fruit tree species of the *Rosaceae* family exhibit gametophytic self-incompatibility, a genetically controlled mechanism enabling styles to reject self-pollen (de Nettancourt, 2001). This trait is governed by the highly polymorphic, multiallelic *S*-locus, in which the pistil *S-ribonuclease* (*S-RNase*) (McClure et al., 1989) and the pollen-expressed *S-haplotype specific F-box* genes reside (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003). When the only *S*-allele in the haploid pollen matches either of the two alleles carried by the diploid pistil tissue, pollen tube growth is arrested and fertilization is impossible. The *S-RNase* gene contains five conserved regions (C1-C3, RC4 and C5) as well as one hypervariable region (RHV), which was shown to be exposed at the surface of the folded protein and play an important role in the discrimination of self from non-self pollen (Ushijima et al., 1998). The *S-RNase* gene also contains two introns which present allele-specific length variations. Self- and mutual incompatibility between cultivars of sweet cherry has been known for a long period (Crane and Lawrence, 1929). Following the description of the first six cherry *S*-alleles (S_1-S_6) (Matthews and Dow, 1969), biochemical and molecular analyses indicated the presence of another 10 *S*-alleles (Bošković et al., 1997; Bošković and Tobutt, 2001).

However, later S_8, S_{15} and S_{11} were shown to be identical with S_3, S_5 and S_7, respectively. A provisional label, S_x, was relabelled as S_{16} (Sonneveld et al., 2003). Three *S*-alleles (S_{23}-S_{25}) were described in Spanish cultivars (Wünsch and Hormaza, 2004) although S_{23} and S_{25} seem to be identical with S_{14} and S_{21}, respectively (Vaughan et al., 2008). Further 12 alleles (S_{17}-S_{22} and S_{27}-S_{32}) have been identified in Belgian and UK wild cherries (De Cuyper et al., 2005; Vaughan et al. 2008). Some wild cherry *S*-alleles (S_{17}, S_{19}, S_{21} and S_{22})

were also detected in Sicilian, German and Hungarian commercial cultivars (Békefi et al., 2003; Marchese et al., 2007; Schuster et al., 2007).

Eight sweet cherry *S*-alleles (S_1, S_4, S_6, S_9, S_{12}, S_{13}, S_{14}- and S_{16}) are also present in sour cherry (Tsukamoto et al., 2008) and many of them experienced a loss-of-function mutation in sour cherry, which might have been maintained latent in a tetraploid species like sour cherry (Tsukamoto et al., 2006). In addition, several alleles (S_{26}, S_{33}-S_{35}, S_{36a}, S_{36b}, S_{36b2}, and S_{36b3}) detected in sour cherry have not been identified in sweet cherry, and hence were presumed to derive from the other species as parent, *P. fruticosa* (Tsukamoto et al., 2008). Cultivars sharing the same *S*-genotype are mutually incompatible and form crossincompatibility groups (CIG). Among the sweet cherry cultivars, 44 CIGs have been established. By combining the results of traditional open field fruit set analyses and molecular analyses, Tobutt et al. (2005) described 26 (I-XXVI) cross-incompatibility groups. The number of the CIGs was later increased to 36 (Schuster et al., 2007). Later, groups XXXVIIXL (Marchese et al., 2007), XLI (Gisbert et al., 2008) and XLII-XLIV (Ipek et al., 2011) were also described. In addition, Békefi et al. (2010) also described a group labelled as XLII but with different genotype and hence the number of the existing groups is currently 45. Cultivars with unique incompatible genotype can be associated with trees belonging to any of the CIGs and used as universal pollen donors.

While hundreds of sweet cherry cultivars and wild accessions have been *S*-genotyped from Eastern Europe to North America, only limited data are available on accessions native to the centre of origin of this species (Ipek et al., 2011). Therefore, this study was carried out to determine the *S*-genotype of old Turkish landrace sweet cherry cultivars and some local genotypes selected from populations growing wild in the Black Sea regions of Turkey. New allele-specific PCR assays were elaborated to ensure reliable allele assignment in a germplasm of great genetic diversity. Many *S-RNase* alleles were partially sequenced and analysed from the aspects of *S*-allele evolution and phylogenetic relationships within the *Prunus* genus.

2. AIMS OF OUR STUDY

Our study was carried out to reach the following objects:

1. Determination of the *S*-genotype of 30 landrace Turkish cultivars and 17 local genotypes selected from populations growing wild at the Black Sea coast.
2. Molecular analysis of new cherry *S-RNases*, and determination of the partial gene sequence of them.
3. To review of the *S*-allele set of the analyzed germplasm and to characterize the variability of the *S*-locus.
4. Factors contributing to the formation of the great genetic diversity in gene centre were also considered.
5. To develop new allele-specific PCR assays to ensure reliable allele assignment in a germplasm of great genetic diversity.
6. Exploration of the putative mutations which occurred in the *S-RNase* gene and to analyze the evolutionary / phylogenetic relationship of them in the *Prunus* genus.

3. MATERIALS AND METHODS

3.1. Plant material

A total of forty seven Turkish sweet cherry accessions were used for the experiments; those include 30 landrace cultivars and 17 local genotypes selected from populations growing wild at the Black Sea coast. Buds of each accession were collected from these 47 accessions at the sweet cherry germplasm collection held at the Atatürk Central Horticultural Research Institute, Yalova, Turkey.

