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Correspondence between maturity date and molecular variations in a *NAC* transcription factor of diploid and polyploid *Prunus* species

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Abstract: The maturity date (MD) of *Prunus* stone fruit has been long known to be a quantitatively inherited trait. A *NAC*-type gene indicated as *PpNAC1* (*ppa008301m*) has been found recently to be a strong candidate of a major gene influencing MD in peach. A 9-bp insertion in this gene resulted in earlier MD in two segregating peach populations. This study was carried out to test whether this mutation in the *PpNAC1* gene can be used as a reliable functional marker for MD in a wide range of peach cultivars of various origins and phenotypic characters. A total of 125 peach cultivars were examined using a 3 × 3 custom chi-square contingency table according to their *NAC* genotype and MD (early, midseason, and late). Cramér's V equaled 0.478 and the Goodman–Kruskal index (l) was 0.37, indicating an extremely strong correlation between MD and *NAC* genotype. In addition, we determined 15 sequences from 10 cultivars of five *Prunus* species including peach, almond, apricot, sour cherry, and European plum with *E*-values ranging from 9e-88 to 2e-74, supporting their homology to *PpNAC1*. A total of 69 single nucleotide polymorphisms and two insertion/deletions were detected in the coding region of the partial *NAC* domain sequences with three mutations putatively inducing nonconservative amino acid replacements and a nonsense mutation in specific alleles of early ripening apricot and sour cherry cultivars. The results are discussed with focus on the putative molecular mechanisms of mutations in the *NAC* genes, crop evolutionary perspectives, and the opportunities for designing cost-efficient markers to predict MD in *Prunus* breeding programs.

Key words: Fruit maturity, molecular markers, NAC transcription factor, *Prunus*, ripening time, stone fruits

1. Introduction

The maturity date (MD) of stone fruit species (*Prunus*) is an important agronomic trait determined by the complex processes of fruit development and ripening. These processes involve the coordinated regulation of several metabolic pathways influencing texture, flavor, aroma, and appearance (Giovannoni, 2004). Choosing the optimal harvest date is essential in order to reach the best fruit quality at consumption. In addition, a wide range of ripening period could allow market growth by extending the length of the production season (Eduardo et al., 2011, Pirona et al., 2013).

Most fruit quality traits, including MD, are quantitatively inherited; therefore, several quantitative trait locus (QTL) analyses have been carried out in *Prunus* species using both intra- and interspecific populations. In peach, QTLs controlling fruit ripening have been mapped on different chromosomes, with a major QTL located on linkage group 4 (LG4) (Dirlewanger et al., 1999, 2012; Quilot et al., 2004). Eduardo et al. (2011) showed that the QTL detected on LG4 (*qMD4.1*) behaves as a Mendelian trait and has pleiotropic effects determining different pomological characteristics, such as fruit weight, juice titratable acidity, and soluble solid content. QTLs for MD were detected in four genomic regions of LG1, LG4, LG6, and LG7 by Romeu et al. (2014) with the major QTL also being located on LG4. A second cluster of QTLs on LG1 colocalized with the most significant QTL for chilling requirement, which highlights the outstanding influence of this locus on reproductive phenology in peach. Three major QTLs were detected by Nuñez-Lillo et al. (2015) and they all colocalized on LG4 between 31.0 and 42.0 cM. A major MD QTL was also identified in the collinear region of sweet cherry (*P. avium* L.) and apricot (*P. armeniaca* L.) genomes, suggesting a common regulation mechanism of fruit ripening in related *Prunus* species (Dirlewanger et al., 2012; Salazar et al., 2016).

Two segregating peach populations were genotyped to narrow the MD locus into a 220-kb region of the peach genome (Pirona et al., 2013). Among the 25 annotated genes within this interval, a *NAC*-type gene indicated as *PpNAC1* (*ppa008301m*) was found to be a strong

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candidate gene for controlling MD in peach. The Sanger resequencing of this candidate gene uncovered an in-frame 9-bp insertion in early MD cultivars, resulting in a tandem duplication of three amino acids (AAs) in the last exon of the C terminal domain. The allelic variants cosegregated with the MD of all individuals in both F2 populations: the early ripening individuals had the 9-bp insertion while the late ripening genotypes carried the reference allele. This insertion resulted in the duplication of a threonine – aspartic-acid – proline stretch with possible impact on protein structure and function. This allele variant was suggested to be used as a functional marker to determine MD in peach breeding programs (Pirona et al., 2013).

NAC (*NAM*, *ATAF1*, *ATAF2*, *CUC2*) transcription factors constitute one of the largest plant-specific protein families (Olsen et al., 2005). Their common structural element is the highly conserved *NAC*-domain of the N-terminal region comprising approximately 150 AAs. The C terminal part is highly diverse and it does not contain any known protein domains; however, it frequently contains simple AA repeats and it is rich in serine, threonine, proline, glutamine, or their acidic residues (Ooka et al., 2003; Olsen et al., 2005). They are implicated in various processes of plants, such as embryogenesis (Aida et al., 1997), biotic and abiotic stress responses (Wang et al., 2013; Sun et al., 2015), flowering (Sablowski and Meyerowitz, 1998), and fruit development and maturation (Shan et al., 2012). Six *NAC*-type proteins were described in banana; from these, *MaNAC1* and *-2* were upregulated by ethylene in peel and pulp, consistent with the increase in hormone production, suggesting that these two transcription factors are possibly involved in the fruit ripening process.

In peach, more than 1500 transcription factors have been identified in the past decade (about 5.55% of the protein coding genes) (Bianchi et al., 2015). Slow ripening (SR) peach fruits do not mature normally and remain on the tree even after leaf fall, a trait determined by a single gene (*Sr/sr*) with recessive homozygotes showing the SR phenotype (Ramming, 1991). QTL mapping of the progeny of two heterozygous (*Sr*) parents revealed that the SR and MD traits are located in the same genomic region of LG4, including the *ppa008301m* (*PpNAC1*) gene. A marker developed for this locus cosegregated with the SR trait and showed no amplification in the case of the *srsr* genotypes, suggesting that there is a large deletion in the MD region including the ppa008301m sequence. Hence, it is possible that the absence of the product of the *PpNAC1* gene causes the SR phenotype (Eduardo et al., 2015). These results all confirm that LG4 has a major effect on biochemical processes related to fruit maturation in peach.

The aim of our study was to test whether the formerly described 9-bp insertion in the *PpNAC1* gene can be used as a reliable functional marker for MD in a wide range of peach cultivars of various origins and phenotypic characters. We also wanted to check the presence of a homologous gene in other stone fruit species and characterize sequence variations to measure the possibility of marker development based on this locus in diploid and polyploid *Prunus* species.

