Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2019) 43: 464-477
© TÜBİTAK
doi:10.3906/tar-1803-3

Molecular characterization of apple (*Malus* × *domestica* Borkh.) genotypes originating from three complementary conservation strategies

Anca BUTIUC-KEUL[1,2], **Ana COSTE**[3], **Anca FARKAS**[1,2], **Victoria CRISTEA**[2,4], **Valentina ISAC**[5], **Adela HALMAGYI**[3,*]

[1]Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babes-Bolyai University, Cluj-Napoca, Romania

[2]Laboratory of Plant Biology, Genetics, Microbiology, and Biotechnology, Center of Systems Biology, Biodiversity, and Bioresources, Babeş-Bolyai University, Cluj-Napoca, Romania

[3]National Institute of Research and Development for Biological Sciences, Branch Institute of Biological Research, Cluj-Napoca, Romania

[4]'Al. Borza' Botanical Garden, Babes-Bolyai University, Cluj-Napoca, Romania

[5]Research Institute for Fruit Growing, Piteşti, Romania

Received: 05.03.2018 • **Accepted/Published Online:** 20.04.2019 • **Final Version:** 02.10.2019

Abstract: Apple (*Malus* × *domestica* Borkh.) genotypes originating from different plant collections (field collection, in vitro plant collections undergoing or not undergoing cryopreservation) were screened and characterized by SSR markers. Shoot tips excised from plants grown in vitro were successfully cryopreserved by encapsulation-dehydration. The highest regrowth frequency (69%, cultivar Goldrush) of cryopreserved apices was achieved after 24 h of osmoprotection in 0.5 M sucrose, 3 h of desiccation, and 24% water content of alginate beads. No differences in morphological characteristics including shoot length and number and length of roots were observed between controls and plants recovered after cryopreservation. SSR markers were used for calculation of genetic similarities between plants from the field collection, in vitro-micropropagated plants, or plants regenerated after liquid nitrogen storage. The set of microsatellite markers showed a low level of polymorphism among the studied genotypes, which could be distinguished by a specific combination of alleles generated by CH03g07, CH05c02, CH05d11, and CH05e03 primers. The CH03g07, CH05c02, CH05d11, CH05e03, GD96, GD147, and GD162 SSR markers exhibited low levels of polymorphism, while CH04AE07, CH04g10, GD100, and GD142 were nonpolymorphic. The Dice coefficient confirmed the effectiveness of SSRs for distinguishing between plants from ex situ collections and preserved plants. No major differences between ex situ plants, micropropagated plants, and plants recovered after cryopreservation were observed.

Key words: Cryostorage, encapsulation-dehydration, microsatellites, SSR markers

1. Introduction

Germplasms of temperate perennial fruit species are usually conserved as whole plants in the field. Ex situ preservation of plant genetic resources plays an important role in the maintenance of biodiversity, although preservation of field collections carries the risk of infections, pests, diseases, or environmental disasters (Panis and Lambardi, 2005). Biodiversity is fundamental to both biotechnology and sustainable agriculture (Singh, 2000). As a consequence, to secure the maintenance of plant genetic resources over the years, alternative conservation approaches have been developed. To overcome the difficulties of grafting in woody species, tissue culture techniques have been applied for mass propagation, including various tissue types such as shoot tips (Butiuc-Keul et al., 2010; Feng et al., 2013), dormant buds (Höfer, 2015), or in vitro axillary buds (Condello et al., 2011). The role of in vitro techniques within ex situ conservation strategies for trees has been well documented (Cruz-Cruz et al., 2013), and among them conservation by slow growth has been successfully applied for plants of both temperate and tropical species (Lambardi et al., 2007). There are many different virus diseases in fruit trees, which may produce visible symptoms in some varieties but remain latent in others, leading to significant quantitative and qualitative yield reduction (Nemeth, 1986). Apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), and apple stem grooving (ASGV) are some of the most common viral pathogens (Desvignes et al., 1999); therefore, screening of plant material for viruses before induction of in vitro cultures is highly significant. The maintenance of in vitro culture stability over a long period of time is of fundamental importance as it enables multiplication of healthy plant material; therefore, assessment of genetic integrity of regenerated

* Correspondence: adela.halmagyi@icbcluj.ro

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plants after long-term in vitro culture is desirable (Harding, 2004). Cryopreservation in liquid nitrogen (–196 °C) as a long-term conservation method was applied to a diverse range of vegetatively propagated plant species, including fruit trees (Engelmann, 2008; Reed, 2008; Benelli et al., 2013). Encapsulation-dehydration is based on desiccation of alginate-coated plant material (shoot tips, somatic embryos, cell suspensions) in the presence of sucrose or other osmotically active substances followed by evaporative desiccation (Wang et al., 2005; Halmagyi and Deliu, 2006; Barraco et al., 2011).