3.2. DNA-based analysis

3.2.1. DNA isolation

Genomic DNA was extracted from buds using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA concentrations and purification parameters were measured using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

3.2.2. Consensus PCR analysis

PCR was conducted using cherry consensus primer pairs of the fluorescently (6-FAM) labelled PaConsI-F, PaConsI-R amplifying the first intron; and PaConsII-F, PaConsII-R for amplification of the second intron (Sonneveld et al., 2003).

To amplify the microsatellites within the first and second introns of *S*$_{13}$-*RNase*, the primer pairs (with 6-FAM label on the forward primer), *S*$_{13}$-1SSR and *S*$_{13}$-2SSR, were used according to Marchese et al. (2010).

For the amplification of the 3$_{rd}$ exon of the gene from the C3 to the C5 region, the reverse complement of the EMPC3consRD (Sutherland et al., 2004), labelled as EM-PC3consFD, was used in combination with EM-PC5ConsRD.

Approximately 20–80 ng of genomic DNA was used for PCR amplification in a 25 μl reaction volume, containing 10× DreamTaq™ Green buffer (Fermentas-Thermo Scientific, Burlington, Canada) which contains KCl and $(NH_4)_2SO_4$ at a ratio optimized for robust performance of DreamTaq™ DNA Polymerase in PCR with final concentrations of 4,5 mM $MgCl_2$, 0.2 mM of dNTPs 0.2 μM of the adequate primers and 0.06 U of Dream*Taq* DNA

polymerase (Fermentas). PCR was carried out in a PTC 200 thermocycler (MJ Research, Quebec, Canada) according to Sonneveld et al. (2003).

The second intron PCR products were separated by electrophoresis in 1,2 % TAE or TBE agarose gels (1,5 h at 100V) and DNA bands were visualized by the ethidium bromide staining. Fragment lengths were estimated by comparison with the 1 kbp DNA ladder (Promega, Madison, USA).

3.2.3. Allele specific PCR analysis

Allele-specific PCR analysis was carried out to selectively amplify the S_1-S_7, S_9-S_{10}, S_{12}-S_{14} and S_{16} *S-RNase* alleles and *phenylalanine ammonia-lyase* (PAL) as internal control according to the protocol described for the primers (Sonneveld et al., 2001; 2003). New allele-specific primers were designed to detect S_{17}-S_{19}, S_{25}, S_{34} and S_{37} alleles.

Allele-specific PCR was carried out with DreamTaq DNA polymerase (Fermentas) using a program for 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 50-63 °C (according to the protocol described for the allele-specific primers), 1 min and 30 s at 72 °C, followed by 5 min at 72 °C. The annealing temperature applied for the newly developed S_{17}-S_{19}, $S_{21/25}$, S_{34} and S_{37} specific primers was 50 °C.

3.2.4. Fragment lenght analysis, cloning, sequencing of PCR products and sequence analysis

To determine the exact size of the *S-RNase* first intron region fragments under 500 bp, the fluorescently labelled products were run in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Genomic DNA fragments were extracted and purified from the 1,2 % TAE agarose gels using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham Ontario, Canada) and cloned into a pGEM-T Easy (Promega, Madison, USA) or rather pTz-57R/T (Fermentas-Thermo Scientific, Burlington, Canada) plasmid vector. The ligated plasmid vectors were transformed into JM109 or DH5α competent *E. coli* cells preparation with Z-Competent™ E. coli Transformation Kit (Zymo Research Corp., Irvine, California, USA).

Plasmid DNA was isolated with the EZ-10 Spin Column Plasmid DNA Kit (Bio Basic Inc., Markham Ontario, Canada) and sequenced in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). For each fragment, the

nucleotide sequences of two or three clones were determined in both directions with sequencing primer M13.

DNA and deduced amino acid sequences were compared using NCBI BLASTN 2.2.27+ software (Zhang et al., 2000) and CLUSTAL W program (Thompson et al., 1994). The aligned sequences were edited with BioEdit v.7.0.9.0. (Ibis Biosciences, Carlsbad, Kalifornia, USA). Restriction enzyme sites were predicted using the TACG 3.2 program at Biology WorkBench (http://seqtool.sdsc.edu). A phylogenetic tree was generated using the maximum parsimony method with MEGA version 5.1 (Tamura et al., 2011).

3.2.5. Cloning and sequencing of the S_{7m}-*RNase* allele with unclarified function

Three accessions, 'Artvin-45', 'Eryatağı Amasya 0849' and 'Turfanda Kara' amplified a fragment of 376 bp in the first intron PCR and another of approximately 3000 bp for the second intron region. Since no alleles have been published with such sizes, these were hypothesized to be new alleles in sweet cherry.

We amplified the third exon of the *S-RNase* gene using EM-PC3consFD and EM-PC5consRD primer pairs. The amplicons of the 3rd exon region of the unknown *S-RNase* allele (later labelled as S_{7m}) of 'Turfanda Kara' (S_4S_{7m}) were screened using a colonial PCR and products were digested with *Eco*RV (Fermentas-Thermo Scientific, Burlington, Kanada). Only those colonies were used for plasmid preparation, which were not cut into the calculated sizes (81 and 165 bp, added to the lengths between the cloning site and annealing site of M13 primers), indicating the S_4-*RNase*.

