Determination of (in)compatibility genotypes of Hungarian sweet cherry (*Prunus avium* L.) accessions by PCR based methods

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Summary: Sweet cherries *(Prunus avium* L.*)* are generally self-incompatible and pollinator cultivars are needed in orchards for reliable yield. In Hungary, choosing the appropriate cross-compatible cultivar pairs has so far been based on traditional test-crosses in the field. In recent years PCR-based methods that allow the identification of the *S*-alleles responsible for compatibility have been elaborated. We have determined the *S*-allele constitution of 24 cultivars and four selections important to Hungarian growers and breeders using PCR-based methods developed at Horticulture Research International, East Malling. The 28 accessions had various pairs of 9 alleles including one new allele, S_x. They could be assigned to 12 of the existing incompatibility groups or to a new group (S_4S_{12}) for which the designation 'Group XXVII' is proposed. The cultivars 'Krupnoplodnaja' and 'Rita' had novel genotypes, S_5S_9 and S_5S_x, respectively and can be placed into group O that holds universal pollen donors. The genotype of the cultivar 'Hedelfingeni Orias' grown in Hungary was found to be S_3S_4 and therefore different from the cultivar 'Hedelfingen' that is widespread in Western Europe.

Key words: incompatibility, sweet cherry, *S*-alleles

Introduction

Self-incompatibility in sweet cherry and cross-incompatibility between various cultivar pairs has been known for a long time *(Gardener,* 1914; *Kobel & Steinegger,* 1933; *Crane & Brown,* 1937). These phenomena were attributed to a multi-allelic locus, *S*, by *Crane & Lawrence* (1929). The alternative forms of *S*-alleles are designated S_1, S_2, S_3, etc. Cultivars bearing the same combination of *S*-alleles can not fertilise each other and constitute an incompatibility group. The cultivars within the same incompatibility group are cross-incompatible but compatible with cultivars in all other groups and can fertilise each other if they bloom at the same time. Although sweet cherry is basically self-incompatible, *Lewis & Crowe* (1954) using X-irradiated pollen for nominally incompatible pollinations obtained some self-compatible selections. In the selection JI 2420 the S_4 allele had mutated to S_4' *(Lewis & Crowe,* 1954; *Matthews,* 1970) and in JI 2434 the S_3 allele to S_3' *(Bošković* et al., 2000) where the prime symbol indicates a mutation in the pollen part.

Until recently sweet cherry compatibility was studied mostly with traditional test crossings. Thus in Hungary, *Maliga* (1952) tested the cross-compatibility of 'Badacsonyi Orias' and 'Germersdorfi Orias' (considered the same as 'Schneiders Späte Knorpelkirsche') with other Hungarian cultivars and determined two inter-incompatible cultivar pairs. Likewise *Brózik* (1962) carried out compatibility studies in 'Germersdorfi Orias' and determined suitable pollinators. *Brózik & Nyéki* (1975) specified groups of Hungarian cultivars that are not only cross-compatible but bloom simultaneously. These authors later determined some incompatibility groups of Hungarian cultivars *(Brózik & Nyéki* 1980). *Nyéki* (1989) classified various Hungarian and foreign cultivars into incompatibility groups, work completed by *Nyéki & Szabó* (1995). *Apostolné* (1994) clarified compatibility relationships of several new Hungarian cultivars. *Szabó* et al. (2002) summarised the knowledge about Hungarian sweet cherry compatibility.

Matthews & Dow (1969), drawing on test crossing results from the John Innes Institute and overseas, published the *S*-genotype of some 160 cultivars. This classic work became an important compilation for the scientists studying sweet cherry incompatibility. They reported six *S*-alleles and assigned cultivars into 13 incompatibility groups. Later, using stylar protein analysis, *Bošković & Tobutt* (2001) re-examined the *S*-genotypes of the cultivars to be found in the work of *Matthews & Dow* (1969): genotypes of some cultivars were corrected and some additional cultivars were assigned to incompatibility groups.

The work of *Bošković & Tobutt* (1996) demonstrated that the products of sweet cherry *S*-alleles are stylar ribonucleases, the so called *S*-RNases. This finding led to molecular techniques of genotyping and so revolutionised

cherry incompatibility studies. Analysing *S*-RNases extracted from the style resulted in the findings of new *S*-alleles and incompatibility groups *(Bošković* et al. 1997; *Bošković & Tobutt* 2001).