Apple (*Malus* × *domestica* Borkh.) is considered the most economically important fruit crop in the temperate zone (Sansavini et al., 2004). Since the first cryopreservation report on shoot tips (Kuo and Lineberger, 1985), various protocols have been developed for apple, such as two-step freezing (Wu et al., 1999), encapsulation-vitrification (Paul et al., 2000), droplet-vitrification (Kushnarenko et al., 2009; Halmagyi et al., 2010), the dormant-bud technique (Lambardi et al., 2011), and encapsulation-dehydration (Niino and Sakai, 1992; Feng et al., 2013). It is known that apple trees have a gametophytic self-incompatibility mechanism, which enforces outbreeding and a high level of heterozygosity (Kitahara et al., 2005). Genetic analysis by molecular markers could solve the problem of genetic identity of vegetatively propagated woody species like apple trees. Microsatellite (simple sequence repeat, SSR) DNA became a popular tool for fingerprinting germplasm collections (Cipriani et al., 2008). SSR markers have been successfully applied not only for evaluation of genetic diversity within germplasm collections (Guarino et al., 2006; Pereira-Lorenzo et al., 2007; Sikorskaite et al., 2012) but also for apple tree cultivars (Galli et al., 2005; Pérez-Romero et al., 2015; Ganopoulos et al., 2017) or for parent identification (Kitahara et al., 2005; Király, 2013). Although molecular characterization by SSR markers of apple cultivars from the United States (Hokanson et al., 1998) and some European countries (Galli et al., 2006; Patocchi et al., 2009; Sikorskaite et al., 2012; Király, 2013; Pérez-Romero et al., 2015; Ganopoulos et al., 2017) was accomplished, to the best of our knowledge there are no records regarding molecular characterization by SSR markers of apple genotypes commonly bred in Romania originating from 3 complementary conservation strategies. Structural changes in genomic DNA from leaves of apple trees grown in a field collection and in vitro-grown plants have been investigated using surface-enhanced Raman spectroscopy (Muntean et al., 2011a, 2011b).

The main objective of this study was molecular characterization by SSR markers of plant material (6 apple genotypes) originating from 3 conservation strategies: 1) a field collection (ex situ), 2) an in vitro collection (3 years of in vitro conservation), and 3) a cryocollection (plants regenerated from cryopreserved shoot apices). Encapsulation-dehydration cryopreservation method-related elements (e.g., sucrose concentration, water content of the alginate beads, desiccation duration) have been analyzed and morphological and physiological parameters were assessed to characterize plant recovery after cryopreservation. Selection of a predetermined set of marker loci will enable us to link up the profiles of our apple cultivars with those from other laboratories, resulting in a comprehensive database for European apple genotypes.

2. Materials and methods

2.1. Plant material

Initiation of in vitro apple (*Malus* × *domestica* Borkh., cultivars Goldrush, Rebra, Romus3, Romus4, Idared, and Florina) tissue cultures was accomplished from buds harvested from mature plants of a field collection (Mărăcineni Pitești, Romania). In vitro plants were grown on Murashige-Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with vitamins (Lee and De Fossard, 1977), 1.2 mg L^{-1} *N6*-benzyladenine (BA), 0.004 mg L^{-1} α-naphthalene-acetic acid (NAA), 30 g L^{-1} dextrose, and 7 g L^{-1} agar (Vălimăreanu et al., 2010). The plants were grown at 24 ± 1 °C under a 16-h light photoperiod with a light intensity of 40 µmol m^{-2} s^{-1} photosynthetic active radiation. By monthly transfer of nodal stem segments (1.5 cm in length) with two leaves on the above-mentioned medium the plants were maintained for 3 years. For cryopreservation by encapsulation-dehydration (Figures 1a–1e), shoot tips (apical meristem covered by 2–3 leaf primordia) approximately 0.2–0.3 cm in length were excised from in vitro plants (30 days after transfer) under a stereomicroscope in aseptic conditions (Figure 1a).

2.2. Serological DAS-ELISA tests

Leaf samples (collected in April and May from the field collection) were used to detect the following viruses: apple chlorotic leaf spot (ACLSV), apple mosaic virus (ApMV), apple stem grooving (ASGV), apple proliferation phytoplasma (ApP), and apple stem pitting virus (ASPV). DAS-ELISA serological tests (Bioreba commercial kit) for virus identification were performed following the protocol described by Clark and Adams (1977). The absorbance was measured at 405 nm using the PR 2100 microplate reader. The tested plant material was used for further initiation of in vitro cultures.