4. RESULTS AND DISCUSSION

4.1. Variability of the *S*-locus of Turkish cherries

Twelve *S-RNase* alleles (S_1-S_7, S_9-S_{10} and S_{12}-S_{14}) were found in the 47 Turkish sweet cherry accessions out of the 13 alleles described by Sonneveld et al. (2003) from international cultivars; only S_{16} was not detected. Some wild cherry alleles, S_{17}-S_{19} and $S_{21/25}$ (Wünsch and Hormaza, 2004; De Cuyper et al., 2005) as well as S_{31} (Vaughan et al., 2008) were also detected (Figure 1). In addition, S_{34}, known as a "sour cherry allele" was identified in sweet cherry with a new (S_{37}) and a doubtful allele (provisionally labelled as S_{7m}). A total of 26 different *S*-genotypes were assigned to the 47 accessions, and one new cross-incompatibility group, XLV (S_2S_{18}) was established between the cultivars, 'Tezce 0912' and 'Yakacık' (the selection, Coll. 16 is also included in this group, which might be important during designing parental combinations in a breeding program).

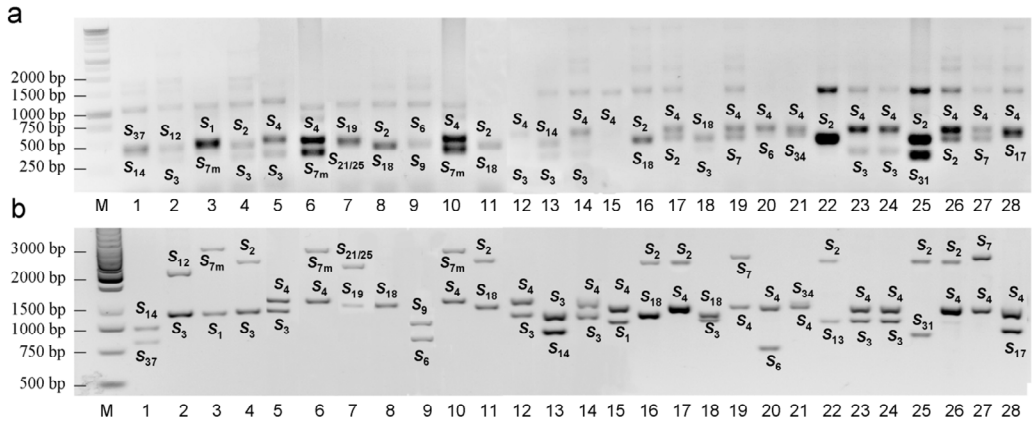

Figure 1. *S*-PCR analysis of 31 Turkish cherry accessions conducted using the PaConsI-F and PaConsI-R (**a**) and PaConsII-F and PaConsII-R (**b**) consensus primers amplifying the first and second introns of the Prunus SRNase gene, respectively. M: 1kb marker (Promega, Mannheim, Germany), 1. 'Artvin-5' ($S_{14}S_{37}$), 2. 'Artvin-43' (S_3S_{12}), 3. 'Artvin-45' (S_1S_{7m}), 4. 'Aydın Kirazı' 0890 (S_2S_3), 5. 'Elifli' (S_3S_4), 6. 'Eryatağı Amasya 0849' (S_4S_{7m}), 7. 'Tabanlı' ($S_{19}S_{21/25}$), 8. 'Tezce 0912' (S_2S_{18}), 9. 'Turfanda' (S_6S_9), 10. 'Turfanda Kara' (S_4S_{7m}), 11. 'Yakacık' (S_2S_{18}), 12. Coll. 1 (S_3S_4), 13. Coll. 12 (S_3S_{14}), 14. Coll. 13 (S_3S_4), 15. Coll 14 (S_1S_4), 16. Coll. 16 (S_2S_{18}), 17. Coll. 19 (S_2S_4), 18. Coll. 20 (S_3S_{18}), 19. Coll. 34 (S_4S_7), 20. Coll. 36 (S_4S_6), 21. Coll. 38 (S_4S_{34}), 22. Coll. 43 (S_2S_{13}), 23. Coll. 54 (S_3S_4), 24. Coll. 56 (S_3S_4), 25. Coll. 59 (S_2S_{31}), 26. Coll. 68 (S_2S_4), 27. Coll. 69 (S_4S_7) and 28. Coll. 71 (S_4S_{17}).

4.2. *S*-genotype revisions and methodological developments

Recently, *S*-genotypes have been proposed for 22 cultivars also tested in the present study using consensus and allele-specific PCR (Ipek et al., 2011). Nineteen *S*-genotypes were confirmed and three of them were improved. Ipek et al. (2011) described 'Kara Gevrek' as a

triploid cultivar but only the alleles S_3 and S_5 were confirmed in the present study. Although triploid genotypes are found rarely in wild populations and show vigorous growth, a diploid genome is more consistent with an old cultivar known to crop reliably. The S_2S_3 genotype of 'Aydın Siyahı' was corrected to S_2S_{10} based on allele-specific PCR and S_{10} was also confirmed by DNA sequencing. In case of 'Tabanlı', neither of the two alleles (S_2 and S_{10}) proposed by Ipek et al. (2011) was amplified by allele-specific primers in the present study. However, sequence analysis detected two wild cherry alleles, S_{19} and $S_{21/25}$. If the analysed samples in the two studies were indeed representatives of the same cultivar 'Tabanlı', cross-amplification of S_2 and S_{10} and wild cherry alleles should be checked carefully, although we did not experienced it in our study.