Recently, PCR-based methods have been developed that enable *S*-genotypes of sweet cherry to be determined from vegetative material such as leaves and buds. Some authors *(Tao* et al., 1999; *Wiersma* et al, 2001) designed consensus primer pairs to amplify the two intron regions (1st and 2nd intron) and separate certain *S*-alleles on the basis of their intron length polymorphism. *Sonneveld* et al. (2003) has also developed consensus primers that are more generally useful. *Sonneveld* et al. (2001 and 2003) designed allele-specific primer pairs: each can detect one particular *S*-allele so they are ideal for confirming *S*-allele scores derived from consensus primer analysis.

Results of recent *S*-genotyping of sweet cherry cultivars have been summarised by *Tobutt* et al. (2001). Thirteen *S*-alleles (S_1 to S_{16}, excluding S_8, S_{11} and S_{15}, which are duplicates) and 26 incompatibility groups are known at the moment in sweet cherry, along with a group O of unique genotypes which are universal pollen donors and the SC group containing self-compatible cultivars *(Tobutt* et al., in press).

Our aim was to clear up compatibility relationships among various old and novel cultivars important in Hungary and new selections by means of *S*-allele analysis. In the interest of completeness, results of 'Alex', 'Aida' and 'Peter' are included, although they have recently been genotyped by *Sonneveld* et al. (2003).

Material and method

Plant materials

The accessions analysed were growing in the experimental field of the Research Institute for Fruitgrowing and Ornamentals in Erd *(Table 1)*. Shoots bearing dormant buds have collected from the nuclear stock plantation (cultivars) and from research trial plots (selections). Cultivars used as standards came from the collection of Horticulture Research International, East Malling *(Table 2)*. Genomic DNA was extracted from buds according to a miniprep version of the CTAB method *(Doyle & Doyle,* 1987) as modified by *Sonneveld* et al. (2001).

Table 1 Cherry accessions analysed and their parentages where known

Cultivars	Country of origin	Mother	Father	
Alex	Hungary	Van	Cherry Self Fertile 46 (JI 2538?)*	Brózik, S., Apostol, J. (2000)
Botond	Hungary	?	?	
Germersdorfi 1	Hungary			
Germersdorfi 3	Hungary			
Hedelfingeni Orias	Hungary			
Katalin	Hungary	Schneiders Späte Knorpel	Podjebrad	Brózik S., Apostol J. (2000)
Kavics	Hungary	Schneiders Späte Knorpel	Budakalászi helyi fekete	Brózik S., Apostol J. (2000)
Krupnoplodnaja	Hungary	?	?	
Linda	Hungary	Hedelfingeni Orias	Schneiders Späte Knorpel	Brózik S., Apostol J. (2000)
Margit	Hungary	Schneiders Späte Knorpel	o.p.	Brózik S., Apostol J. (2000)
Münchebergi Korai	Germany	Flamentiner(?)	Márki korai	Brózik S., Apostol J. (2000)
Solymári Gömbölyű	Hungary			
Szomolyai Fekete	Hungary			
Trusenszkaja 2	Russia			
Valerij Cskalov	Russia			
Vera	Hungary	Ljana (Trusenszkaja 6)	Van	Brózik S., Apostol J. (2000)
candidate cultivars (novelties) from Budapest, Research Institute for Fruitgrowing and Ornamentals				
III-42/114 (Carmen)		Yellow Dragan	H 203	Brózik S., Apostol J. (2000)
IV-3/41 (Anita)		Trusenszkaja 2	H 3	Brózik S., Apostol J. (2000)
IV-5/62 (Rita)		Trusenszkaja 2	H 2	Brózik S., Apostol J. (2000)
IV-6/5 (Péter)		Bigarreau Burlat	Stella	Brózik S., Apostol J. (2000)
IV-6/12 (Sándor)		Bigarreau Burlat	Stella	Brózik S., Apostol J. (2000)
IV-6/39 (Pál)		Bigarreau Burlat	Stella	Brózik S., Apostol J. (2000)
IV-13/20 (Aida)		Moldvai Fekete	H 236	Brózik S., Apostol J. (2000)
IV-13/51 (Tünde)		Yellow Dragan	Bigarreau Burlat	Brózik S., Apostol J. (2000)
hybrids, breeding lines from Budapest, Research Institute for Fruitgrowing and Ornamentals				
IV-5/5		Trusenszkaja 2	H 1	Apostol (pers. comm.)
IV-6/66		Bigarreau Burlat	Stella	Apostol J, Brózik S (1998)
IV-6/240		?	?	
IV-13/120		Yellow Dragan	Bigarreau Burlat	Apostol (pers. comm.)

