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Characterisation of indigenous apple accessions with respect to polymorphism of *ACS1* and *ACO1* genes

Slađana Marić[1*], Ivana Glišić[1], Nebojša Milošević[1], Sanja Radičević[1], Milena Đorđević[1], Tatjana Vujović[2]

[1]*Department of Pomology and Fruit Breeding, Fruit Research Institute, Čačak, Kralja Petra I/9, 32000 Čačak, Republic of Serbia*
[2]*Department of Fruit Physiology, Fruit Research Institute, Čačak, Kralja Petra I/9, 32000 Čačak, Republic of Serbia*

**Corresponding author: smaric@institut-cacak.org*

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A B S T R A C T

Ethylene is the simplest signalling molecule with a hormone-like function that plays a major role in many developmental processes, including ripening of climacteric apple fruit. The allelic polymorphisms of *ACS1* and *ACO1* genes, encoding for ACC synthase and ACC oxidase, which catalyse the last two steps in the ethylene biochemical pathway, were analysed in nineteen indigenous apple accessions grown in individual growers' orchards in the regions of central and southwestern Serbia. A polymorphism was detected using the polymerase chain reaction (PCR) for the *ACS1* gene and the additional enzymatic digestion of the PCR product with *Bam*H1 and *Rsa*I for the *ACO1* gene. The *ACS1-1* and *ACS1-2* alleles of the *ACS1* gene, as well as the *a* and *c* alleles of the *ACO1* gene were identified. The polymorphisms observed upon PCRs and digestion with restriction enzymes were generated in two genotypes for both genes, i.e. the *ACS1* gene – *ACS1-1/1* and *ACS1-1/2*, and the *ACO1* gene – *aa* and *ac*. Out of nineteen apple accessions, sixteen were homozygous for the allele *ACS1-1* and three were heterozygous (*ACS1-1/2*); regarding the *ACO1* genotype, fourteen were homozygous for the allele *a* and five were heterozygous (*ac* allelic constitution). The molecular survey in the current study provides an increase in the number of apple accessions with potential to be used as parents in breeding programmes, aiming to obtain high quality cultivars that retain fruit texture during long storage. Therefore, the heterozygous accessions for the *ACS1* gene – 'J-LuN/1', 'Kraljica' and 'Šumatovka', may have an important position in future breeding programmes.

Keywords: *Malus* × *domestica* Borkh., ACC synthase, ACC oxidase, ethylene, allele, autochthonous genotype.

И З В О Д

Етилен је најједноставнији сигнални молекул са хормонском функцијом који утиче на бројне процесе током растења и развића, укључујући и дозревање климактеричног плода јабуке. Алелни полиморфизам *ACS1* и *ACO1* гена који кодирају 1-аминоциклопропан-1-карбоксилат (ACC) синтазу и ACC оксидазу, кључне ензиме у биосинтези етилена, анализиран је код деветнаест аутохтоних генотипова јабуке гајених у засадима индивидуалних произвођача на подручју појединих територијалних јединица централне и југозападне Србије. Полиморфизам *ACS1* гена је детектован применом ланчане реакције полимеразе (PCR метода), док је дигестија PCR производа рестрикционим ензимима *Bam*H1 и *Rsa*I коришћена за детектовање алела *ACO1* гена. У испитиваном материјалу идентификована су по два алела *ACS1* (*ACS1-1* и *ACS1-2*) и *ACO1* (*a* и *c*) гена. Уочени полиморфизам након PCR амплификације и рестрикционе анализе омогућио је идентификовање по две алелне конституције *ACS1* гена – *ACS1-1/1* и *ACS1-1/2*, као и *ACO1* гена – *aa* и *ac.* Од деветнаест анализираних аутохтоних генотипова јабуке, шеснаест су хомозиготи за алел *ACS1-1* (*ACS1-1/1*), док су три генотипа хетерозиготи (*ACS1-1/2*); у погледу *ACO1* алелне конституције, четрнаест генотипова су хомозиготи за алел *a* (*aa* алелна конституција) и пет генотипова су хетерозиготи (*ac* алелна конституција). Молекуларно истраживање у оквиру овог рада пружа нови квалитет у области очувања и одрживог коришћења генетичких ресурса, резултирајући повећањем броја генотипова који могу бити интересантни за будући оплемењивачки рад на стварању нових сорти јабуке добрих складишних способности. У том погледу, генотипови хетерозиготни за *ACS1* ген – 'Ј-ЛуН/1', 'Краљица' и 'Шуматовка', могу представљати важан почетни материјал за савремене програме оплемењивања, као и програме увођења у производњу аутохтоних генотипова јабуке жељених агрономских особина.