First, S_{25} was assigned to the cultivar 'Tabanlı' because BLASTN analysis revealed 100% identity between the 400 bp fragment of the first intron PCR product from 'Tabanlı' and S_{25} of 'Taleguera Brillante' (Wünsch and Hormaza, 2004). However, it must be mentioned that S_{25} seems to be identical with S_{21} over a great part of the second and third exon regions (Vaughan et al., 2008), and hence 'Tabanlı' may carry the S_{21} if the two labels are synonyms. An allele specific primer has been designed from S_{25} second intron sequence and gave positive signal.

Interestingly, the DNA sequence of S_{21} from a Belgian (De Cuyper et al., 2005) and a German (Schueler et al., 2006) wild cherry showed many indels and nucleotide substitutions over a long stretch of the second intron. The sequence of 'Tabanlı' $S_{21/25}$ completely matched that of the German wild cherry S_{21}-allele. Our allele-specific primer is expected to amplify only the Turkish/German allele and not the Belgian allele since it was designed from a region showing major differences between the two.

Twelve *S*-genotypes remained unresolved from the total of 47 Turkish accessions after consensus and available allele-specific PCR assays (Sonneveld et al., 2001; Sonneveld et al., 2003). It was associated with the present of wild and formerly unknown cherry alleles. We developed allele-specific primers for S_{17}, S_{18}, S_{19}, $S_{21/25}$, S_{34} and S_{37}, all of which worked reliably. Hence these alleles can be offered for the verification of *S*-genotypes assigned from consensus PCR, especially in a more diverse germplasm, e.g. local cultivars from the Middle East.

Our results demonstrate the limitation of using exclusively consensus primers for the reliable determination of *S*-genotypes. The chance of occurrence of functionally different *S-RNase* alleles with matching intron lengths is quite considerable in a diverse germplasm as was shown in the case of the S_9- and S_{31H}-*RNases* in Eastern European almonds (Halász et al., 2008). To achieve reliability and avoid the redundant allele-specific reactions, we propose a

two-step analysis by using first consensus PCR (pre-screening) and then using allele-specific primers to confirm doubtful alleles presumed from the consensus PCR (validation).

4.3. Insights into the *S-RNase* allele evolution

Trans-specific evolution (i.e. alleles from different species can be more similar than alleles in the same species) was apparently shown for the *S-RNases* in the *Solanaceae*, *Maloideae* and *Prunoideae* (Ioerger et al., 1990; Sassa et al., 1996; Sutherland et al., 2008). In the present study we found two trans-specific *S-RNase* alleles. Either of the two, S_{37}, is a newly identified allele in sweet cherry, which showed 99 % identity with the *P. speciosa* S_{13}-*RNase* with only two SNPs in the exon regions and the deletion of a large part of second intron. *P. speciosa* S_{13} was previously identified in only two individuals growing on a small island, Kouzu, in Japan (Kato et al., 2007). Since the Turkish accessions carrying S_{37} is native to the province Artvin, Turkey, this allele must have been inherited from the common ancestor of the *Pseudocerasus* (*P. speciosa*) and *Eurocerasus* (*P. avium*) sections (Bailey, 1927; Rehder, 1958).

Another example of trans-specific evolution was provided by the wild cherry S_{31}-*RNase* allele detected in Coll. 59. This too shared considerable similarity with a *P. speciosa* allele (AB289900), *P. armeniaca* S_{36}, *P. armeniaca* S_{20} and *P. mume* S_{32}. The intron sizes and exon-intron structure of S_{31} were very similar to those of S_{20} from the Hungarian apricot cultivar 'Ceglédi Piroska' (unpublished results).

A phylogenetic analysis reliably mirrored the presumed evolutionary distances among species of the sections *Cerasus* (*P. avium* and *P. speciosa*) and *Prunophora* (*P. armeniaca* and *P. mume*). Most variations were silent and hence they are not expected to degrade *S-RNase* function; apricot S_{20} was indeed confirmed to be fully functional (Halász, 2007). The variations among the DNA sequences of the trans-specific alleles might be correlated with the time after their divergence.

Although species divergence times within the *Rosaceae* family (http://paleodb.org) are still not available, silent mutations within the *S-RNase* gene are not exposed to natural selection and hence might be used as a molecular clock if rate constancy is assumed. The ongoing evolution of *Prunus S-RNases* was also captured in the nucleotide variations of the S_1, S_3, S_4 S_6 and S_{10} alleles.

The Turkish landrace cultivars and wild cherries compared with the database sequences (representing mainly old European cultivars) declared some intronic and non-synonymous

SNPs. The most characteristic example of *S*-allele evolution was provided by S_{7m}. Based on PCR assays and first intron sequence it seemed to be a new sweet cherry allele.

However, the C3-C5 region of the *S*-RNase protein was fully identical with that of S_7. The second intron is also supposed to be different from S_7 as its second intron region is approximately 3 kb instead of the 2385 bp described for S_7 (Sonneveld et al., 2003). In addition, the S_7 allele-specific PCR was also negative.

Although many amino acid replacements were revealed between C1 and C2, this region has not yet been demonstrated to be responsible for allele-specificity. This region of *Pyrus pyrifolia* S_3*-RNase* is not included in any of the four regions (PS1-PS4), in which positive selection may operate (Ishimizu et al., 1998). However, the same region in *Prunus S-RNase* seems to be more variable; and hence further analysis is required to clarify the functional identity of the allelic products of S_7 and S_{7m} and estimate the contribution of the region C1-C2 to allele specificity.