*an accession received from the John Innes Institute, possibly as 1411/46 (Apostol, pers. comm.); 1411/46 is the same as JI 2538 (Matthews and Lapins, 1967)

PCR with consensus primers

Preparation of a 25 µl PCR amplification reaction, its content, final concentrations, the number of PCR cycles and temperatures were performed according to the protocol described by *Sonneveld* et al. (2003). The consensus primers used were: 2nd intron, PaConsII-F and PaConsII-R; 1st intron, PaConsI-F and PaConsI-R *(Sonneveld* et al., 2003). A negative control (water) was included. PCR reactions were carried out in a PTC-200 thermal cycler (MJ Research).

PCR products were separated by electrophoresis, for the 2nd intron on a 1.3% agarose gel for 16h at 60V and for the 1st intron on a 2.0% agarose gel for 17h at 60V. Molecular weight ladders, 1kb+, were included. After electrophoresis the gels were stained in a 0.5 µl/ml ethidium-bromide solution for 1h. Photographs were taken on an ultraviolet transilluminator. Provisional genotypes deduced by comparing banding patterns of the Hungarian cultivars with those of the standard cultivars.

PCR with allele-specific primers

After provisional genotypes had been deduced from the amplification patterns of the consensus primers separate PCR reactions were set up with different allele-specific primers *(Sonneveld* et al., 2001, 2003) and annealing temperatures according to the protocol described by *Sonneveld* et al. (2003) *(Table 3)*. In order to avoid false negatives in PCR reactions an additional internal control primer pair was used in each reaction *(Sonneveld* et al., 2003). PCR products were run on a 1.5% agarose gel for 1h at 90V. Then the gels were handled as described in the analysis of the 2nd intron above.

Table 2 Cherry cultivars used as standards and their *S*-genotypes

Cultivar	Genotype
Early Rivers	S_1S_2
Victor	S_2S_3
Lapins	S_1S_4'
Napoleon	S_3S_4
Late Black Bigarreau	S_4S_5
Colney	S_5S_6
Charger	S_1S_7
Inge	S_4S_9
Orleans 171	S_7S_{11}
Schneiders Späte Knorpelkirsche	S_3S_{12}
Noble	S_6S_{13}
Dikkeloen	S_5S_{14}

Results

Electrophoretic banding patterns of the accessions after amplification with the 2nd intron primers are shown in *Figure 1*. Examining the patterns of the standard cultivars it showed that the alleles $S_1/S_3/S_{13}$ and S_2/S_5 are not easily distinguishable. After 1st intron analysis *(Figure 2)* these alleles could be distinguished. On the basis of the 1st and 2nd intron analyses together it was possible to determine the *S*-genotypes of the Hungarian accessions. Finally, results were confirmed with allele-specific PCR. *S*-allele scores and assignment of analysed accessions into incompatibility groups are given in *Table 3*.

IV-5/62 ('Rita') appears to have a new allele, S_x. Its 2nd intron length is approximately 2300 bp and the 1st intron is appr. 420 bp as estimated from its patterns. Although this