Кључне речи: *Malus* × *domestica* Borkh., ACC синтаза, ACC оксидаза, етилен, алел, аутохтони генотип.

1. Introduction

Apple (*Malus* × *domestica* Borkh.) is the main deciduous fruit crop of temperate regions of the world, with an annual production of more than 87 million tonnes in 2019 (FAOSTAT, 2021), ranking third after citrus and banana. In addition, according to the Food and Agriculture Organisation of the United Nations, apple is also an important fruit crop in the Republic of Serbia, whose average production in the period

2015–2019 (434,172 tonnes) reached the production of plum (429,209 tonnes) as the main fruit species in the country. Based on an analysis of the range of apple cultivars, only a few cultivars dominate the world production although more than 7,500 cultivars have been named and released (Kellerhals, 2009). Furthermore, a very limited number of cultivars have been used as parents in breeding programmes worldwide, i.e. 'Cox's Orange Pippin', 'Golden Delicious', 'Jonathan' and 'McIntosh' dominated mostly in earlier programmes, whilst 'Gala' and 'Fuji' are now the most commonly used parental cultivars (Noiton and Alspach, 1996; de Albuquerque Jr. et al., 2011). Apple breeding programmes need new germplasm, such as indigenous genotypes which appear to carry useful traits (Marić et al., 2016).

Additionally to breeding work, the Fruit Research Institute, Čačak also has a tradition of assessing genetic variability in indigenous apple genotypes in both *ex situ* and on-farm collections. In the Republic of Serbia, this evaluation has been intensified during the last fifteen years, and revealed the richness and diversity in biological properties of the material (Marić et al., 2005a, 2007, 2013, 2016; Mratinić, 2005; Mratinić and Fotirić-Akšić, 2011). Since indigenous genotypes carry useful genes, better genotypic and phenotypic characterisation should be conducted. There has been an increasing awareness of the value of old cultivars in recent years.

Fruit quality is determined by a large series of physiological changes which are genetically programmed to render the fruit more attractive to impact consumer appreciation, i.e. the fruit becomes softer, sweeter, more coloured and more aromatic throughout the phase of ripening (Giovannoni, 2001; Busato et al., 2016; Milošević et al., 2019). Apple fruits are climacteric in nature; therefore, physiological and biochemical processes are governed by the enzymes whose activity is triggered and coordinated by ethylene. The level of ethylene is tightly controlled by the activity of enzymes that catalyse the last two reaction steps in the biochemical pathway well-known as Yang cycle: 1-aminocyclopropane-1-carboxylate (ACC) synthase (EC: 4.4.1.14; the *ACS* gene) and ACC oxidase (EC: 1.14.17.4; *ACO* gene) (Yang, 1985). ACC synthase is a very important constituent of the cycle, generally accepted as a rate limiting factor in ethylene production; manipulating this step can influence its production, which affects the rate of ripening (Nybom et al., 2008, 2013; Dougherty et al., 2016).