4.4. Crop evolutionary perspectives

Since the Black Sea coast of Turkey receives a great amount of rainfall (the eastern part of a region that receives 2,200 millimetres annually) (Sensoy, 2003), genotypes growing wild were screened for resistance to fruit cracking in 2003 and labelled as the Coll. series. These selections are similar in fruit size to commercial cultivars and may create the genetic basis of a new cherry breeding program in Turkey. Since these accessions were selected from wild populations, it is not surprising that some previously identified wild cherry alleles, S_{17}, S_{18} and S_{31} (De Cuyper et al., 2005; Vaughan et al., 2008) were also present in this germplasm.

However, other wild cherry alleles including S_{18}, S_{19} and $S_{21/25}$ were also identified in Turkish landrace cultivars. This is the first study to report the S_{18}-allele in cultivated sweet cherry ('Tezce 0912' and 'Yakacık'), which indicates that Turkish sweet cherry cultivars represent an essentially broader gene pool as compared with currently grown international cultivars. A similar phenomenon was seen in case of apricot (Halász et al., 2010) indicating great genetic resources are available in Turkey for *Prunus*.

Our analysis detected some aspects of the connections between sweet and sour cherry germplasm. The selection Coll. 38 was clarified to carry an allele, S_{34}, which has been only detected in sour cherry until now. Tsukamoto et al. (2008) hypothesized that this allele, together with the S_{33} and S_{35}, might have been contributed by ground cherry (*P. fruticosa*), which is a parent of sour cherry (Iezzoni and Hancock, 1996; Tavaud et al., 2004).

Our study clarified the origin of this allele from sweet cherry by showing that this allele is carried by a wild cherry collected around the Black Sea coast, a region where sour cherry is also abundant. Our finding is further supported by the remark of Tsukamoto et al. (2008), who mentioned a survey of a *P. fruticosa* collection that has resulted in the identification of multiple accessions possessing the S_{33}- and S_{35}-haplotypes but no individual was found to carry the S_{34}-haplotype.

Sequence variations between the sour and sweet cherry S_{34} may reflect the time that passed after their divergence and be associated with the tetraploid genome of sour cherry where a paralogous gene might have experienced an accelerated evolution (Tsukamoto et al., 2006).

Recently, Marchese et al. (2010) have described that intra-allelic variations of the microsatellite regions located in both introns of the cherry S_{13}-*RNase* gene can be used to distinguish sweet, wild and sour cherries. In sweet and wild cherries, sizes of the first intron SSR regions ranged from 263 to 273 bp, and those of the second intron ranged from 316 to 322 bp. S_{13} was carried by only one accession in the tested germplasm, Coll. 43. The sizes of the SSR regions in the first and second introns were 251 and 310 bp, respectively.

These SSR variants were exclusively found in sour cherry by Marchese et al. (2010). These data confirm that allele pools of sweet and sour cherry in the Black Sea region are overlapping and show that species identification based on S_{13} SSR variation may be misleading when applied to trees native to the centre of origin of these species.

The 251-bp variant was carried by only two sour cherry cultivars, including the Hungarian ‘Favorit’ (Marchese et al., 2010). One of its parents was an old Hungarian landrace cultivar, ‘Pándy’. Many unproven hypotheses are known regarding the origin of Pándy, one of which reports that a Turkish military officer brought it from Turkey to Hungary during the time of Turkish occupation in Hungary (Faust and Surányi, 1997). This origin of ‘Pándy would give an explanation for the collective occurrence of such a rare SSR variant in the Hungarian sour cherry cultivar ‘Favorit’ and a Turkish wild cherry. A detailed analysis of the Turkish and Hungarian sweet and sour cherries may shed light on the historical link between these germplasm.

5. NEW SCIENTIFIC RESULTS

1) We determined the *S*-genotypes of 47 landrace Turkish cultivars and 17 local genotypes selected from populations growing wild at the Black Sea coast. The results can be used all over the world in the breeding programs of cultivars resistant to fruit cracking.
2) We identified a new *S-RNase* allele in sweet cherry (S_{37}) and a new cross incompatibility group (CIG) with S_2S_{18} genotype (Group XLV).
3) We proved that the S_{34}-*RNase* allele identified in sour cherry originated from *Prunus avium*, and not the *Prunus fruticosa* parent species.
4) The detected great genetic diversity in the gene centre of sweet cherry can be associated with the hybridization among cultivated and wild sweet cherries as well as sour cherry.
5) We developed new allele-specific primers to detect some *S*-alleles. These primers increase the reliability of the detection of *S*-genotype in case of germplasm of great genetic diversity.
6) The variations among the DNA sequences not exposed to natural selection of the trans-specific alleles could be correlated with putative phylogenetic relationships within the *Prunoideae* subfamily. These mutations might be used in phylogenetic analysis.