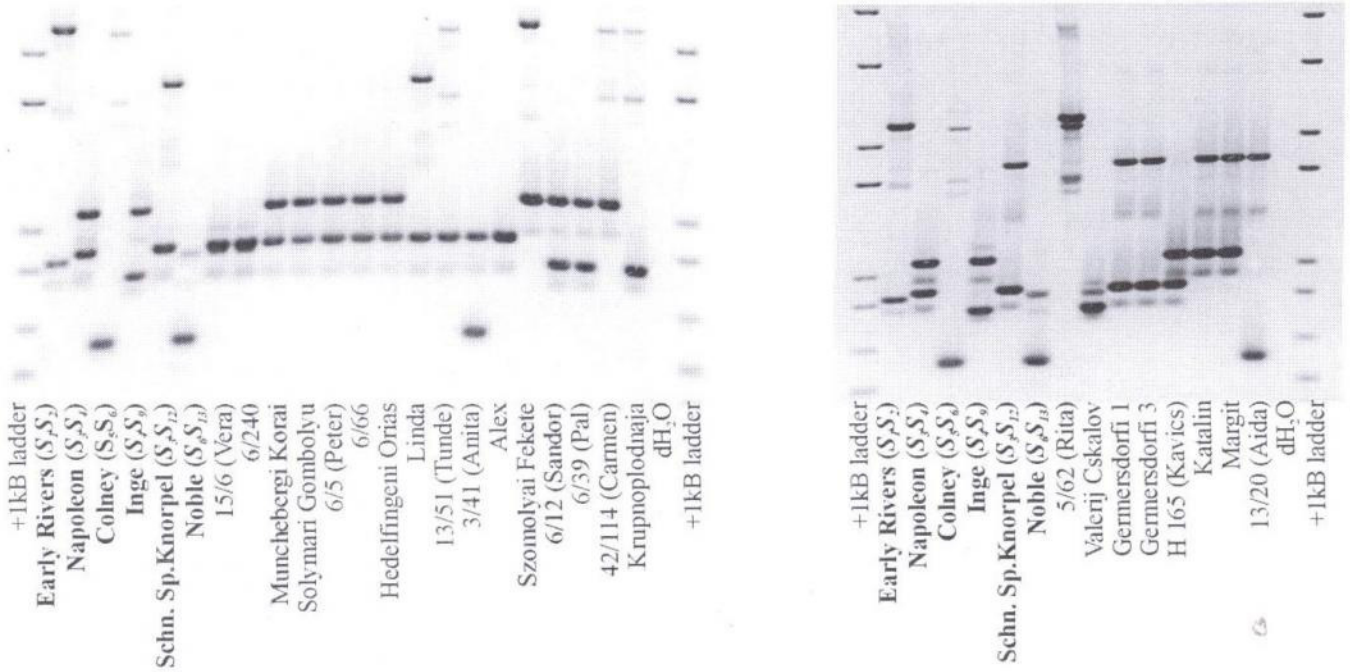

Figure 1 PCR amplification of the accessions with consensus primers for the 2nd intron (cultivars in bold: standards)