2. Materials and methods

2.1. Plant material

The plant material and the data for MD were collected at the Experimental and Research Farm of Szent István University (Budapest, Hungary), the Research Stations of the National Food Chain Safety Office (Tordas, Hungary), and the National Agricultural Research and Innovation Centre Fruticulture Research Institute (Érd, Hungary). A total of 125 peach cultivars were examined showing considerable variations in their origin, phenotypic traits, and MDs, ranging from very early (the middle of June) to very late (the beginning of October) (Table S1). Peach vegetative buds were collected from November 2015 to February 2016 and were stored at –20 °C before processing. In addition, 18 cultivars of different *Prunus* species (almond, apricot, peach, plum, and sour cherry) were chosen for fragment length determination on a capillary sequencer and among them 10 were analyzed for partial DNA sequencing (*NAC* sequence analysis) based on the alterations in their MD (Table S2).

2.2. DNA extraction and polymerase chain reaction (PCR) analysis

Genomic DNA extraction from fully expanded young leaves was carried out using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The quantity and quality of DNA were analyzed by NanoDrop ND-1000 spectrophotometer (Bio-Science, Budapest, Hungary).

For each sample, PCR amplification in a reaction volume of 25 µL contained 20–50 ng of genomic DNA, 10X DreamTaq Green Buffer (Fermentas, Szeged, Hungary) with final concentration of 4.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of the NAC-INDEL specific primers (Pirona et al., 2013; forward: 5′-AGAACTCAGCGGGTTGATAACT-3′; reverse: 5′-TGCACCCCTACTCGATTTCT-3′), and 0.75 U of DreamTaq DNA Polymerase (Fermentas). The PCR protocol was used as described by Pirona et al. (2013) with some modifications: the amplification program consisted of an initial denaturation step of 95 °C for 2 min; 40 cycles of 92 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s; and a final extension of 72 °C for 5 min. The PCR products were separated on 2% TAE agarose gels at 100 V for 2 h and DNA bands were stained with ethidium bromide. Fragment sizes were estimated by comparison with the 1 kb + DNA ladder (Promega, Madison, WI, USA).

For fragment length analysis, the PCR products amplified by fluorescently labeled forward primer (NAC-

INDELF, 5′6-FAM) were run in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For data analysis, ABI Peak Scanner 1.0 software and GS500 LIZ size standard were used.

PCR products were cloned into the pTZ57R/T plasmid vector using the InsTAclone PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA) and sequenced by ABI 3500 XL Genetic Analyzer (Applied Biosystems). For each fragment, the nucleotide sequences of three clones were determined in both directions.

2.3. Bioinformatics

The identified *NAC* sequences were used as a query sequence for MegaBLAST analysis (Morgulis et al., 2008). An alignment of 15 *Prunus NAC* sequences was carried out using the CLUSTAL W program (Thompson et al., 1994) in MEGA5.1 (Tamura et al., 2011) and the alignment was manually curated. The alignments are presented using BioEdit v.7.0.9.0 (Hall, 1999). Sequence data from this article were submitted to the EMBL/GenBank databank under accession numbers from KX650377 to KX650390 and MF464013.

2.4. Statistical analysis

Because *NAC* genotypes and MD are categorical data, we analyzed the distributions of data using a 3 × 3 custom chi-square contingency table and calculated Cramér's V (Cramér, 1946) as $(\chi^2/N)^{1/2}$, where χ^2 is from the above likelihood ratio test and N is the total number of assessed cultivars, which is a measure of the strength of association among *NAC* genotypes and maturity data, and the Goodman–Kruskal index (l) of predictive association (http://vassarstats.net/newcs.html). The results of Cramér's V tests were evaluated according to the following criteria: <0.10 = no relationship, 0.10 to <0.20 = weak association, 0.20 to <0.25 = moderate association, 0.25 to <0.30 = moderately strong association, 0.30 to <0.35 = strong association, 0.35 to <0.40 = very strong association, 0.40 to <0.45 = extremely strong relationship, 0.45 to <0.99 = two variables probably measuring the same concept, 1.00 = perfect relationship; independent variables will predict the dependent variable (Baker et al., 2008). For each cell in the contingency table, percentage deviation was calculated as (observed – expected frequencies)/expected frequency × 100.

3. Results

3.1. Correspondence between maturity date and NAC genotype of peach

Pirona et al. (2013) revealed that fragment length polymorphisms due to an insertion/deletion mutation in a peach *NAC* gene (*ppa008301m*) cosegregated with the MD locus in two mapping populations. Our study was designed to check its general validity and provide firm statistical support for the effect of *ppa008301m* by genotyping a wide range of peach cultivars. The NAC-INDEL-specific forward primer designed by Pirona et al. (2013) was fluorescently labeled and used for PCR amplification of 125 peach cultivars with known MD. Then amplicons were sized accurately on a capillary sequencer.

The fragment length analysis of 125 peach cultivars resulted in polymorphic chromatograms representing three distinct genotypes: 1) homozygous for the 192-bp reference allele, 2) homozygous for the 201-bp allele, and 3) heterozygous cultivars carrying both the 192-bp and 201-bp alleles (Figure S1). Altogether 33 cultivars (26.4% of the examined cultivars) were homozygous for the 201-bp allele. From these, 25, 5, and 3 cultivars belonged to the early (MD on Julian days 165–203), midseason (204–237 days), and late (238–274 days) ripening categories (Table S1), respectively. From the 33 cultivars homozygous for the 192-bp allele (26.4%), 19 had MD recorded after the 3rd ten days of August, while 12 and 2 were characterized by mid-season and early-midseason ripening time, respectively. The remaining 59 cultivars (47.2%) were heterozygous with 35 ripening in the midseason while 17 and 7 cultivars have early and late MD, respectively.

For statistical support, we conducted a 3 × 3 contingency table analysis for independence according to the three categories of MD (early, midseason, and late) and three genotypes (192/192 bp, 192/201 bp, and 201/201 bp) and used the chi-square test for significance analysis. The association of the *NAC* genotype and MD was significant ($c^2 = 57.2$, df = 4, $P \leq 0.0001$) and differences between the observed and expected frequencies indicated considerable distortions as 192/192 homozygotes were less frequent in the early and midseason MD categories and 201/201 homozygotes were underrepresented in the middle and late season categories (Table 1). The frequency of the heterozygous cultivars peaked in the midseason category. Cramér's V equaled 0.478 while the Goodman–Kruskal index (l) was 0.37.