REFERENCES

1. Bailey, L.H. (1927): The standard cyclopedia of horticulture. Macmillan, London.
2. Békefi, Z., Vaughan, S., Tobutt, K. (2010): Determination of incompatibility (*S*) genotypes of sweet cherries in the Hungarian gene-bank by a PCR-based method. Acta Agron. Hung., 58: 377–384.
3. Békefi, Zs., Tobutt, K.R., Sonneveld, T. (2003): Determination of (in)compatibility genotypes of Hungarian sweet cherry (*Prunus avium* L.) accessions by PCR based methods. Int. J. Hort. Sci., 9: 37–42.
4. Bošković, R., Tobutt, K.R. (2001): Genotyping cherry cultivars assigned to incompatibility groups, by analysing stylar ribonucleases. Theor. Appl. Genet., 103: 475–485.
5. Bošković, R., Russell, K., Tobutt, K.R. (1997): Inheritance of stylar ribonucleases in cherry progenies, and reassignment of incompatibility alleles to two incompatibility groups. Euphytica, 95: 221–228.
6. Crane, M.B., Lawrence, W.J.C. (1929): Genetical and cytological aspects of incompatibility and sterility in cultivated fruits. J. Pomol. Hort. Sci., 7: 276–301.
7. De Candolle, A. (1894): Termesztett növényeink eredete. K.M. Természettudományi Társulat, Budapest.
8. De Cuyper, B., Sonneveld, T., Tobutt, K.R. (2005): Determining selfincompatibility genotypes in Belgian wild cherries. Mol. Ecol., 14: 945–955.
9. de Nettancourt, D. (2001): Incompatibility in angiosperms. Springer-Verlag, New York.
10. Entani, T., Iwano, M., Shiba, H., Che, S.F., Isogai, A., Takayama, S. (2003): Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen-expressed F-box gene with allelic-diversity. Genes Cells, 8: 203–213.
11. Ercisli, S. (2004): A short review of the fruit germplasm resources of Turkey. Genet. Res. Crop Evol., 51: 419–435.
12. Faust, M., Surányi, D. (1997): Origin and dissemination of cherry. Hort. Rev., 19: 263–317.
13. Gisbert, A.D., Badenes, M.L., Tobutt, K.R., Llacer, G., Romero, C. (2008): Determination of the *S*-allele composition of sweet cherry (*Prunus avium* L.) cultivars grown in the southeast of Spain by PCR analysis. J. Hortic. Sci. Biotech., 83: 246–252.
14. Halász, J. (2007): A kajszi önmeddőségét meghatározó *S*-allél-rendszer molekuláris háttere. Doktori Értekezés, BCE KTK, Budapest (Kézirat).
15. Halász, J., Fodor, Á., Hegedűs, A., Pedryc, A. (2008): Identification of a new self-incompatibility allele (S_{31}) in a Hungarian almond cultivar and its reliable detection. Sci. Hortic.-Amsterdam, 116: 448–451.
16. Halász, J., Pedryc, A., Ercisli, S., Yilmaz, K.U., Hegedus, A. (2010): *S*-genotyping supports the genetic relationships between Turkish and Hungarian apricot germplasm. J. Amer. Soc. Hort. Sci., 135: 410–417.
17. Iezzoni, A.F., Hancock, A.M. (1996): Chloroplast DNA variation in sour cherry. Acta Hort., 410: 115–120.
18. Ioerger, T.R., Clark, A.G., Kao, T.H. (1990): Polymorphism at the selfincompatibility locus in *Solanaceae* predates speciation. P. Natl. Acad. Sci. USA, 87: 9732–9735.
19. Ipek, A., Gulen, H., Akcay, M.E., Ipek, M., Ergin, S., Eris, A. (2011): Determination of self-incompatibility groups of sweet cherry genotypes from Turkey. Gen. Mol. Res., 10: 253–260.
20. Ishimizu, T., Endo, T., Yamaguchi-Kabata, Y., Nakamura, K.T., Sakiyama, F., Norioka, S. (1998): Identification of regions in which positive selection may operate in *S*-RNase of *Rosaceae*: Implication for *S*-allele-specific recognition sites in *S*-RNase. FEBS Lett., 440: 337–342.
21. Kato, S., Iwata, H., Tsumura, Y., Mukai, Y. (2007): Distribution of *S*-alleles in island populations of flowering cherry, *Prunus lannesiana* var. *speciosa*. Genes Genet. Syst., 82: 65–75.
22. Lai, Z., Ma, W., Han, B., Liang, L., Zhang, Y., Hong, G., Xue, Y. (2002): An F-box gene linked to the self-incompatibility (*S*) locus of *Antirrhinum* is expressed specifically in pollen and tapetum. Plant Mol. Biol., 50: 29–42.