In apple, *ACS* and *ACO* genes are encoded by multigene families, and 19 *ACS* genes and three *ACO* genes have been reported so far (Binnie and McManus, 2009; Li et al., 2013). Among these genes, *ACS1* and *ACO1* are predominantly expressed in ripening apple fruit (Harada et al., 2000; Wakasa et al., 2006; Li et al., 2013). Additionally, Li et al. (2013) reported that *ACS1*, together with *ACS7* and *ACS8*, is responsible for the burst of ethylene production within System 2 (McMurchie et al., 1972 provided an accurate description of Systems 1 and 2). Regarding the identified number of alleles of *ACS1* and *ACO1* genes, two and five alleles, respectively, have been revealed so far. The difference between the two alleles of the *ACS1* gene (*ACS1-1* and *ACS1-2*) is reflected in the presence of a short interspersed element (SINE) in the promoter region, whose homozygosity is known to correlate with the delayed ethylene production in apple genotypes (Sunako et al., 1999). Discrimination of *ACO1* alleles is based on the amplification of the entire gene and subsequent digestion with restriction enzymes (Marić et al., 2005a,b; Marić and Lukić, 2014) or on the fragment amplification that encompasses part of the third and the fourth exons, including the third intron which shows a large length polymorphism (Costa et al., 2005). Therefore, among *ACO1* alleles, A and B, firstly revealed by Castiglione et al. (1999), correlate with the alleles *a* and *b,* reported by Marić and Lukić (2014), and the alleles *ACO1-2* and *ACO1-1*, published by Costa et al. (2005), as well as with the alleles *c, d* and *n*, found by Marić et al. (2005b), Marić and Lukić (2014) and Marić (2016). To date, the allele *d* of the *ACO1* gene has been confirmed only in nine wild *Malus* species (Marić, 2016; Marić et al., 2019).

A few preharvest and postharvest treatments, including the application of chemicals to inhibit ethylene production or block ethylene receptors, as well as controlled atmosphere in storage chambers as an alternative, are used to prolong fruit storage ability (Mhelembe et al., 2020). These methods require additional costs, hence new low ethylene producing cultivars can help solve this problem. Actually, the objective of many breeding programmes worldwide is to maintain fruit texture during storage in order to provide year-round high-quality apples to consumers (Zhu and Barritt, 2008). Accordingly, for predicting and maximising apple fruit quality and shelf life, new parental genotypes and the integration of marker-assisted selection into conventional breeding programmes are of crucial importance (Marić and Lukić, 2014; Marić et al., 2016).

Over the past decade, the work at the Fruit Research Institute, Čačak has mainly been focused on the characterisation of indigenous apple genotypes from its *ex situ* collection (Marić et al., 2005a, 2007, 2013, 2016). To avoid the loss of the Serbian indigenous apple material, it is indispensable that the collection and characterisation of the existing landraces be continued, aiming to document, preserve and encourage the use of these genotypes. Therefore, in this work, we characterised for the first time the allelic polymorphism of genes involved in ethylene biosynthesis (*ACS1* and *ACO1* genes) in nineteen apple accessions grown in different regions of central and southwestern Serbia.

2. Materials and methods

2.1. Plant material and isolation of genomic DNA

Nineteen indigenous apple genotypes (Table 1), corresponding to old cultivars or landraces of unknown origin, were sampled in individual growers' orchards in central and southwestern Serbia, i.e. the regions of Čačak (villages Jezdina, Ostra and Prislonica), Užice (village Trnava), Kraljevo (village Samaila), Lučani (village Negrišori), Gornji Milanovac (town) and Aranđelovac (village Progoreoci). The name given to a particular apple landrace was formed on the basis of geographical determinants (city, municipality and village) and number, which was assigned according to the ripening time of the genotype in the specific region. Fresh leaves of apple genotypes were collected at the beginning of May 2021, frozen in liquid nitrogen and stored at -80°C. Frozen leaf samples were ground with

four ball-bearings (2 mm in diameter) in a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) and genomic DNA was isolated according to the CTAB mini prep method (Doyle and Doyle, 1987), with the extraction buffer modified with 0.5% β-mercaptoethanol and 2% polyvinylpyrrolidone (PVP 40). Isolated DNA was dissolved in TE buffer (10 mM Tris, pH8.0 and 1 mM EDTA), treated with RNase A (Invitrogen, Groningen, the Netherlands) and kept at -20°C until use for PCRs.