2.3. Encapsulation, osmoprotection, desiccation, cryopreservation, and recovery

The excised apices were individually encapsulated in alginate beads by transferring them from a solution of 3% sodium alginate in Ca^{2+}-free MS to a solution of 100 mM $CaCl_2$ and MS mineral salts, where they remained for 20 min under continuous stirring. After polymerization, the beads (approximately 0.4–0.5 cm in diameter) were

Figure 1. Cryopreservation of apple shoot tips by encapsulation-dehydration. (a) Shoot tips on filter paper humidified with MS liquid medium; (b) Na-alginate coated shoot tips; (c) shoot recovered from cryopreserved shoot tip; (d) shoots recovered from cryopreserved shoot tips (interrupted circles represent dead shoot tips; cultivar Florina 6 weeks after rewarming); (e) plants recovered from cryopreserved shoot tips (cultivar Florina 3 months after rewarming). Bars represent 1 cm.

washed with liquid MS medium (without $CaCl_2$) to eliminate excess $CaCl_2$ (pH 5.7) (Figure 1b). To increase tolerance to liquid nitrogen (LN), encapsulated shoot tips were incubated in MS medium containing sucrose (0.25, 0.50, 0.75, and 1.00 M) for 24 h on a rotary shaker (98 rpm) at 24 ± 1 °C. Osmoprotected beads were then dehydrated in laminar air flow for up to 5 h. During desiccation, the environmental conditions of the room were monitored for temperature (24 ± 1 °C) and relative humidity (38 ± 4%). At 1-h intervals, dehydrated beads were placed in 2-mL cryovials (5 beads/cryovial) and immersed in liquid nitrogen (–196 °C) contained in a 25-L Dewar flask, where the samples remained for 24 h. Rewarming was performed by immersion of cryovials in a water bath (38 °C) for 2 min. Regrowing of shoot tips was performed on the same medium as used for in vitro plant growth but with 3.5 g L^{-1} agar at the same temperature and growth conditions as mentioned for plant multiplication.

2.4. Water content of alginate beads

To determine the optimum desiccation time for high regrowth frequencies of encapsulated shoot tips following cryopreservation, the amount of water loss in the beads was monitored. For dry weight determination, 10 beads were weighed in 3 replications and dried at 60 °C until constant weight was attained. The percentage of water content was calculated after every hour of desiccation and was expressed on a fresh weight basis using the following formula:

Moisture content (MC) (%) = [(Fresh weight – Dry weight) / Fresh weight] × 100

2.5. SSR markers

Genomic DNA was isolated from leaves of apple cultivars using the CTAB method (Doyle and Doyle, 1987). Molecular characterization was performed by SSR markers and three categories of plant material were analyzed: 1) plants from a field collection, 2) plants from an in vitro collection, and 3) plants from a cryocollection. For each cultivar, 3 clones (noted as v1, v2, v3 for in vitro collection and c1, c2, c3 for plants from cryocollection) were analyzed and compared to plant material from the field collection. For microsatellite marker analysis, 11 primer pairs shown to be specific to polymorphic microsatellite loci of *Malus* ×

domestica and recommended by the ECPGR *Malus/Pyrus* working group were selected: CH03g07, CH04AE07, CH04g10, CH05c02, CH05d11, CH05e03 (Liebhard et al., 2002), GD96, GD100, GD142, GD147, and GD162 (Hokanson et al., 1998) (Table 1). PCR amplifications were performed in 0.2-mL tubes containing 2 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP, 1.5 U of Taq, and 25 ng of genomic DNA in a final volume of 25 µL. The amplification program was as follows: 1) T = 94 °C, 4 min; 2) T = 94 °C, 50 s; 3) primer alignment at 56 °C, 50 s; 4) elongation T = 72 °C, 50 s; steps 2–4 were repeated 45 times. Amplicons were separated on 2.0% agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. SSR analyses were performed twice to confirm the repeatability of the scored bands. Genetic similarities between the 3 categories studied were measured by the Dice coefficient (Dice, 1945) with Past software (Hammer et al., 2001), and the neighbor-joining tree was constructed using the same software. Allelic polymorphism information content (PIC) was calculated by the following formula: $PIC = 1 - \sum Pi^2$, where Pi is the frequency of the allele calculated for each SSR marker (Anderson et al., 1993).

Table 1. SSR primers used for PCR amplification.

Primer	Sequence 5'–3'
CH03g07	aataagcattcaaagcaatccg tttttccaaatcgagtttcgtt
CH04AE07	ttgaagatgtttggctgtgc tgcatgtctgtctcctccat
CH04g10	caaagatgtggtgtgaagagga ggaggcaaaaagagtgaacct
CH05c02	ttaaactgtcaccaaatccaca gcgaagctttagagagacatcc
CH05d11	cacaacctgatatcggggac gagaaggtcgtacattcctcaa
CH05e03	cgaatattttctactctgactggg caagttgttgtactgctccgac
GD96	cggcgaaagcaatcacct gccagccctctatggttccaga
GD100	acagcaaggtgttgggtaagaaggt tgcggacaaaggaaaaaaaaaagtg
GD142	ggcacccaagcccctaa ggaacctacgacagcaaagttaca
GD147	tcccgccatttctctgc aaaccgctgctgctgaac
GD162	gaggcaagtgacaaagaaagatg aaaatgtaacaacccgtccaagtg