3.2. NAC sequence variations in *Prunus* species

Since a major QTL determining MD was also found in the syntenic region of apricot and sweet cherry genomes (Dirlewanger et al., 2012), we wanted to screen for sequence variations in the corresponding *NAC* gene in several *Prunus* species. PCR was carried out on genomic DNA from a range of almond, apricot, European plum, and sour cherry cultivars using the NAC-INDEL specific primer pair (Pirona et al., 2013). The successful amplification indicated the presence of the *NAC*-domain containing sequence in other stone fruit species, as well (Figure S2). The fluorescently labeled NAC-INDEL-specific primer pair was then used to amplify DNA from such samples to look for intraspecific small-scale size variations. However, the majority of the analyzed accessions amplified only one

Table 1. Percentage deviations of the observed to expected chi-square cell frequencies in the *NAC* genotype and maturity date contingency table.

NAC genotype Maturity date (Julian days)	192/192	192/201	201/201
165–203	–82.8	–18.1	115.2
204–237	–12.6	42.6	–63.6
238–274	148.2	–48.9	–60.8

fragment with almond cultivars (Tétényi keményhéjú and Tuono) showing the 192-bp peach reference allele size. Apricot (Ceglédi óriás, Ceglédi Piroska, Corlate, Goldrich, Harmat, Kech-pshar, Magyar kajszi C.235, Pannónia, Rózsakajszi C.140, Samarkandskyi rannii, and Shalakh) and European plum (President and Stanley) cultivars amplified a single 188-bp fragment, while sour cherry (Korai pipacsmeggy and Piramis) had a 189-bp sized amplicon. Only Kántorjánosi 3, a sour cherry cultivar, had two differently sized fragments of 189 and 192 bp. In contrast to peach, cultivars of almond, apricot, and plum with considerably differing MD did not show consequent size polymorphism in the C terminal part of the *NAC* gene. The amplicons were cloned and sequenced to check the homology of the amplified fragments with the peach reference allele ppa008301m and detect the presence or absence of replacement mutations.

We have determined 15 sequences from 10 cultivars of five *Prunus* species including peach, almond, apricot, sour cherry, and European plum. After homology searches in the GenBank database, the *E*-value of those sequences ranged from 9e-88 to 2e-74. Almond sequences showed the greatest similarity to peach, while sequences from other species were more similar to *P. mume NAC*. It is interesting to note that the *P. dulcis* (*Pdu*) Tuono (KX650378) and *P. cerasus* (*Pce*) Kántorjánosi 2 (KX650379) sequences were identical. A common sequence was also carried by two sour cherry cultivars, Piramis (KX650386) and Kántorjánosi 3 (KX650390). The *P. armeniaca* (*Par*) Korai zamatos 1 (KX650380) sequence was also found in a late ripening apricot cultivar, Corlate. This cultivar was shown to carry another allele (MF464013), as well. Two sequences were identified in European plum with the *P. domestica* (*Pdo*) Stanley 1 (KX650377) sequence also occurring in President, a late MD cultivar.

Sequences were aligned with the *Prunus persica* ppa008301m cDNA sequence. The early MD peach cultivar Springtime contained the 9-bp insertion while the sequence from the very late ripening, blood-fleshed peach Vérbarack was identical to the reference genome sequence (Figure 1). Although the 9-bp insertion of the early ripening peach cultivars were not present in sequences from other *Prunus* accessions, several variations were detected. Sequence alignment of other *Prunus* species showed high levels of similarity and some characteristic alterations compared to the peach reference allele (Table 2).

A total of 69 single nucleotide polymorphisms (SNPs) were detected in the coding region of the partial *NAC* domain sequences (Table 2) with 23 synonymous, 45 nonsynonymous, and one nonsense single base substitutions. In addition, 30 and 12 SNPs resulted in conservative and semiconservative AA replacements, respectively. Only three mutations induced nonconservative AA replacements in specific apricot and sour cherry alleles. A nonsense mutation was also detected in a sour cherry cultivar, Korai pipacsmeggy. A 1-bp insertion occurred in 5 sour cherry sequences and a 4-bp deletion in apricot, plum, and 4 of the 5 sour cherry sequences.

4. Discussion

A 9-bp insertion in the C terminal domain of a peach *NAC* gene was suggested to be associated with early MD in two segregating populations (Pirona et al., 2013). We wanted to clarify the influence of the *PpNAC1* gene by genotyping 125 peach cultivars characterized by different MD categories and validate its application as a reliable molecular marker. Significance analysis and Cramér's V test indicated that the two variables (*NAC* genotype and maturity category) are probably measuring the same concept (Baker et al., 2008). This extremely strong correlation was further supported by the Goodman–Kruskal index (l), indicating substantial increase in the probability of correct prediction of MD when *NAC* genotype is considered. These data support the use of this marker in marker-assisted selection for MD. However, there were some outlier cultivars in both the early (two cultivars homozygous for the 192-bp allele) and the late (three cultivars homozygous for the 201-bp allele) MD groups, indicating that other loci may also contribute to MD determination. This is reasonable since NAC proteins are part of a complex network (Shan et al., 2012; Nuñez-Lillo et al., 2015).

We have determined the partial *PpNAC1* sequence from Vérbarack, a blood-fleshed cultivar (with heavy