2.2. PCR analysis and allele identification for ACS1 genotyping

Approximately 100 ng of DNA was used for the PCR amplification of the *ACS1* genomic fragment in a final volume of 25 µl containing 1× PCR reaction buffer, 200 µM dNTPs, 0.2 µM ACS1–5'F and ACS1–5'R primers (Sunako et al., 1999), 1.5 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany). Amplification was carried out in a Mastercycler® nexus gradient (Eppendorf, Hamburg, Germany) thermal cycler using the following conditions: an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 2.5 min, and a final 10 min extension at 72°C. *ACS1* allele identification was based on the separation of PCR products in a 1.5% agarose gel in the Biometra Horizon 11.14 system (Analytik Jena GmbH, Jena, Germany) for about 4 h at 70 V cm^{-1}; 1 Kb plus DNA ladder (Invitrogen) was included for the sizing of fragments. Visualisation of DNA bands was performed by ethidium bromide staining and under ultraviolet light of the BIO-PRINT-1500/26M imaging system (Vilber Lourmat, Collégien, France).

2.3. PCR analysis and allele identification for ACO1 genotyping

PCRs were carried out in a 50 µl volume with 100 ng genomic DNA, 1× PCR reaction buffer, 0.2 mM of each dNTP, 0.1 µM M11 and M12 primers (Castiglione et al., 1999), 1.5 mM $MgCl_2$ and 0.625 U *Taq* DNA polymerase (Qiagen). Amplification conditions in Mastercycler® nexus gradient (Eppendorf) were: 92°C for 3 min, followed by 5 cycles of 92°C for 1 min, 65°C for 1 min and 72°C for 1.5 min, then 35 cycles of 92°C for 1 min, 60°C for 1 min and 72°C for 1.5 min, with a final 10-min extension step at 72°C. The PCR products were checked in a 1.5% agarose gel for 3 h at 70 V cm^{-1}. In order to reveal the allelic polymorphism of *ACO1* gene, the PCR product was digested with *Bam*H1 and *Rsa*I (Fermentas, Termo Scientific, Waltham, USA) as follows: an aliquot of 25 µl PCR product was incubated for 12 h at 37°C with 0.2 µl restriction enzyme (10 U $µl^{-1}$) and 2.8 µl 10× buffer. DNA digested *ACO1* fragments were separated by electrophoresis in 2% agarose gel (70 V cm^{-1} for 4 h); detection, visualisation and sizing of *ACO1* fragments were performed according to the methodology described in the above paragraph.

3. Results and discussions

This study reports a genotyping survey of nineteen old apple cultivars and landraces of unknown origin for two most important genes – *ACS1* and *ACO1* involved in the ethylene biosynthetic pathway, among which fifteen were published here for the first time.

3.1. ACS1 genotyping of old cultivars and landraces

The identification of *ACS1* alleles was based on the PCR amplification of the promoter region of this gene using the ACS1-5´F/R primers reported by Sunako et al. (1999). Amplification products of approximately 510 bp and 650 bp, corresponding to the *ACS1-1* and *ACS1-2* alleles, respectively, can be clearly distinguished when separated with agarose gel electrophoresis. The allelic variation of this locus was based on the 162 bp SINE insertion in the promoter region, with a concomitant deletion of 24 bp (Sunako et al., 1999). Therefore, the observed polymorphism (*ACS1-1* and *ACS1-2* alleles) generated two allelic constitutions of the *ACS1* gene in assessed indigenous apple accessions (Table 1). Out of nineteen apple genotypes, sixteen were homozygous for allele *ACS1-1* (*ACS1-1/1*) and three were heterozygous (*ACS1-1/2*). The obtained allelic pattern in several cultivars and landraces is shown in Figure 1.