2.6. Assessment of recovery following cryopreservation and molecular characterization

Regrowth of shoot tips was evaluated 30 days after liquid nitrogen treatment and was expressed as the percentage of single apices that developed shoots. Encapsulated explants treated with sucrose, desiccated but not cryopreserved, were used as controls. Ten to 12 shoot tips were used for each of the 3 replications per treatment. The results were expressed as mean percentages ± standard deviation (SD). Data were analyzed by ANOVA using Tukey's test for data comparison. Since cryopreserved shoot tips show a lag phase in their recovery following liquid nitrogen treatment, the assessment of morphological characteristics was performed after different durations (3 weeks after transfer to regeneration medium for control shoots, and 4 weeks after transfer to regeneration medium for cryopreserved shoots).

3. Results

3.1. Screening for viruses

The DAS-ELISA serological test results were negative, showing that the genotypes are free of the mentioned viruses (Table 2) and could be used as donors for induction of healthy in vitro cultures.

3.1. Effects of sucrose on shoot regrowth from encapsulated and osmoprotected shoot tips

Comparison of recovery frequencies for the 6 genotypes showed a significant decrease in regrowth of shoot tips with increased sucrose concentrations. Osmotic dehydration was excessive in case of 1.00 M sucrose, causing stress to shoot tips, with significantly negative effects on shoot regrowth in all cultivars (Table 3). Lower sucrose concentrations (0.25 M and 0.50 M) resulted in recovery frequency of up to 91%, suggesting that 24 h of exposure to these concentrations did not negatively influence shoot recovery (Table 3).

3.2. Cryopreservation and changes in the water content of alginate beads during desiccation

Due to low regrowth percentages obtained with 1.00 M, only 0.25 M, 0.50 M, and 0.75 M sucrose concentrations were considered in the following experiments (Figures 2a–2f). The most efficient treatment for shoot development following cryopreservation was osmoprotection in 0.50 M sucrose in combination with 3 or 4 h of desiccation according to cultivar. The highest regrowth percentage (69%, cultivar Goldrush) was achieved at 24% MC of the alginate beads after 3 h of dehydration (Figure 2a). A desiccation time of 4 h following osmoprotection in 0.50 M sucrose also led to high regrowth rates for cultivars Rebra (64% at 20% MC), Idared (61% at 21% MC), and

Table 2. DAS-ELISA serological tests.

Virus	Positive control (mean)	Negative control (mean)	Cultivar Goldrush	Cultivar Rebra	Cultivar Romus3	Cultivar Romus4	Cultivar Idared	Cultivar Florina
ACLSV	1.142	0.234	0.222	0.209	0.218	0.278	0.233	0.229
ASGV	1.000	0.231	0.216	0.205	0.213	0.241	0.235	0.201
ASGV	0.627	0.259	0.239	0.291	0.265	0.243	0.220	0.230
ApP	0.912	0.213	0.190	0.188	0.192	0.219	0.212	0.195
ASPV	1.185	0.357	0.305	0.253	0.267	0.243	0.199	0.308

Table 3. Shoot regrowth from encapsulated, osmoprotected, noncryopreserved shoot tips.

Cultivar	Shoot regrowth (% ± SD)*				
	Sucrose (M)				
	0	0.25	0.50	0.75	1.00
Goldrush	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	91.6 ± 1.0^{a}	86.1 ± 1.5^{a}	61.1 ± 1.5^{b}
Rebra	100.0 ± 0.0^{a}	94.4 ± 1.1^{a}	86.1 ± 1.5^{a}	77.7 ± 2.0^{b}	63.8 ± 2.5^{c}
Romus3	100.0 ± 0.0^{a}	91.6 ± 1.7^{a}	88.8 ± 1.5^{a}	75.0 ± 1.7^{b}	58.3 ± 3.4^{c}
Romus4	97.2 ± 0.5^{a}	94.4 ± 0.5^{a}	83.3 ± 2.0^{a}	72.2 ± 2.0^{b}	66.6 ± 1.7^{bc}
Idared	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	91.6 ± 1.0^{a}	83.3 ± 2.0^{a}	58.3 ± 2.6^{c}
Florina	100.0 ± 0.0^{a}	97.2 ± 0.5^{a}	86.1 ± 1.5^{a}	86.1 ± 2.0^{a}	61.1 ± 3.0^{c}

Following excision, shoot tips were encapsulated in Na-alginate, osmoprotected in sucrose, and transferred to fresh medium for shoot regrowth. *Values represent mean ± standard deviation (SD). Values followed by the same letter within a row are not significantly different ($P \leq 0.05$).

Florina (58% at 19% MC) (Figures 1c–1e, 2b, 2e, and 2f). The initial water content of the osmoprotected beads ranging between 59% and 64% decreased to 22%–25% within the first 3 h of air drying and was 17%–19% after 5 h of desiccation (Figure 2).