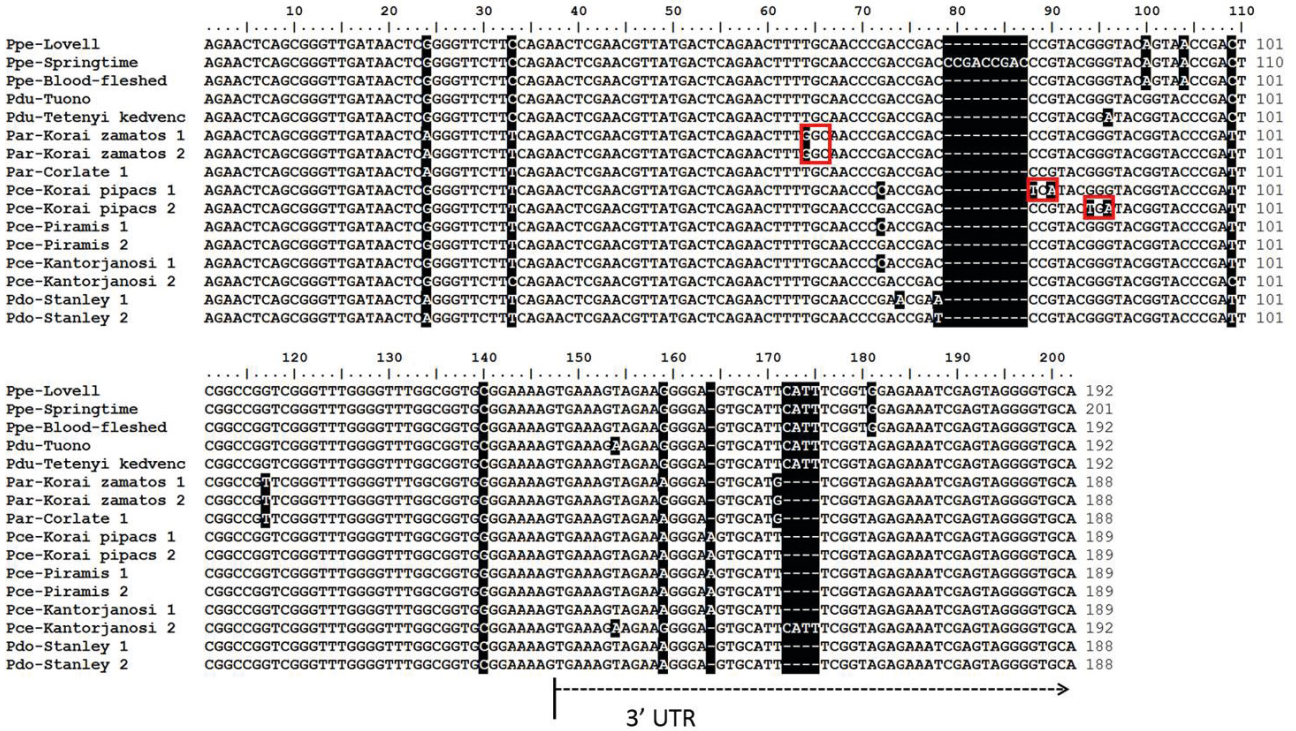

Figure. Alignment of the C terminal domain and 3' untranslated region (UTR) of *Prunus NAC* sequences. Sequence alterations are shown in black boxes; the framed triplets code for nonconservative amino acid replacements or a premature stop codon.

anthocyanin accumulation in the mesocarp) with extremely late MD (Mohácsy, 1954). It has been recently shown that *PpNAC1* forms a heterodimer with another *NAC*-type protein responsible for the blood-flesh phenotype in peach. The major locus of this trait was mapped to linkage group 5 (Shen et al., 2013) and a candidate gene encoding a *NAC* transcription factor was found to be highly upregulated in blood-fleshed peaches compared with non-red-fleshed varieties. This heterodimer activates the *PpMYB10.1* transcription factor responsible for anthocyanin accumulation in fruit mesocarp (Zhou et al., 2015). Since Vérbarack carried the wild-type *NAC1* allele, functional loss of *PpNAC1* due to the 9-bp insertion in the coding region of the gene will not only result in early MD but may also affect the integrity of NAC heterodimers, resulting in anthocyanin-free mesocarp. It may explain why blood-fleshed mesocarp and late MD are associated although their coding genes are located on different chromosomes (Pirona et al., 2013; Zhou et al., 2015).

The 9-bp insertion in the *PpNAC1* gene provides the first evidence of loss-of-function mutation in the C terminal part of the protein. Structural characteristics of the C terminal region of NAC proteins suggest its role as a transcriptional activation domain (Olsen et al., 2005). A 6-bp insertion in the *NAC* domain generated upon the excision of a transposable element resulted in the incorporation of two extra amino acids and abolished NAC protein function in *Petunia* (Souer et al., 1996). A 9-bp insertion in *PpNAC1* might also be a footprint of a transposon or a direct repeat. *FaSt*, a recently identified nonautonomous transposon that occurs frequently in the peach genome generates a 9-bp insertion upon transposition (Halász et al., 2014). However, the sequence of the target site is significantly different from that of the direct repeat in peach *NAC* containing mainly C and G nucleotides compared to *FaSt* TSD rich in A and T. However, the action of another *Mutator*-transposon cannot be ruled out.

The insertion may also be explained by replication slippage due to mispairing between neighboring CCGA repeats. A CCCGTACGGG palindromic sequence was found right after the insertion/deletion (indel) position (Figure 1), indicating that secondary structures may interfere with DNA replication and increase the rate of mutation in this region. Palindromic repeats often cause indels due to hairpin loop-induced template switching in replication (Montgomery et al., 2013). A CATT repeat was also found in the 3' untranslated region (UTR) of *P. persica* (*Ppe*) and *Pdu* alleles (and *Pce* Kantorjanosi 2), while all apricot and plum and five of the sour cherry sequences had this motif only in one copy. In addition, those sour cherry alleles contained a 1-bp insertion 4 bp upstream of the CATT motif. Such alterations reflect that mutations seem to be quite frequent in this region of the gene.

Table 2. Homology and polymorphisms detected in the C terminal domain of NAC protein genes from diploid and polyploid *Prunus* species.

Sequences	Accession number	Best hit against NCBI database	Best hit accession number	BLAST *E* value	Single nucleotide polymorphisms[a]					Total[b]
					Syn.	Nonsynonymous			Nonsense	
						Cons.	Semi-cons.	Non-cons.		
Pdu Tuono	KX650378	*P. persica*	XM_007211500	9e-88	–	S→G	N→T	–	–	4
Pdu Tétényi kedvenc	KX650383	*P. persica*	XM_007211500	9e-88	1	S→G,	N→T	–	–	4
Par Korai zamatos 1	KX650380	*P. mume*	XM_008228454	1e-80	3	S→G, L→F, A→G	N→T	C→G	–	11, 4 bp del.
Par Korai zamatos 2	KX650384	*P. mume*	XM_008228454	7e-79	3	S→G, L→F, A→G	N→T	C→G	–	10, 4 bp del.
Par Corlate	MF464013	*P. mume*	XM_008228454	3e-82	3	S→G, L→F, A→G	N→T	–	–	10, 4 bp del.
Pce Korai pipacsmeggy 1	KX650388	*P. mume*	XM_008228454	2e-74	3	S→G, L→F, A→G	N→T	P→S	–	10, 1 bp ins., 4 bp del.
Pce Korai pipacsmeggy 2	KX650389	*P. mume*	XM_008228454	4e-76	2	S→G, L→F, A→G	N→T	–	TGA	9, 1 bp ins., 4 bp del.
Pce Piramis 1	KX650386	*P. mume*	XM_008228454	9e-78	2	S→G, L→F, A→G	N→T	–	–	8, 1 bp ins., 4 bp del.
Pce Piramis 2	KX650387	*P. mume*	XM_008228454	2e-79	1	S→G, L→F, A→G	N→T	–	–	7, 1 bp ins., 4 bp del.
Pce Kántorjánosi 1	KX650390	*P. mume*	XM_008228454	9e-78	2	S→G, L→F, A→G	N→T	–	–	8, 1 bp ins., 4 bp del.
Pce Kántorjánosi 2	KX650379	*P. persica*	XM_007211500	9e-88	–	S→G,	N→T	–	–	4
Pdo Stanley 1	KX650377	*P. mume*	XM_008228454	3e-77	2	D→E, S→G, L→F	T→N, N→T	–	–	9, 4 bp del.
Pdo Stanley 2	KX650385	*P. mume*	XM_008228454	7e-79	3	S→G, L→F	N→T	–	–	8, 4 bp del.