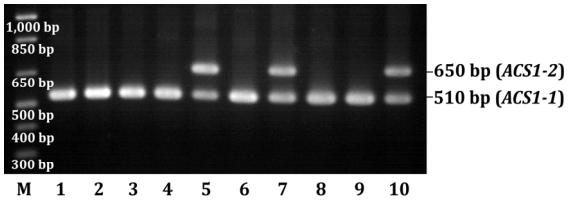

Figure 1. *ACS1* banding pattern of ten indigenous apple accessions analysed on a 1.5% agarose gel and stained with ethidium bromide: 'Prancija Slatka' (lane 1); 'Lubenjaja' (lane 2); 'Vidovača' (lane 3); 'Lepocvetka' (lane 4); 'J-LuN/1' (lane 5); 'Ilinjača' – Trnava (lane 6); 'Kraljica' (lane 7); 'J-GM/1' (lane 8); 'J-ČaP/1' (lane 9); 'Šumatovka' (lane 10); 1Kb plus DNA ladder (lane M)

Table 1.
ACS1- and ACO1-genotyping of indigenous apple cultivars and landraces grown in central and southwestern Serbia

Location	Accession name	Coordinates and altitude	*ACS1* genotype	*ACO1* genotype
Jezdina	Budimka	43°51'653"N; 20°18'200"E; 397 m	*ACS1-1/1*	*ac*
Trnava	Džumurka	43°57'545"N; 19°53'981"E; 539 m	*ACS1-1/1*	*ac*
Jezdina	Ilinjača	43°51'681"N; 20°18'254"E; 396 m	*ACS1-1/1*	*aa*
Trnava	Ilinjača	43°57'476"N; 19°53'987"E; 532 m	*ACS1-1/1*	*aa*
Ostra	J-ČaO/1	43°55'713"N; 20°29'679"E; 319 m	*ACS1-1/1*	*ac*
Prislonica	J-ČaP/1	43°56'218"N; 20°27'036"E; 354 m	*ACS1-1/1*	*ac*
G. Milanovac	J-GM/1	44°01'193"N; 20°24'916"E; 197 m	*ACS1-1/1*	*aa*
Negrišori	J-LuN/1	43°50'282"N; 20°10'581"E; 333 m	*ACS1-1/2*	*aa*
Jezdina	Kolačara	43°51'640"N; 20°18'175"E; 401 m	*ACS1-1/1*	*aa*
Progoreoci	Kraljica	44°20'380"N; 20°24'814"E; 184 m	*ACS1-1/2*	*aa*
Samaila	Lepocvetka	43°46'010"N; 20°30'044"E; 266 m	*ACS1-1/1*	*aa*
Jezdina	Lubenjaja	43°51'140"N; 20°17'917"E; 446 m	*ACS1-1/1*	*aa*
Jezdina	Prancija	43°51'649"N; 20°18'090"E; 383 m	*ACS1-1/1*	*aa*
Jezdina	Prancija Slatka	43°51'090"N; 20°17'992"E; 441 m	*ACS1-1/1*	*aa*
Jezdina	Senabija	43°51'018"N; 20°18'154"E; 499 m	*ACS1-1/1*	*aa*
Trnava	Slatka Kadumana	43°57'514"N; 19°53'993"E; 534 m	*ACS1-1/1*	*aa*
Progoreoci	Strekinja	44°20'412"N; 20°24'090"E; 203 m	*ACS1-1/1*	*aa*
Prislonica	Šumatovka	43°56'257"N; 20°27'050"E; 358 m	*ACS1-1/2*	*aa*
Jezdina	Vidovača	43°51'662"N; 20°18'311"E; 386 m	*ACS1-1/1*	*ac*

In the current study, we used the primers published by Sunako et al. (1999), whereas redesigned primers (ACS1-Pr´F/R) for distinguishing the *ACS1* alleles via an automated sequencer were reported by Mhelembe et al. (2020). The fragment sizes of 202 bp and 339 bp were observed for alleles *a* and *b*, which correlate with *ACS1-1* and *ACS1-2* alleles, respectively. Even though fluorescent sizing is costly, it can reveal variation in the product size not distinguishable by agarose gel electrophoresis. In addition, the same authors tested the fluorescently labelled ACS1-5 primers reported by Sunako et al. (1999) on five cultivars and detected that fragment sizes of 514 bp and 652 bp were consistently observed for the two alleles.