3.3. Morphological characteristics of cryopreservation-derived plants

No significant differences within the same genotype were observed in morphological characters (number of shoots, length of plants, number and length of roots) between noncryopreserved plants and plants regenerated from alginate-encapsulated cryopreserved shoot tips (Table 4). However, significant differences were observed between cultivars in the number of shoots/explant (from 1.3 shoots/explant for Romus4 to 7.6 shoots/explant for Goldrush), as well as in the other tested parameters (Table 4).

3.4. Molecular characterization by SSR markers

Each tested SSR primer generated reliable microsatellite alleles in all cultivars and a total of 18 polymorphic alleles were amplified (Figure 3). The average number of alleles was 1.6, and the allelic composition of each SSR marker is shown in Table 5. Most CHO primers generated polymorphic alleles in all cultivars, except CH04AE07 and CH04g10, which generated one nonpolymorphic allele. The markers generated with CH04AE07 and CH04g10 primers are not polymorphic. The same alleles were generated in all cultivars from the field collection, in vitro collection, and cryocollection. Regarding genetic stability of apple cultivars after in vitro culture or cryopreservation, markers generated with CH03g07, CH05c02, CH05d11, CH05e03, GD147, and GD162 primers showed differences between field-grown plants and in vitro or cryopreserved plants (Table 6). The distribution power of each marker was estimated by the PIC value. The PIC value ranged from 0.10 for the GD100 and GD142 loci to 0.89 for the GD162 locus, while the mean PIC value for all loci was 0.40 (Table 6).

There are no specific alleles associated with different conservation conditions (in vitro and cryopreservation). Cultivars Romus4, Florina, Rebra, and Idared showed the same combination of alleles with 5–7 primers (CH03g07, CH04AE07, CH04g10, CH05d11, GD96, GD100, and GD142), regardless of culture conditions.