23. Marchese, A., Bošković, R., Caruso, T., Tobutt, K. (2010): Intra-allelic variation in introns of the *S_{13}-RNase* allele distinguishes sweet, wild and sour cherries. Tree Genet. Genomes, 6: 963–972.
24. Marchese, A., Tobutt, K.R. Raimondo, A., Motisi, A., Boškovic, R.I., Clarke, J., Caruso, T. (2007): Morphological characteristics, microsatellite fingerprinting and determination of incompatibility genotypes of Sicilian sweet cherry cultivars. J. Hortic. Sci. Biotech., 82: 41–48.
25. Matthews, P., Dow, K.P. (1969): Incompatibility groups: sweet cherry (*Prunus avium*). In: R.L. Knight (Ed.) Abstract Bibliography of Fruit Breeding & Genetics to 1965, *Prunus*, pp. 540–544. Commonwealth Agricultural Bureaux, Farnham Royal.
26. McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F., Clarke, A.E. (1989): Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. Nature, 342: 955–957.
27. Rehder, A. (1958): Manuel of cultivated trees and shrubs hardy in North America. Macmillan, New York.
28. Sassa, H., Nishio, T., Kowyama, Y., Hirano, H., Koba, T., Ikehashi, H. (1996): Self-incompatibility (*S*) alleles of the *Rosaceae* encode members of a distinct class of the T2/*S* ribonuclease superfamily. Mol. Gen. Genet., 250: 547–557.
29. Schueler, S., Tusch, A., Scholz, F. (2006): Comparative analysis of the within-population genetic structure in wild cherry (*Prunus avium* L.) at the self-incompatibility locus and nuclear microsatellites. Mol. Ecol., 15: 3231–3243.
30. Schuster, M., Flachowsky, H., Köhler, D. (2007): Determination of self-incompatible genotypes in sweet cherry (*Prunus avium* L.) accessions and cultivars of the German Fruit Gene Bank and from private collections. Plant Breeding, 126: 533–540.
31. Sensoy, S. (2003): Climate of Turkey. Turkish State Meteorological Service. http://213.139.210.130/2003eng/general/climate/climateofturkey.htm.
32. Sonneveld, T., Robbins, T.P., Bošković, R., Tobutt, K.R. (2001): Cloning of six sweet cherry self-incompatibility alleles and development of allelespecific PCR detection. Theor. Appl. Genet., 102: 1046–1055.
33. Sonneveld, T., Tobutt, K.R., Robbins, T.P. (2003): Allele-specific PCR detection of sweet cherry self-incompatibility (*S*) alleles S_1 to S_{16} using consensus and allele-specific primers. Theor. Appl. Genet., 107: 1059–1070.
34. Sutherland, B.G., Robbins, T.P., Tobutt, K.R. (2004): Primers amplifying a range of *Prunus S*-alleles. Plant Breeding, 123: 582–584.
35. Sutherland, B.G., Tobutt, K.R., Robbins, T.P. (2008): Trans-specific *S-RNase* and *SFB* alleles in *Prunus* self-incompatibility haplotypes. Mol. Genet. Genomics, 279: 95–106.
36. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011): MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28: 2731–2739.
37. Tavaud, M., Zanetto, A., David, J.L., Laigret, F., Dirlewanger, E. (2004): Genetic relationships between diploid and allotetraploid cherry species (*Prunus avium*, *Prunus x gondouinii* and *Prunus cerasus*). Heredity, 93: 631–638.
38. Tobutt, K.R., Sonneveld, T., Bekefi, Z., Bošković, R. (2005): Cherry (in)compatibility genotypes - an updated cultivar table. Acta Hort., 663: 667–671.
39. Tsukamoto, T., Hauck, N., Tao, R., Jiang, N., Iezzoni, A. (2006): Molecular characterization of three non-functional *S*-haplotypes in sour cherry (*Prunus cerasus*). Plant Mol. Biol., 62: 371–383.
40. Tsukamoto, T., Potter, D., Tao, R., Vieira, C.P., Vieira, J., Iezzoni, A.F. (2008): Genetic and molecular characterization of three novel *S*-haplotypes in sour cherry (*Prunus cerasus* L.). J. Exp. Bot., 59: 3169–3185.
41. Ushijima, K., Sassa, H., Tao, R., Yamane, H., Dandekar, A.M., Gradziel, T.M., Hirano, H. (1998): Cloning and characterization of cDNAs encoding *S*-RNases from almond (*Prunus dulcis*): primary structural features and sequence diversity of the *S*-RNases in *Rosaceae*. Mol. Gen. Genet., 260: 261–268.
42. Ushijima, K., Sassa, H., Dandekar, A.M., Gradziel, T.M., Tao, R., Hirano, H. (2003): Structural and transcriptional analysis of the self-incompatibility locus of almond:

identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. Plant Cell, 15: 771–781.
43. Vaughan, S., Bošković, R., Gisbert-Climent, A., Russell, K., Tobutt, K. (2008): Characterisation of novel *S*-alleles from cherry (*Prunus avium* L.). Tree Genet. Genomes, 4: 531–541.
44. Wünsch, A., Hormaza, .JI. (2004): Cloning and characterization of genomic DNA sequences of four self-incompatibility alleles in sweet cherry (*Prunus avium* L.). Theor. Appl. Genet., 108: 299–305.

PUBLICATIONS CONNECTED TO THE DISSERTATION

Articles in impact factored journals:

Szikriszt B., Dogan A., Akcay ME, Ercisli S., Hegedűs A., Halász J. (2012): Molecular typing of the self-incompatibility locus of Turkish sweet cherry genotypes reflects phylogenetic relationships among cherries and other *Prunus* species. Tree Genet. Genomes (11 July 2012), pp. 1-11, ISSN 1614-2942, DOI:10.1007/s11295-012-0543-2. **IF 2.335**

Other journal articles:

Szikriszt, B., Papp, N., Taller, D., Halász, J., Nyéki, J., Szabó, Z., Stefanovits-Bányai, É., Hegedűs, A. (2011): Preliminary evaluation of breeding perspectives of Ukrainian sweet cherry cultivars: nutraceutical properties and self-incompatibility. International Journal of Horticultural Science, 17: 7–11.