[a] Single nucleotide polymorphisms in sour cherry, plum, and apricot *NAC* sequences compared to the peach (Lovell) reference allele.
[b] Total number of sequence alterations in the coding and untranslated regions.
Syn.: synonymous amino acid replacement. Cons.: conservative amino acid replacement. bp: base pairs. ins.: insertion. del.: deletion. *Par*: *Prunus armeniaca*. *Pce*: *P. cerasus*. *Pdo*: *P. domestica*. *Pdu*: *P. dulcis*.

Free-stone French cultivars like Belle Garde and Reine des Vergers originated in the 17th and 19th centuries and became popular in Hungary in the second half of the 19th century. Such cultivars are characterized by MDs in September. Other yellow flesh landrace cultivars (e.g., Crosby, Elberta, Lady Palmerston, Magyar aranyduráncija, Mezőkomáromi duránci, and Württembergi király) were also widely grown in the country with MDs ranging from the end of August to October (Rapaics, 1940; Mohácsy, 1954). It explains the Hungarian name of peach (őszibarack) indicating a ripening time in autumn. In the meantime, breeding programs were initiated in the United States for early ripening peach cultivars. Amsden (genotyped in this study to be heterozygous in the *PpNAC1* locus) was the first cultivar in Missouri state, which was established in Hungary at the end of the 19th century and was available for orchard establishment through 2012 (National Food Chain Safety Office, 2012). The early MD cultivars have taken over the market in many countries and now the majority of peach cultivars ripen during the summer months while the Hungarian name of this fruit has lost its original meaning with the spread of the insertion allele in newly released peach cultivars. Strong putative selection on regions of chromosome 4 around *PpNAC1* was also detected in modern fruit cultivars of peach by Akagi et al. (2016) based on genome-wide single nucleotide polymorphism.

Since the *ppa008301m NAC* gene was confirmed to have major effects on MD in peach, we cloned and sequenced the fragments amplified using the peach PCR primers in a range of *Prunus* accessions with variable MDs. We have identified 2, 2, 2, and 6 partial sequences from almond, apricot, European plum, and sour cherry, each showing significant homology to *PpNAC1*. The 9-bp insertion that has been associated with early MD in peach was not found in other *Prunus NAC* sequences. However, other nucleotide polymorphisms occurred frequently in the analyzed region of the gene, four of which might be supposed to alter the function of the protein.

Each of the three nonconservative mutations and the nonsense mutation was found in early ripening cultivars of apricot (Korai zamatos) and sour cherry (Korai pipacsmeggy). We have identified two sequence variations in Korai zamatos differing in only the 3' untranslated region of the gene. Both sequences had a C → G AA replacement immediately upstream of the insertion in the nonfunctional peach allele, which indicates that this nonconservative AA replacement might be associated with the early MD of Korai zamatos. It is further supported by the fact that Corlate, a late ripening apricot cultivar, was shown to have another allele without this C → G replacement. Korai pipacsmeggy ripens in the middle of June, approximately 3 weeks before Kántorjánosi 3. Interestingly, this cultivar was shown to carry two variations of the *NAC* sequence with one of those containing a P → S nonconservative AA replacement. The C terminal region of NAC proteins is responsible for transcriptional activation (Olsen et al., 2005) and missense mutations were described to result in dysfunctional NAC proteins due to conformational instability or inhibited nuclear transport of the protein (Takada et al., 2001). Since both C and P have special roles to form disulfide bridges and introduce kinks into helices (Patthy, 2008), respectively, such AA interchanges may affect normal protein function.

In *Pce* Korai pipacsmeggy 2, a premature stop codon was introduced in a position 6 bp downstream of the insertion in the *PpNAC1*, resulting in the loss of function of this gene in peach. The truncated protein is expected to miss 18 AAs at the C terminus and, based on the loss of function induced by a small insertion in the early ripening Springtime (Pirona et al., 2013), it is presumed to seriously affect protein function.

These variants were not found in Kántorjánosi 3, a late ripening sour cherry cultivar. The *NAC* sequences isolated from Kántorjánosi 3 contained mainly synonymous SNPs and conserved AA replacements. In Piramis, another early ripening sour cherry cultivar, two slightly different alleles were identified without major sequence alterations and hence they are presumed to have no considerable effects on protein function. However, other regions of the gene must also be screened for putative mutations.

In a diploid species, peach, our analysis provided firm statistical support for the major influence of *PpNAC1* on MD, which is affected by the copy number of the nonfunctional alleles. The identification of nonfunctional *NAC* alleles will shed light on the interaction of alleles accumulated in elevated copy number within polyploid fruit tree genomes. Mutation rates are increased in polyploid genomes if one of the paralogous gene pairs is free of selection pressure, like in the case of *S*-haplotypes in sour cherry (Tsukamoto et al., 2006). The frequency of mutations in sour cherry and European plum sequences was not different from that of apricot, a diploid species (Table 2), indicating that each nonfunctional allele may have a direct effect on MD in polyploid species, as well. Our homology-based approach provided evidence that syntenic regions of diploid almond, apricot, tetraploid sour cherry, and hexaploid European plum genomes carry a homologous *NAC* sequence that has been shown to be a strong candidate gene with major effects on the MD trait in peach and support the QTL mapping data for sweet cherry and apricot (Dirlewanger et al., 2012).