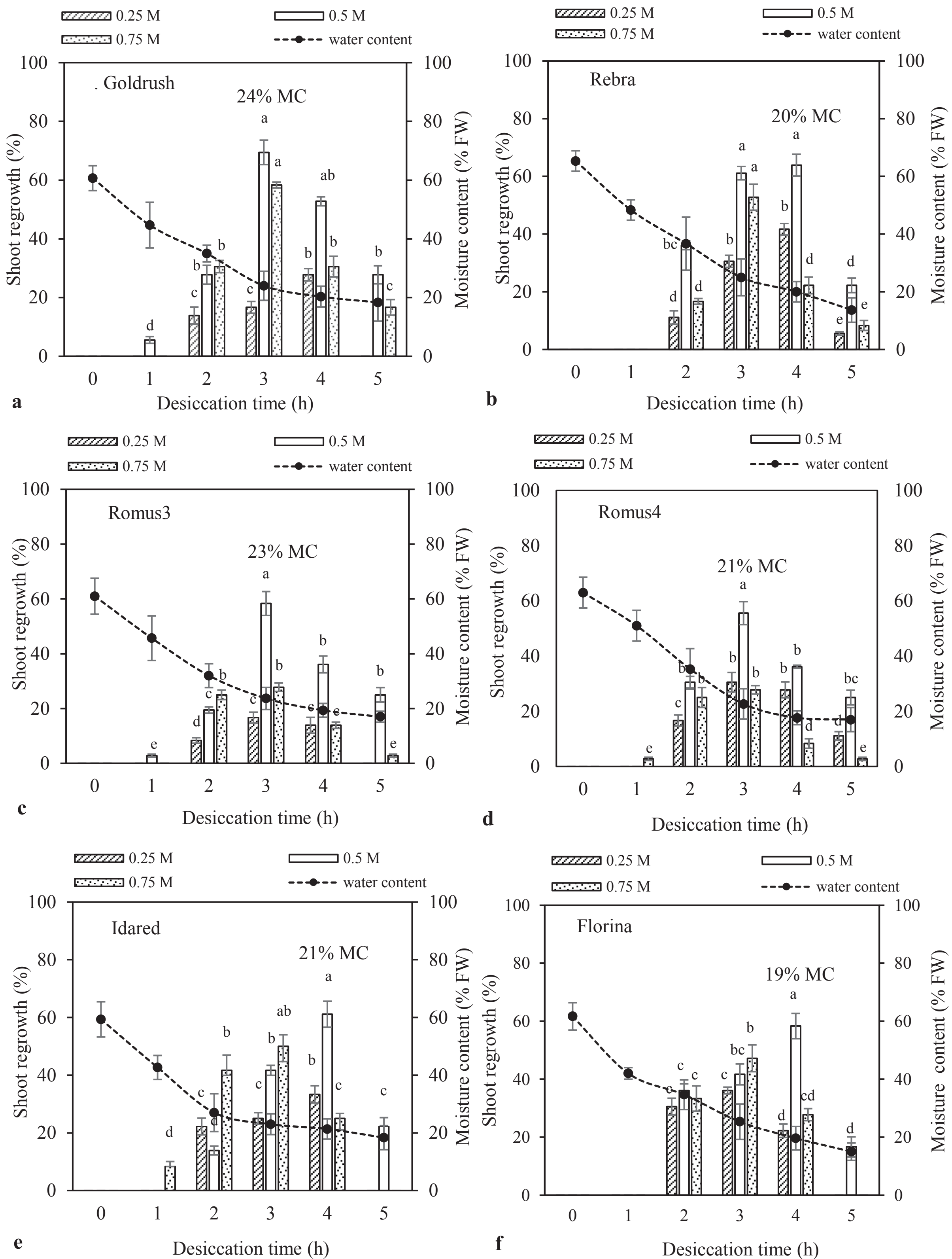

Figure 2. Shoot regrowth from cryopreserved alginate-coated shoot tips as a function of moisture content of the beads, sucrose concentration, and desiccation duration. The water content values are for beads osmoprotected in 0.5 M sucrose. Vertical bars represent standard deviation. Different letters indicate significant differences (P ≤ 0.05).

Table 4. Comparison of morphological characteristics between plants regenerated from noncryopreserved (– LN) and cryopreserved (+ LN) shoot apices.

Cultivar		Morphological characteristics			
		Number of shoots /explant (no. ± SD)*	Length of plants (cm ± SD)*	Number of roots (no. ± SD)*	Length of roots (cm ± SD)*
Goldrush	– LN	7.3 ± 1.5[a]	13.1 ± 0.5[a]	3.0 ± 1.0[a]	1.9 ± 0.4[a]
	+ LN	7.6 ± 0.5[a]	12.7 ± 0.3[a]	2.6 ± 1.5[a]	2.2 ± 0.4[a]
Rebra	– LN	4.6 ± 1.1[b]	10.4 ± 0.7[b]	2.3 ± 1.5[a]	2.7 ± 0.3[a]
	+ LN	4.3 ± 2.0[b]	10.9 ± 1.2[b]	2.3 ± 2.5[a]	2.4 ± 0.6[a]
Romus3	– LN	1.3 ± 0.5[d]	13.8 ± 0.1[a]	1.0 ± 1.0[b]	0.9 ± 0.3[b]
	+ LN	1.6 ± 2.0[d]	13.0 ± 0.3[a]	1.3 ± 1.5[b]	0.8 ± 0.6[b]
Romus4	– LN	3.6 ± 0.5[b]	10.3 ± 1.2[b]	2.6 ± 0.5[a]	2.1 ± 0.4[a]
	+ LN	3.0 ± 1.7[b]	10.7 ± 0.2[b]	2.3 ± 1.5[a]	1.8 ± 0.6[a]
Idared	– LN	3.6 ± 0.5[b]	10.3 ± 1.2[b]	2.6 ± 0.5[a]	2.1 ± 0.4[a]
	+ LN	3.0 ± 1.7[b]	10.7 ± 0.2[b]	2.3 ± 1.5[a]	1.8 ± 0.6[a]
Florina	– LN	4.6 ± 1.1[b]	10.9 ± 1.7[b]	1.3 ± 1.5[b]	2.2 ± 0.4[a]
	+ LN	4.3 ± 1.5[b]	10.7 ± 0.8[b]	1.6 ± 1.5[b]	1.7 ± 0.7[a]

*Values represent mean ± standard deviation (SD). Values followed by the same letter within a column are not significantly different ($P \leq 0.05$).