Szikriszt, B., Hegedűs, A., Halász, J. (2011): Review of genetic diversity studies in almond (*Prunus dulcis*). Acta Agron. Hung., 59(4): 379–395. DOI: 10.1556/AAgr.59.2011.4.9

Szikriszt B., Hegedűs A., Halász J. (2011): A mandula (*Prunus dulcis*) genetikai sokféleségének összefoglaló áttekintése. Kertgazdaság, 43.(4): 77-87.

Pfeiffer, P., Szikriszt, B., Hegedűs, A. (2012): Valós idejű PCR alkalmazása a növénybiológiai kutatásokban. Kertgazdaság, (in press)

Szikriszt, B., Ercisli, S., Hegedűs, A., Halász, J. (2012): Self-incompatibility alleles in Eastern European and Asian almond (*Prunus dulcis*) genotypes: a preliminary study. International Journal of Horticultural Science, 18(2): 23-26.

Conference papers (Abstracts):

Szikriszt B., Hegedűs A., Halász J. (2011): Ukrán cseresznyefajták *S*-genotípusának DNS-alapú vizsgálata. XVII. Növénynemesítési Tudományos Napok, 2011. április 27., Összefoglalók (Szerk.: Óvári, J.), BCE, KeTK, Budapest, p. 99. ISBN 978-963-08-1235-1.

Szikriszt B., Dogan A., Ercisli S., Hegedűs A., Halász J. (2011): Török cseresznyefajták *S*-genotípusának molekuláris jellemzése. XVII. Növénynemesítési Tudományos Napok, 2011. április 27., Összefoglalók (Szerk.: Óvári, J.), BCE, KeTK, Budapest, p. 95. ISBN 978-963-08-1235-1.

Halász, J., Szikriszt, B., Ercisli, S., Yilmaz, K.U., Dogan, A., Szabó, Z., Nyéki, J., Pedryc, A., Hegedűs, A. (2011): *S*-locus genotyping on stone fruits. Flower biology and *S*-incompatibility in fruit species, 22–25 June 2011, San Michele all'Adige (Trento) and Bologna, Italy. Abstract book, 2 pages.

Szikriszt B., Dogan A., Akcay M. E., Ercisli S., Hegedűs A., Halász J. (2012): Az *S*-lókusz variabilitása a géncentrumban: török cseresznye genotípusok termékenyülési kapcsolatának molekuláris jellemzése. XVIII. Növénynemesítési Tudományos Napok, 2012. március 6., Összefoglalók (Szerk.: Veisz, O.), Magyar Tudományos Akadémia, Budapest, p. 52. ISBN: 978-963-8351-38-8.

Halász J., Szikriszt B., Ercizli S., Pedryc A., Hegedűs A. (2012): Az *S*-lókusz variabilitásának jellemzése egy vadon élő török kajszipopulációban. XVIII. Növénynemesítési Tudományos Napok, 2012. március 6., Összefoglalók (Szerk.: Veisz, O.), Magyar Tudományos Akadémia, Budapest, p. 86. ISBN: 978-963-8351-38-8.

J. Halász; B. Szikriszt, S. Ercisli, Z. Szabó, J. Nyéki, M. Tóth, A. Pedryc, A. Hegedűs (2012): The self-incompatibility locus provides insight into the evolution of cultivated fruit trees, 15–18 April 2012, 2nd Global Congress on Plant Reproductive Biology (PRB-2012), Pécs, Hungary. Abstracts, p. 34.

A. Hegedűs, B. Szikriszt, A. Dogan, E. M Akcay, S. Ercisli, J. Halász (2012): self-incompatibility analysis of turkish *Prunus avium* germplasm: information from the centre of origin of cherries, 15–18 April 2012, 2nd Global Congress on Plant Reproductive Biology (PRB-2012), Pécs, Hungary. Abstracts, p. 35.

B. Szikriszt, S. Ercisli, A. Hegedűs, J. Halász (2012): The latest achievements in almond (*Prunus dulcis*) self-incompatibility studies, 15–18 April 2012, 2nd Global Congress on Plant Reproductive Biology (PRB-2012), Pécs, Hungary. Abstracts, p. 66.

J. Halász, B. Szikriszt, S. Ercizli, Z. Szabó, J. Nyéki, M. Tóth, A. Pedryc, A. Hegedűs (2012): Recent progress in fruit tree self-incompatibility studies, 4-6 June 2012, Pannonian Plant Biotechnology Workshops, Advances in plant breeding and plant biotechnology in Central Europe, Debrecen, Hungary. Book of Abstracts and programme: p. 16.

B. Szikriszt, S. Ercizli, A. Pedryc, A. Hegedűs, J. Halász (2012): Self-incompatibility RNase alleles in a wild-growing Turkish apricot population, 4-6 June 2012, Pannonian Plant Biotechnology Workshops, Advances in plant breeding and plant biotechnology in Central Europe, Debrecen, Hungary. Book of Abstracts and programme: p. 60.

B. Szikriszt, S. Ercizli, A. Hegedűs, J. Halász (2012): Variations in almond (*Prunus dulcis*) self-incompatibility alleles: from Eastern Europe to Western Asia. 1-5 July 2012, 2nd Symposium on Horticulture in Europe, Angers, France. Book of abstracts: p. 235.

Supervisor of Scientific Student-Conference (TDK) paper:

TDK-paper: Taller Dénes (Mezőgazdasági Biotechnológus MSc. I. évf.): Ukrán cseresznyefajták funkcionális nemesítési programbeli felhasználhatóságának értékelése. 2011. BCE Genetika és Növénynemesítés Tanszék

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