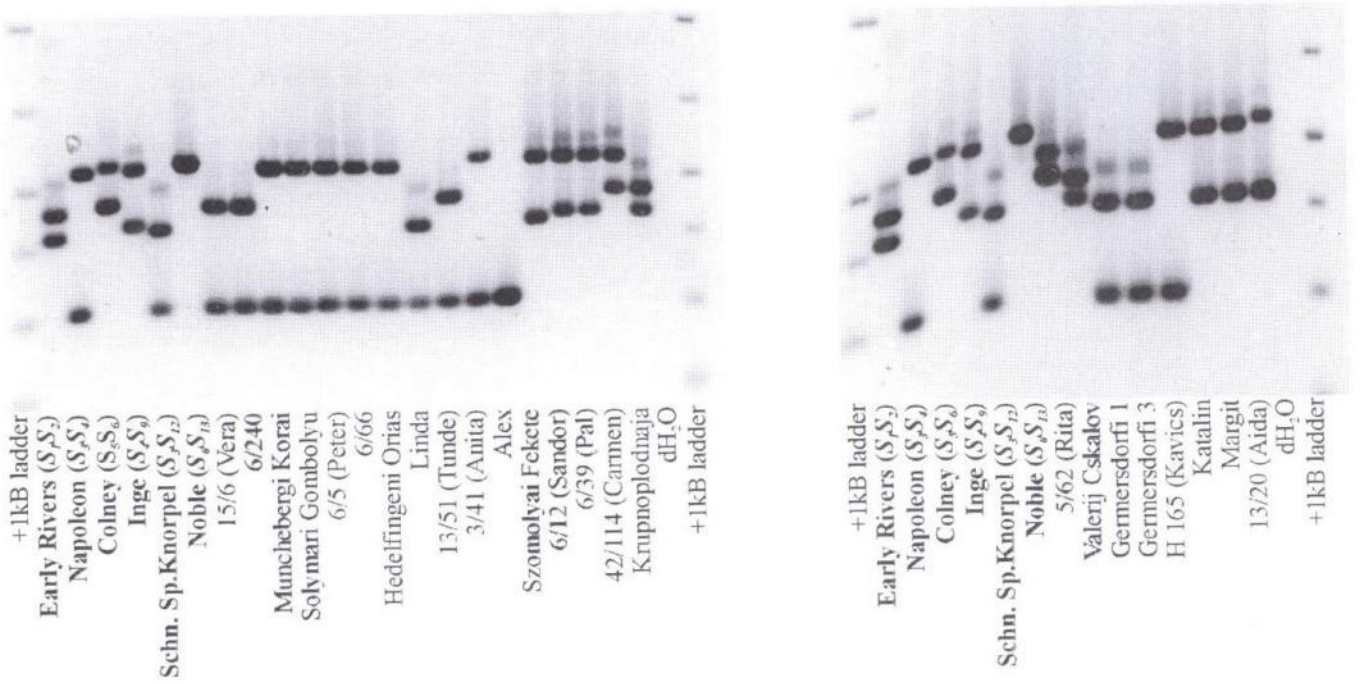

Figure 2 PCR amplification of the accessions with consensus primers for the 1st intron (cultivars in bold: standards)

intron is similar in size to S_2 and S_7, it does not amplify with primers specific for S_2 and S_7. *Figure 3* shows the patterns of IV-5/62 ('Rita') after amplification with the S_2-allele specific primers. As the S_2 band is missing, but the internal control band is present, the unknown allele can not be S_2. We had similar results with S_7 allele-specific primers.

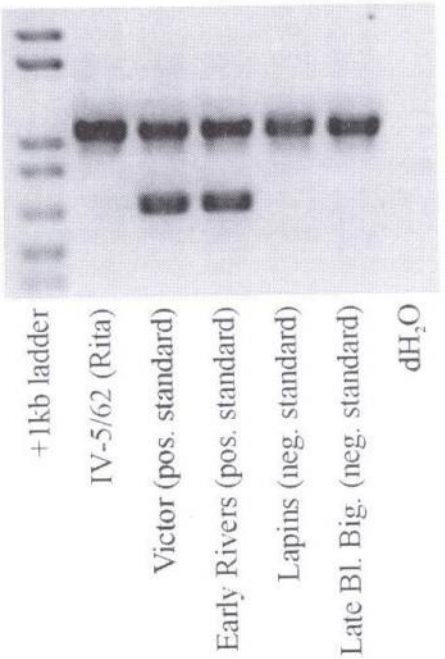

Figure 3 PCR amplification of cultivar 'Rita' with allele-specific primers for S_2

The banding patterns of 'Germersdorfi 1' and 'Germersdorfi 3' were the same. Moreover, they were the same as of 'Schneiders Späte Knorpel', thus belonging to group XXII.

The genotype of 'Valerij Cskalov', a cultivar originating from Russia, proved to be S_1S_9 and can be placed into group XVIII.

Discussion

Traditionally, (in)compatibility relationship of sweet cherry have been studied with test crosses in the field. However, results are greatly affected by weather, tree conditions, quality of pollen, etc. and are often inconsistent. PCR-based methods allow us to eliminate external factors and study (in)compatibility genotypes directly.

According to *Table 3*, of 28 accessions, 26 could be assigned to 12 of the existing incompatibility groups. The most frequent alleles were S_3 (appeared in 16 cultivars) and S_1 (in 14 cultivars) while the least common alleles were S_2, only in 'Szomolyai Fekete', a Hungarian local cultivar and S_x, in 'Rita'.

'Katalin' and 'Margit' have the new genotype S_4S_{12} thus they form a new incompatibility group (XXVII).

The genotypes of 'Krupnoplodnaja' (S_5S_9) and 'Rita' (S_5S_x) are unique, therefore they can be added to Group O.

In 'Rita' we found an allele, S_x, that does not correspond to any of the known alleles (S_1 to S_{16}; *Sonneveld* et al., 2003). Its intron sizes correspond to a new allele found in wild cherries by De Cuyper (pers. comm.) who labelled it S_{22}. This is the first report of the existence of S_{22} allele in