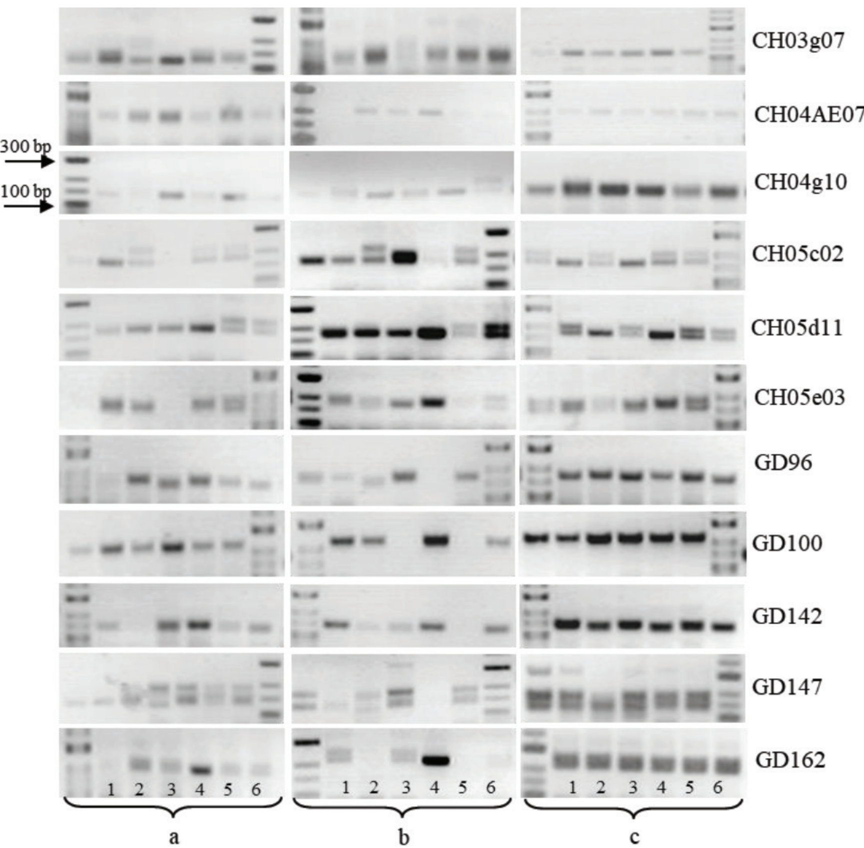

Figure 3. SSR pattern of apple genotypes. (a) Plants from field collection; (b) plants from in vitro collection; (c) plants from cryocollection. Lines in gel: 1) cultivar Goldrush, 2) Rebra, 3) Romus3, 4) Romus4, 5) Idared, 6) Florina. Control bands of DNA ladders at 100 and 300 bp.

Table 5. Distribution of alleles obtained by amplification with SSR primers in plant material originating from field collection (ex situ), in vitro collection (v1, v2, v3), and cryocollection (c1, c2, c3).

Cultivar			SSR primers/allele size (pb)										
			CH03g07	CH04AE07	CH04g10	CH05c02	CH05d11	CH05e03	GD96	GD100	GD142	GD147	GD162
Goldrush	Field collection	Ex situ	140	200	140	180	180	-	160	220	150	150	220:200
	In vitro collection	v1	140	-	140	180	180	200	160	220	150	200:150	280:220
		v2	140	200	140	180	180	200	160	220	150	200:150	280:220
		v3	140	200	140	180	180	200	160	220	150	200:150	280:220
	Cryocollection	c1	140	200	140	220:180	220:180	200:160	160	220	150	200:150	280:220
		c2	140	200	140	220:180	220:180	200:160	160	220	150	200:150	280:220
		c3	140	200	140	180	220:180	200:160	160	220	150	200:150	280:220
Rebra	Field collection	Ex situ	150:140	200	140	180	180	160	160	220	-	150	220:200
	In vitro collection	v1	150:140	200	140	180	180	160	160	220	150	150	-
		v2	150:140	200	140	180	180	160	160	220	150	150	220:200
		v3	150:140	200	140	180	180	160	160	220	150	150	220:200
	Cryocollection	c1	140	200	140	220:180	180	200:160	160	220	150	200:150	280:220
		c2	150:140	200	140	220:180	180	160	160	220	150	200:150	280:220
		c3	150:140	200	140	180	180	160	160	220	150	200:150	280:220
Romus3	Field collection	Ex situ	150:140	200	140	220:180	180	160	160	220	150	200:150	220:200
	In vitro collection	v1	-	200	140	220:180	180	160	160	-	150	200:150	280:220
		v2	140	200	140	220:180	180	160	160	220	150	200:150	280:220
		v3	140	200	140	220:180	180	160	160	220	150	200:150	280:220
	Cryocollection	c1	140	200	140	220:180	220:180	200:160	160	220	150	200:150	280:220
		c2	140	200	140	220:180	220:180	160	160	220	150	200:150	280:220
		c3	140	200	140	220:180	180	160	160	220	150	200:150	280:220

Table 5. (Continued).

Romus4	Field collection	Ex situ	140	200	140	-	180	160	160	220	150	200:150	200
	In vitro collection	v1	140	200	140	180	180	200	160	220	150	200:150	200
		v2	140	200	140	180	180	160	160	220	150	200:150	200
		v3	140	200	140	180	180	200	160	220	150	200:150	200
	Cryocollection	c1	140	200	140	180	180	200:160	160	220	150	200:150	220:200
		c2	140	200	140	180	180	200:160	160	220	150	200:150	200
		c3	140	200	140	180	180	200:160	160	220	150	200:150	200
Idared	Field collection	Ex situ	150:140	200	140	220:180	220:180	-	160	220	150	200:150	220:200
	In vitro collection	v1	140	200	140	220:180	220:180	200	160	-	-	-	-
		v2	140	200	140	220:180	220:180	-	160	220	150	200:150	220:200
		v3	140	200	140	220:180	220:180	200	160	220	150	200:150	220:200
	Cryocollection	c1	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200
		c2	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200
		c3	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200
Florina	Field collection	Ex situ	140	200	140	220:180	220:180	200:160	140	220	150	200:150	220:200
	In vitro collection	v1	140	200	140	220:180	220:180	200:160	160	220	150	200:150	200
		v2	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200
		v3	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200
	Cryocollection	c1	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200
		c2	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200
		c3	140	200	140	220:180	220:180	200:160	160	220	150	200:150	220:200

Table 6. Number and size of alleles and polymorphism information content (PIC) values of SSR markers.