Among the assessed cultivars and landraces, the *ACS1* genotypes for the four cultivars ('Budimka', 'Kraljica', 'Strekinja' and 'Šumatovka') were published in our previous studies (Marić et al., 2005a, b). The results for 'Budimka' and 'Strekinja' (both *ACS1-1/1*) were consistent with those reported, but not for 'Kraljica' and 'Šumatovka'. The current study revealed the heterozygosity of the *ACS1* gene (*ACS1-1/2*) in 'Kraljica' and 'Šumatovka', while the homozygosity of these cultivars for the *ACS1-1* allele was published by Marić et al. (2005a). Namely, Marić et al. (2005a) analysed the autochthonous genotypes from the *ex situ* apple collection of Fruit Research Institute, Čačak, whilst the material in this study was sampled in the on-farm collection, indicating that the origin of many old cultivars/landraces is undocumented and their names confusing, due to the occurrence of homonyms and synonyms. On the contrary, the cultivar 'Ilinjača' sampled from different locations showed the same allelic constitution of the *ACS1* gene.

As we expected, *ACS1-1* was the most frequent allele in this indigenous material, occurring at a frequency of 91.7% (excluding one 'Ilinjača', i.e. this cultivar was counted once; and assuming that all genotypes were diploid), while lower frequency for the *ACS1-2* allele was found (8.3%). Other studies that reported the *ACS1* genotyping of apples in germplasm collections, including indigenous genotypes, also found a high frequency of the *ACS1-1* allele and an overrepresentation of the *ACS1-1/1* allelic constitution. Out of 24 autochthonous genotypes assessed by Marić et al. (2005a), 21 were homozygous for allele *ACS1-1* and three were heterozygous. Nybom et al. (2013) reported that among 127 apple cultivars (out of which 45 probably originated in Sweden), 73 were homozygous for *ACS1-1*, 46 were heterozygous and eight were homozygous for the *ACS1-2* allele. In the latest study, Mhelembe et al. (2020) stated that 102 accessions were homozygous for allele *a* (correlates with allele *ACS1-1*), 100 were heterozygous and 22 were homozygous for allele *b* (correlates with allele *ACS1-2*).

The frequency of the *ACS1-2* allele has substantially increased in newly released apple cultivars, indicating that this allele due to its dramatically reduced transcriptional activity has been favoured for the breeding of low ethylene producing cultivars within modern programmes (Nybom et al., 2008, 2012). Therefore, knowledge of *ACS1* genotypes will lead to the choice of parents for crossing, since accessions with *ACS1-2/2* genotypes would create progenies also homozygous for the *ACS1-2* allele, but in cases where heterozygous (*ACS1-1/2*) parents are crossed with each other or with *ACS1-2/2* homozygotes, the progenies will segregate. New cultivars homozygous for the *ACS1-2* allele are absolutely essential for both apple exporters and small-scale growers who are unable to provide sophisticated postharvest facilities to be faced with storage challenges. From this point of view, including some desirable biological traits, the heterozygous accessions

('J-LuN/1', 'Kraljica' and 'Šumatovka') analysed in this study may have an important position in future breeding programmes.

3.2. ACO1 genotyping of old cultivars and landraces

The amplification of the *ACO1* gene by using M11 and M12 primers (Castiglione et al., 1999) resulted in a PCR product of approximately 1,600 bp (Figure 2a). The size of the PCR fragment was consistent with the *ACO1* product reported for autochthonous and foreign cultivars (Marić et al., 2005a; Marić and Lukić, 2014), promising selections singled out at the Fruit Research Institute, Čačak (Marić et al., 2005b) and seedlings of an interspecific F_1 progeny from the cross 'Fiesta' × 'Totem' (Fernández-Fernández et al., 2008) and *Malus* species (Marić, 2016; Marić et al., 2019).