Table 3 S-allele results of the accessions analysed

Cultivar	Consensus primers, preliminary results genotype	Allele-specific primers, confirmation												Final results	
		S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_9	S_{10}	S_{12}	S_{13}	S_{14}	S_{16}	genotype	incomp.group
Vera	S_1S_3	+		+											S_1S_3	II
IV-6/240	S_1S_3	+		+											S_1S_3	II
13/120	S_1S_3	+		+											S_1S_3	II
Münchebergi Korai	S_3S_4			+	+										S_3S_4	III
Solymári Gömbölyű	S_3S_4			+	+										S_3S_4	III
Hedelfingeni Orias	S_3S_4			+	+										S_3S_4	III
Kavics	S_3S_4			+	+										S_3S_4	III
Botond	S_3S_4			+	+										S_3S_4	III
5/5	S_4S_5				+	+									S_4S_5	V
III-42/114 (Carmen)	S_4S_5				+	+									S_4S_5	V
IV-3/41 (Anita)	S_3S_6			+			+								S_3S_6	VI
IV-13/51 (Tünde)	S_3S_5			+		+									S_3S_5	VII
Szomolyai Fekete	S_2S_4		+		+										S_2S_4	XIII
Trusenszkaja 2	S_5S_6					+	+								S_5S_6	XV
Valerij Cskalov	S_1S_9	+							+						S_1S_9	XVIII
Linda	S_3S_{12}			+							+				S_3S_{12}	XXII
Germersdorfi 1	S_3S_{12}			+							+				S_3S_{12}	XXII
Germersdorfi 3	S_3S_{12}			+							+				S_3S_{12}	XXII
IV-13/20 (Aida)	S_6S_{12}						+				+				S_6S_{12}	XXIV
Katalin	S_4S_{12}				+						+				S_4S_{12}	XXVII (new)
Margit	S_4S_{12}				+						+				S_4S_{12}	XXVII (new)
Krupnoplodnaja	S_5S_9					+			+						S_5S_9	O
IV-5/62 (Rita)	S_5S_2? S_5S_7? S_5S_x?		-			+		-							S_5S_x*	O
IV-6/5 (Péter)	S_3S_4'			+	+										S_3S_4'	SC
IV-6/66	S_3S_4'			+	+										S_3S_4'	SC
IV-6/12 (Sándor)	$S_4'S_9$				+				+						$S_4'S_9$	SC
IV-6/39 (Pál)	$S_4'S_9$				+				+						$S_4'S_9$	SC
Alex	S_3S_3'			+											S_3S_3'	SC

*S_x is probably S_{22}

sweet cherry. As the female parent of 'Rita' has the genotype S_5S_6, this allele should come from selection 'H 2', the paternal parent of 'Rita', that originates from open pollination of 'Schneiders Späte Knorpelkirsche' (Apostol, pers. comm.).

In this work it was shown that 'Germersdorfi 1' and 'Germersdorfi 3' have the same incompatibility genotype as 'Schneiders Späte Knorpelkirsche', but whether they are indeed the same cultivar, as believed among Hungarian scientists, would require fingerprinting e.g. by microsatellites. The relationship to 'Germersdorfer', an other German cultivar, could also be checked. *Störtzer* et al. (1992) state that 'Germersdorfer' is the synonym name for 'Schneiders Späte Knorpelkirsche'.

'Alex'shows only a single band in the S_3 position with 1st and 2nd intron consensus primers. From its PCR results and parentage *Sonneveld* et al. (2003) deduced that 'Alex', which is self-compatible, is S_3S_3'.

Self-compatibility cannot be proved by these PCR methods alone as S_4 and S_4' give the same amplification products with the primers used. We can deduce self-compatibility from amplification pattern if its parentage is known. Thus the S_4/S_4' band in 'Sándor', 'Pál' and in the hybrid 'IV-6/66' – which have 'Stella', S_3S_4' as a father parent, indicates the self-compatibility allele S_4'. Self-compatibility of all accessions in Group SC *(Table 3)* was confirmed with selfings in the field (data not shown).

On the basis of genotyping and parentage of IV-13/51 ('Tünde') and 'IV-13/120' it was possible to determine the genotype of their maternal parent. Thus 'Yellow Dragan' must be S_1S_5. PCR analysis of this cultivar confirmed its proposed genotype (Schuster, pers. comm.).

According to our experiments, the cultivar grown in Hungary under the name 'Hedelfingeni Orias' was found to have the genotype S_3S_4, and not S_3S_5 as previously reported for 'Hedelfingen' grown in Western Europe. Therefore it is assumed that 'Hedelfingeni Orias' and 'Hedelfingen' are different cultivars. Again it would be useful to confirm this with microsatellite fingerprinting. To avoid mistakes we propose distinguishing this clone as 'Hedelfingen O'.

On the basis of our results and with knowledge of blooming times cherry growers will be able to choose the ideal cross-compatible cultivar combinations for their orchards. The breeder will also benefit, in being able to choose cross-compatible cultivar combinations for his/her crossings.

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