In conclusion, although the function of the mutant alleles must be checked in future, the detected alterations are likely to influence the MD of the analyzed apricot

and sour cherry cultivars due to the high synteny among *Prunus* genomes (Dondini et al., 2007; Olmstead et al., 2008; Dirlewanger et al., 2012). Korai zamatos and Korai pipacsmeggy are characterized by the earliest MD among apricot and sour cherry cultivars, respectively (Tóth, 1997; Halász et al., 2005). In the case of a diploid species like peach, firm conclusions can be drawn for the MD category of a cultivar based on a simple allele-typing in the *PpNAC1* locus. It can be used efficiently in a wide range of peach cultivars regardless of their origin. In polyploid species like sour cherry and European plum, the identification of additional alleles and the determination of complete gene sequences should be carried out to identify all presumably nonfunctional sequence variations in the locus, find out the interaction of several alleles, and characterize their contributions to fruit MD. Our results showed that the description of sequence variations in the syntenic locus in other *Prunus* species would provide an opportunity for designing reliable and cost-efficient molecular diagnostic assays (allele-specific PCR, PCR-RFLP, high-resolution melting analysis) to predict MD at the early seedling stage.

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Table S1. Name, origin, most important pomological traits, ripening period in Hungary, maturity date, and NAC genotype of peach cultivars analyzed in the study.

Cultivar	Origin	Most important pomological traits[a]	Ripening period in Hungary	Maturity date (Julian day)	*NAC* genotype		
					Homozygote (192 bp)	Heterozygote (192/201 bp)	Homozygote (201 bp)
Primissima Delbard	France	P, R, W, C	2nd ten days of June	165			*
Royal April	USA	P, R, Y, C	2nd ten days of June	165			*
Madeleine Pouyet	France	P, R, W, C	2nd ten days of June	167			*
Spring Lady	USA	P, R, Y, S	2nd ten days of June	169		*	
Springold	USA	P, R, Y, C	3rd ten days of June	175		*	
Starcrest	France	P, R, Y, C	3rd ten days of June	177			*
Redwing	USA	P, R, W, F	3rd ten days of June	179			*
Tena	Czech	N, R, Y, C	3rd ten days of June	180			*
Springtime	USA	P, R, W, C	2nd half of June - beg. of July	180			*
Rubirich	USA	P, R, Y, C	2nd half of June - beg. of July	180			*
Big Haven	USA	N, R, Y, C	2nd half of June - beg. of July	181			*
Springcrest	France	P, R, Y, S	1st ten days of July	182			*
Teska	Czech	P, R, Y, C	1st ten days of July	183			*
Zhao Xia	China	P, R, W, C	1st ten days of July	186		*	
Fenix	Czech	P, R, Y, C	1st ten days of July	188		*	
Favorita Morettini	Italy	P, R, Y, F	1st ten days of July	190	*		
Gloria Red	Hungary	P, R, Y, C	1st ten days of July	191		*	
Nikitskyi 85	Russia	N, R, Y, S	1st ten days of July	191			*
Luna	Czech	P, R, W, F	2nd ten days of July	192		*	
Tenira	Czech	P, R, Y, S	2nd ten days of July	192		*	
June Star	USA	N, R, Y, F	2nd ten days of July	193			*
Stark Early Glo	USA	P, R, Y, F	2nd ten days of July	194		*	
Arany csillag	Hungary	P, R, Y, C	2nd ten days of July	195			*
Cardinal	USA	P, R, Y, S	2nd ten days of July	196			*
Dixired	USA	P, R, Y, C	2nd ten days of July	196			*
Nectagrand	Italy	N, R, Y, C	2nd ten days of July	196			*
Amsden	USA	P, R, W, C	2nd ten days of July	196		*	
Vistarich	USA	P, R, Y, C	2nd half of July	196		*	
Early Red	USA	P, R, Y, C	2nd ten days of July	197		*	
Early White Giant	USA	P, R, W, C	2nd ten days of July	197			*
Early White Giant	USA	P, R, W, C	2nd ten days of July	197		*	
Meigue Pantao	China	P, F, W, C	2nd ten days of July	197	*		
Piroska	Hungary	P, R, W, F	2nd ten days of July	197		*	
Jerseyland	USA	P, R, Y, S	2nd ten days of July	198			*
Regina	USA	P, R, Y, F	2nd ten days of July	198		*	
Earliglo	USA	N, R, Y, C	2nd ten days of July	199		*	
Sunrise	USA	P, F, Y, -	2nd ten days of July	199		*	
Weinberger	Italy	N, R, Y, S	2nd ten days of July	199			*
Albatros	South Africa	P, R, W, C	2nd ten days of July	200			*

Table S1. (Continued).

Mariska	Hungary	P, R, W, S	2nd ten days of July	200			*
Tercie	Czech	P, R, Y, C	2nd ten days of July	200			*
Caldesi 2000	Italy	N, R, W, S	2nd ten days of July	201			*
Sunhaven	USA	P, R, Y, S	3rd ten days of July	202		*	
Early Redhaven	USA	P, R, Y, S	3rd ten days of July	203			*
Zhao Hui	China	P, R, W, C	3rd ten days of July	205	*		
Redhaven Bianca	Italy	P, R, W, F	3rd ten days of July	206		*	
Harblese	Canada	N, R, Y, C	3rd ten days of July	207			*
Krasava	Czech	P, R, W, C	3rd ten days of July	207		*	
Genadix 7	France	P, R, W, F	3rd ten days of July	208		*	
Pegaso	Italy	N, R, Y, F	3rd ten days of July	210		*	
Big Top	USA	N, R, Y, C	3rd ten days of July	210		*	
Redhaven	USA	P, R, Y, F	3rd ten days of July	210		*	
Harco	USA	N, R, Y, F	3rd ten days of July	211		*	
Russian flat	Russia	P, F, W, C	3rd ten days of July	211		*	
Nikitskyi flat	Russia	P, F, W, F	3rd ten days of July	211		*	
Teresa	Czech	P, R, Y, C	3rd ten days of July	212		*	
Sunbeam	USA	P, R, Y, S	End of July	212		*	
Royal Summer	USA	P, R, Y, F	End of July - beg. of August	212		*	
Flamin' Fury	USA	P, R, Y, F	Beginning of August	213		*	
Royal Time	USA	P, R, Y, S	1st ten days of August	213	*		
Maura	USA	P, R, W, F	1st ten days of August	215	*		
Nektár H	Hungary	P, R, W, F	1st ten days of August	215		*	
Rikakusuimitsu	China	P, R, W, F	1st ten days of August	216		*	
Moravia	Czech	P, R, Y, F	1st ten days of August	218		*	
Flamingo	Czech	P, R, Y, F	1st ten days of August	219		*	
Hale Haven	USA	P, R, Y, F	1st ten days of August	219			*
Kanto-5	China	P, R, Y, C	1st ten days of August	219		*	
Condor	USA	P, R, W, F	1st ten days of August	219		*	
Paraszt Mariska	Hungary	P, R, W, C	1st ten days of August	219		*	
Redskin	USA	P, R, Y, F	1st ten days of August	219	*		
Rubinovyi 8	Russia	N, R, Y, F	1st ten days of August	219	*		
Fairhaven	USA	P, R, Y, F	1st ten days of August	220		*	
Ford	USA	P, R, W, F	1st ten days of August	220		*	
July Elberta	USA	P, R, Y, F	1st ten days of August	220	*		
Carson	USA	P, R, Y, C	1st ten days of August	222		*	
Alitop	Italy	N, R, Y, F	2nd ten days of August	223			*
Krümcsangin	Russia	N, R, Y, F	2nd ten days of August	223		*	
Lednická Zlutá	Czech	P, R, Y, C	2nd ten days of August	224		*	
Meystar	France	P, R, W, F	2nd ten days of August	225		*	
Flavortop	USA	N, R, Y, F	2nd ten days of August	226		*	
63-15-33	China	P, R, W, C	2nd ten days of August	226	*		
Incorico Pierri	Italy	P, R, W, F	2nd ten days of August	227		*	