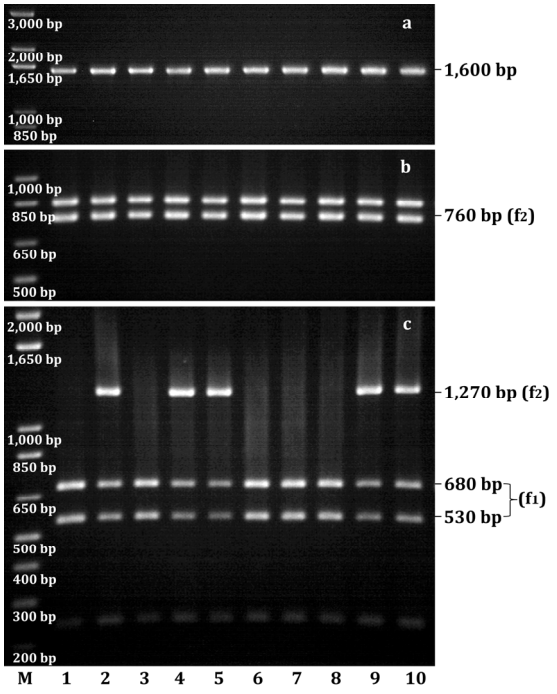

Figure 2. The *ACO1* PCR products (a) and DNA fragments obtained upon restriction analysis with *Bam*H1 (b) and *Rsa*I (c) in ten indigenous apple accessions, separated on a 2% agarose gel and stained with ethidium bromide: 'Prancija Slatka' (lane 1); 'Budimka' (lane 2); 'Lubenjaja' (lane 3); 'Džumurka' (lane 4); 'J-ČaO/1' (lane 5); 'Ilinjača' – Trnava (lane 6); 'Kraljica' (lane 7); 'J-GM/1' (lane 8); 'J-ČaP/1' (lane 9); 'Vidovača' (lane 10); 1Kb plus DNA ladder (lane M)

Given that a monomorphic PCR product was obtained, further identification of *ACO1* alleles was based on its restriction analysis conducted by digestion with *Bam*H1 [yielded a monomorphic fragment of 830 bp and a segregating fragment of 760 bp (f_2); another segregating fragment of 700 bp (f_1) was not observed] and *Rsa*I [yielded a monomorphic fragment of 230 bp and two segregating fragments of 530 and 680 bp (considered as f_1 fragment) and 1,270 bp (f_2)] enzymes. According to Marić and Lukić (2014), the observed polymorphism upon digestion with *Bam*H1 and *Rsa*I was interpreted. The current results confirmed two alleles (*a* and *c*) and two allelic constitutions (*aa* and *ac*). Out of nineteen indigenous apple accessions (Table 1), fourteen were homozygous for allele *a* and five were heterozygous (*ac* allelic constitution). Figure 2b–c summarises the outcome of the *ACO1* PCR product digestion with restriction enzymes, while the relationship between the segregating fragments, the alleles and the allelic constitutions is presented in Table 2.

Table 2.
Relationship between the f_1 and f_2 restriction fragments, obtained upon digestion of the PCR product with *Bam*H1 and *Rsa*I enzymes, the deduced alleles and the allelic constitutions of the *ACO1* gene

Restriction enzyme		*ACO1* allele	Allelic constitution of *ACO1* gene
***Bam*H1**	***Rsa*I**		
f_2 /	f_1 /	*a*	*aa*
f_2 /	f_2 /	*c*	*cc*
f_2 /	f_1f_2	/	*ac*

The identification of *ACO1* alleles in the current study was performed according to our previous studies, i.e. PCR amplification of the entire gene, which encompasses four exons and three introns, followed by digestion with two restriction enzymes (Marić et al., 2005a,b; Marić and Lukić, 2014; Marić, 2016; Marić et al, 2019). This manner made it possible to reveal four alleles (*a, b*, *c* and *d*) of the ripening-specific *ACO1* gene, whose identity was confirmed by cloning and sequencing of their full-length nucleotide sequences (Marić and Lukić, 2014; Marić, 2016). The same authors reported that molecular characterisation of identified alleles revealed a 62 bp deletion in the third intron of alleles *b* and *d*, but not in alleles *a* and *c*; this deletion might affect their expression profiles. On the other hand, the identification of the two *ACO1* alleles (*ACO1-1* and *ACO1-2* corresponding to alleles *b* and *a*, respectively), published by Costa et al. (2005), Zhu and Barritt (2008) and Nybom et al. (2013), was based on the PCR amplification of the fragment that comprises the region of the third intron with part of the third and the fourth exons.