Table S1. (Continued).

Elvira	France*	P, R, Y, F	2nd ten days of August	227		*	
Harbringer	Canada	P, R, Y, C	2nd ten days of August	227			*
Big Bang	France	N, R, Y, C	2nd ten days of August	228		*	
Suncrest	USA	P, R, Y, F	2nd ten days of August	228		*	
Babygold 6	USA	P, R, Y, C	2nd ten days of August	230		*	
Champion	USA	P, R, W, F	2nd ten days of August	231		*	
Köncsögi kopasz	Hungary	N, R, -, -	2nd ten days of August	231	*		
Chinese flat	China	P, F, W, -	2nd ten days of August	232	*		
Tapodi-féle	Hungary	P, R, Y, C	2nd ten days of August	232	*		
Collins	USA	P, R, Y, -	3rd ten days of August	233	*		
Zhong Shan Zao Lu	China	P, R, W, F	3rd ten days of August	233		*	
Stark Red Gold	USA	N, R, Y, F	3rd ten days of August	234		*	
Fantasia	USA	N, R, Y, F	3rd ten days of August	235			*
Zsoltij	Russia	N, R, Y, F	3rd ten days of August	236	*		
Cresthaven	USA	P, R, Y, F	3rd ten days of August	238		*	
Jinfeng	China	P, R, Y, C	3rd ten days of August	238	*		
Royal Pride	USA	P, R, Y, F	3rd ten days of August	238			*
Zee Lady	USA	P, R, Y, F	3rd ten days of August	238	*		
Andross	USA	P, R, Y, C	3rd ten days of August	240		*	
Kisapáthy 1	Hungary	P, R, W, F	3rd ten days of August	240	*		
Kisapáthy 2	Hungary	P, R, Y, F	3rd ten days of August	240	*		
Szegedi arany	Hungary	P, R, Y, F	3rd ten days of August	241	*		
Diana	Czech	P, R, Y, F	3rd ten days of August	242	*		
Harry-Harry	-	P, R, Y, F	3rd ten days of August	242	*		
Klamt	USA	P, R, Y, C	3rd ten days of August	242		*	
Fayette	USA	P, R, Y, F	3rd ten days of August	242		*	
Elberta	USA	P, R, Y, F	3rd ten days of August	243	*		
Inka	Poland	P, R, Y, F	3rd ten days of August	243		*	
Redcal	USA	P, R, Y, F	3rd ten days of August	243	*		
Harken	Canada	P, R, Y, F	1st ten days of September	244			*
Ruzsa	Hungary	-, R, -, -	1st ten days of September	245		*	
Vega	Czech	N, R, Y, C	1st ten days of September	245	*		
Gracia	Czech	P, R, Y, F	1st ten days of September	247	*		
Orion	Italy	N, R, Y, F	1st ten days of September	248			*
Sudanell	Spain	P, R, Y, C	1st ten days of September	248	*		
Gladys	USA	P, R, W, C	1st ten days of September	250	*		
Michelini	Italy	P, R, W, F	1st ten days of September	251	*		
Merill Sundance	USA	P, R, Y, F	1st ten days of September	252	*		
Shipley	USA	P, R, W, F	1st ten days of September	252		*	
Kései bronzos Elberta	Hungary	P, R, Y, F	2nd ten days of September	257	*		
Fairlane	USA	N, R, Y, C	2nd ten days of September	260	*		
Vérbarack 1	Hungary	P, R, R, F	End of Sep - beginning of Oct	270	*		
Vérbarack 2	Hungary	P, R, R, F	End of Sep - beginning of Oct	274	*		

Table S2. Species, cultivar, maturity date, and genotyping assays for *Prunus* accessions used in the study.

Species	Cultivar	Maturity date	Analysis[1]
P. cerasus	Piramis	Early	Fl, S
P. cerasus	Érdi jubileum	Midseason	Fl
P. cerasus	Korai pipacs	Midseason	S
P. cerasus	Kántorjánosi 3	Late	S
P. domestica	Stanley	Early	Fl, S
P. domestica	President	Late	Fl
P. dulcis	Tuono	Early	Fl, S
P. dulcis	Tétényi kedvenc	Late	S
P. dulcis	Tétényi keményhéjú	Late	Fl
P. armeniaca	Harmat	Early	Fl
P. armeniaca	Samarkandskyi-rannii	Early	Fl
P. armeniaca	Korai zamatos	Early	Fl, S
P. armeniaca	Ceglédi Piroska	Midseason	Fl
P. armeniaca	Ceglédi óriás	Midseason	Fl
P. armeniaca	Magyar kajszi C.235	Midseason	Fl
P. armeniaca	Pannónia	Midseason	Fl
P. armeniaca	Zard	Midseason	Fl
P. armeniaca	Pisana	Late	Fl
P. armeniaca	Rózsakajszi C.1406	Late	Fl
P. armeniaca	Corlate (GNT 10/10)	Late	Fl, S
P. armeniaca	Kech-pshar	Very late	Fl
P. persica	Springtime	Early	S
P. persica	Blood-fleshed	Very late	S

[1]Fl: Fragment length determination on capillary sequencer, S: partial DNA sequencing.