Four alleles (*a, b*, *c* and *d*) and nine allelic constitutions (*aa*, *bb*, *cc*, *dd*, *ab*, *ac*, *ad*, *bd* and *cd*) were reported in 24 indigenous apple genotypes (Marić et al., 2005a), 28 foreign cultivars (Marić and Lukić, 2014) and nine *Malus* species (Marić, 2016; Marić et al., 2019). Therefore, allele *a* was the most frequent allele in indigenous and foreign genotypes (average 74.5%), while alleles *b* and *c* occurred at an average frequency of 11.7% and 13.8%, respectively. Among the *Malus* species, allele *d* was one of the most frequent *ACO1* alleles (66.7%), while *a*, *b* and *c* were less frequent (16.7%, 5.5% and 11.1%, resp.). The current study also revealed a high occurrence of allele *a* (86.1%) and a frequency of 13.9% for allele *c*, which was remarkably similar in comparison with the occurrence of this allele in the previously studied material.

Contrary to the allelic constitution of the *ACS1* gene, the *ACO1* genotypes for 'Budimka', 'Kraljica', 'Strekinja' and 'Šumatovka' were in agreement with the results heretofore reported by Marić et al. (2005a, b). Since this study revealed that 'Kraljica' and 'Šumatovka' differed in *ACS1* allelic constitution in comparison to previous reports for these cultivars, the results suggest that the names of both cultivars were probably used

very often and could be the cases of homonyms. Certainly, further work through the application of the most suitable and reliable tool for cultivar identification and clearing up synonyms or homonyms, such as microsatellite markers, will elucidate the molecular profiles of these cultivars.

So far, the functional relevance of *ACO1* polymorphisms has not been explained, although Castiglione et al. (1999) suggested that the allelic forms of this gene might correlate with a wide range of ethylene production during apple fruit ripening. Marić et al. (2005b) underlined the possible positive role of allele *b* in a long storage life of apple fruits, as well as the need for progeny testing and further analysis of the genes involved in ethylene perception. Progeny studies revealed that *ACS1* and *ACO1* independently affect the internal ethylene concentration, as well as that seedlings homozygous for *ACS1-2* and *ACO1-1* (correlates with allele *b*) showed to have the lowest ethylene production and superior shelf-life (Costa et al., 2005; Zhu and Barritt, 2008). Nybom et al. (2013) reported that no effects on firmness at harvest following cold storage or softening rate were associated with the *ACO1* alleles.

In addition to these two genes involved in ethylene biosynthesis, other ripening-related genes, which could be useful for genotyping the accessions within the breeding programme, are also important and they affect the overall storability of apple fruits, including *ACS3* (*ACS1* accelerator; Wang et al., 2009), *PG1* (polygalacturonase; post-harvest softening; Wakasa et al., 2006) and *Exp7* (expansion; fruit softening; Costa et al., 2008; Nybom et al., 2012).

4. Conclusions

The preservation and characterisation of apple germplasm need to be the strategy for the valorisation of the genetic resources linked with the history and tradition of the territory of the Republic of Serbia. This study is a further genotyping survey of indigenous apple material for the genes involved in ethylene biosynthesis (*ACS1* and *ACO1* genes), which have not been published to date. Therefore, out of nineteen apple accessions, the *ACS1* and *ACO1* allelic constitutions for fifteen accessions are published here for the first time. This molecular survey provides an increase in the number of apple accessions with potential to be used as parents in breeding high quality cultivars with low ethylene production and long-term storage capability. In addition, genotypic data presented together with phenotypic data (the work in progress) will be a valuable resource for important apple traits that could be found in on-farm collections.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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