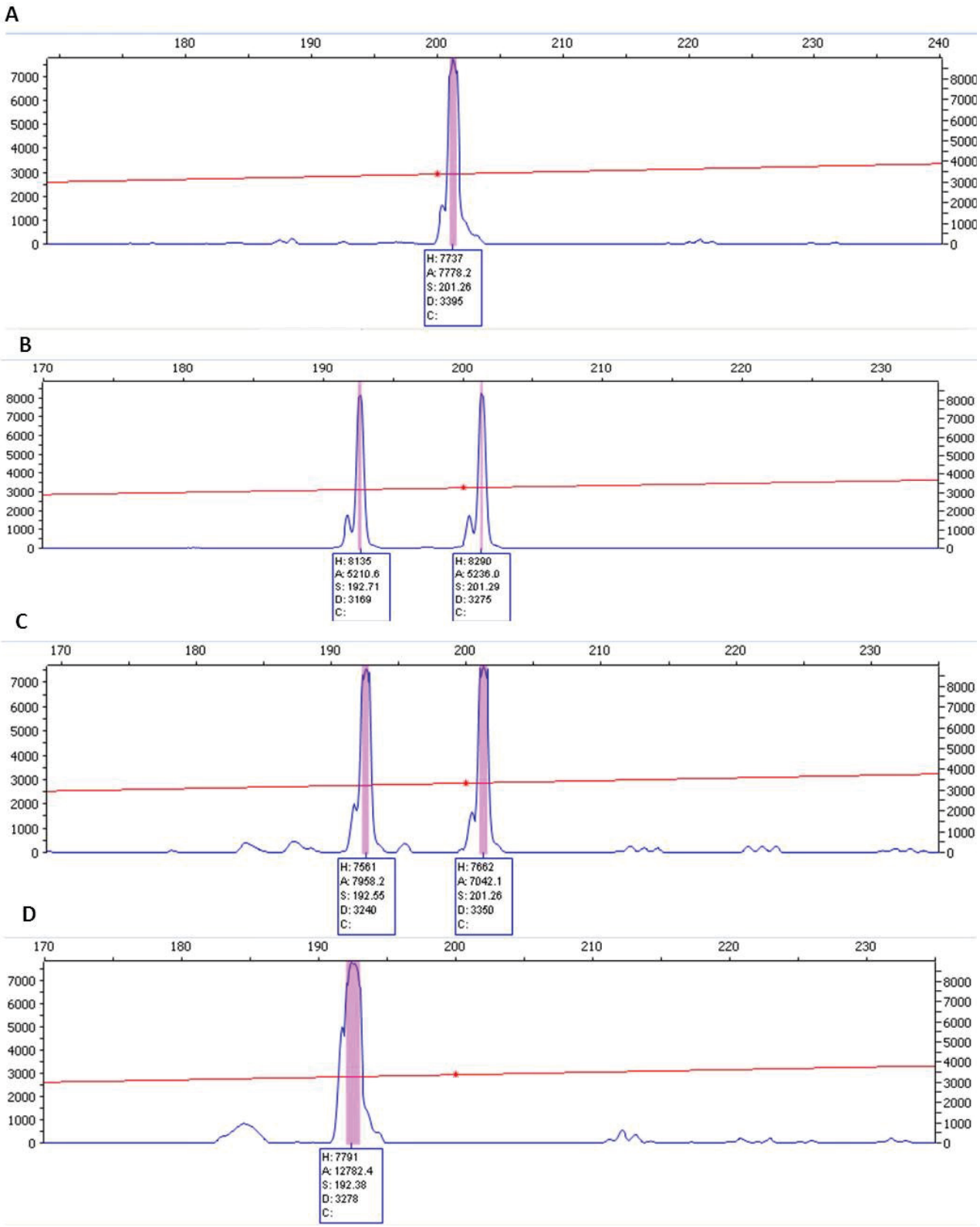

Figure S1. Characteristic chromatograms of four peach cultivars with different maturity dates. A) The early ripening (end of June) Springtime is homozygous for the 201-bp allele containing a 9-bp INDEL. B) and C) The two midseason cultivars, Big Top (end of July) and Babygold 6 (mid-August) are heterozygous, carrying both the 192-bp and 201-bp alleles. D) The late ripening (end of September) cultivar Vérbarack carries only the 192-bp reference allele.

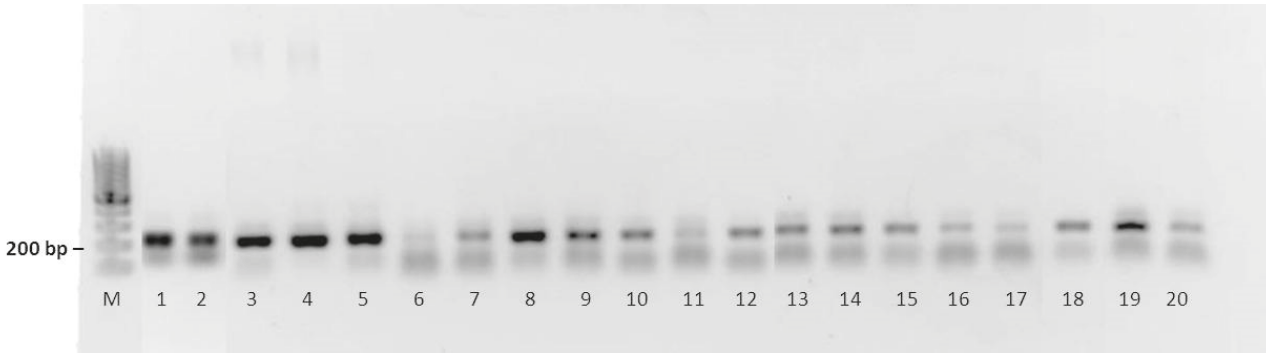

Figure S2. PCR analysis of 20 cultivars of different *Prunus* species with NAC-INDEL specific primer pair. Samples are (from the left): M – GeneRuler 100-bp ladder, 1 – *P. cerasus* Érdi jubileum, 2 – *P. cerasus* Piramis, 3 – *P. domestica* Stanley, 4 – *P. domestica* President, 5 – *P. dulcis* Tétényi keményhéjú, 6 – *P. dulcis* Tuono, 7 – *P. persica* Springtime, 8 – *P. persica* Vérbarack, 9 – *P. armeniaca* Pisana, 10 – *P. armeniaca* Zard, 11 – *P. armeniaca* Korai zamatos, 12 – *P. armeniaca* Harmat, 13 – *P. armeniaca* Corlate, 14 – *P. armeniaca* Kech-psar, 15 – *P. armeniaca* Samarkandskji-rannii, 16 – *P. armeniaca* Pannónia, 17 – *P. armeniaca* Ceglédi piroska, 18 – *P. armeniaca* Magyar kajszi C235, 19 – *P. armeniaca* Ceglédi óriás, 20 – *P. armeniaca* Rózsakajszi C1406.