SSR name	Alleles (no.)	Size (bp)	PIC
CH03g07	2	150:140	0.75
CH04AE07	1	200	0.00
CH04g10	1	140	0.00
CH05c02	2	220:180	0.67
CH05d11	2	220:180	0.70
CH05e03	2	200:160	0.77
GD96	1	160	0.00
GD100	1	220	0.10
GD142	1	150	0.10
GD147	2	200:150	0.42
GD162	3	280:220:200	0.89

Cultivar Goldrush showed the same alleles with 6 primers (CH03g07, CH04AE07, CH04g10, GD96, GD100, and GD142) in plant material from the field collection, in vitro collection, or cryocollection (Figure 4). SSR markers were used for calculation of genetic similarities between the 3 conservation strategies. A neighbor-joining tree was constructed based on the SSR profiles by calculating the Dice coefficient in order to confirm the effectiveness of SSRs in distinguishing between the plants from ex situ collection and preserved plants. Three major clusters were identified using the neighbor-joining dendrogram. Cultivars Romus4 and Goldrush belong in the first cluster, Rebra and Romus3 belong in the second cluster, and Florina and Idared belong in the third cluster, which are very similar according to this set of SSR markers. The Dice coefficient between plants belonging to the same cultivar ranged from 0.075 to 0.15 in Romus4, 0.06 to 0.14 in Goldrush, 0.11 to 0.17 in Rebra, 0.05 to 0.11 in Romus3, and 0.00 to 0.01 in cultivars Idared and Florina. Thus, most of the cultivars showed genetic similarities after 3 years of in vitro culture or cryopreservation. There are several exceptions, as some cryopreserved plants (c1) of Romus3 and Rebra showed similarities with Goldrush, and some in vitro plants (v1) of Florina were more similar to Idared (Figure 4). The most stable genotypes after 3 years of micropropagation (in vitro collection) and cryocollection were cultivars Florina, Goldrush, and Romus4.

4. Discussion

The DAS-ELISA serological test is commonly used to screen large populations and has been successfully applied for the detection of apple viruses (EPPO, 2015). Plant viruses, which are distributed worldwide wherever apple species are cultivated (EPPO, 2007), are causing economic loss; therefore, to provide disease-free material the donor plant needs to be screened for pathogenic viruses (Pradhan et al., 2016). The tested genotypes were free from the most common apple viruses (Table 2).

Biotechnological approaches involving in vitro tissue culture (Dobránszki and Teixeira da Silva, 2010), cryopreservation (Halmagyi et al., 2010), and development of molecular markers (Li et al., 2014) were applied to *Malus* species and cultivars. We showed that high sucrose concentrations (1.00 M) alter shoot regrowth of encapsulated and osmoprotected apple shoot tips even in the absence of cryopreservation (Table 3). Moreover, our study revealed that shoot regrowth following cryopreservation is influenced, besides sucrose concentration, by the water content of alginate beads and desiccation duration, leading to regrowth frequencies of up to 69% (cultivar Goldrush) (Figure 2). Sucrose treatment enhanced the regrowth of cryopreserved tissue for other woody plant species (Shatnawi et al., 2007; Condello et al., 2009). The regrowth percentages of alginate-coated apple shoot tips following cryopreservation were significantly different according to the tested sucrose concentrations and desiccation times (Figure 2). Obviously, the regrowth of encapsulated apices was dependent on residual water content of the beads; however, optimal regrowth conditions (composition of culture medium, temperature, light exposure) should also be considered. In this study with apple genotypes, the highest regrowth after cryopreservation was obtained when the water content ranged between 19% (cultivar Florina) and 24% (cultivar Goldrush), which is close to that previously reported in the cryopreservation of apple species (Niino and Sakai, 1992; Paul et al., 2000; Feng et al., 2013). Other studies showed that water content requirement for successful cryopreservation and subsequent regeneration of viable shoots in woody species varied between 19% (Padrò et al., 2012) and 37% (Le Bras et al., 2014). However, additional treatments were required to raise regrowth percentages following cryopreservation. For instance, it was reported that the addition of glycerol to beads and loading solution increased the regrowth of hawthorn apices after rewarming (Kami et al., 2009). At the same time, it was suggested that a standardization of alginate beads in terms of volume and water content should reduce the variability in physical and thermal features (Block, 2003). In our study, no morphological differences were observed in the shoots recovered after cryopreservation when compared to unfrozen controls (Table 4). Similar results were reported by Yi et al. (2015) regarding the morphological stability and characteristics of plants recovered from cryopreserved dormant apple winter buds.

The aim of long-term germplasm conservation is to minimize the appearance of variations, while proper

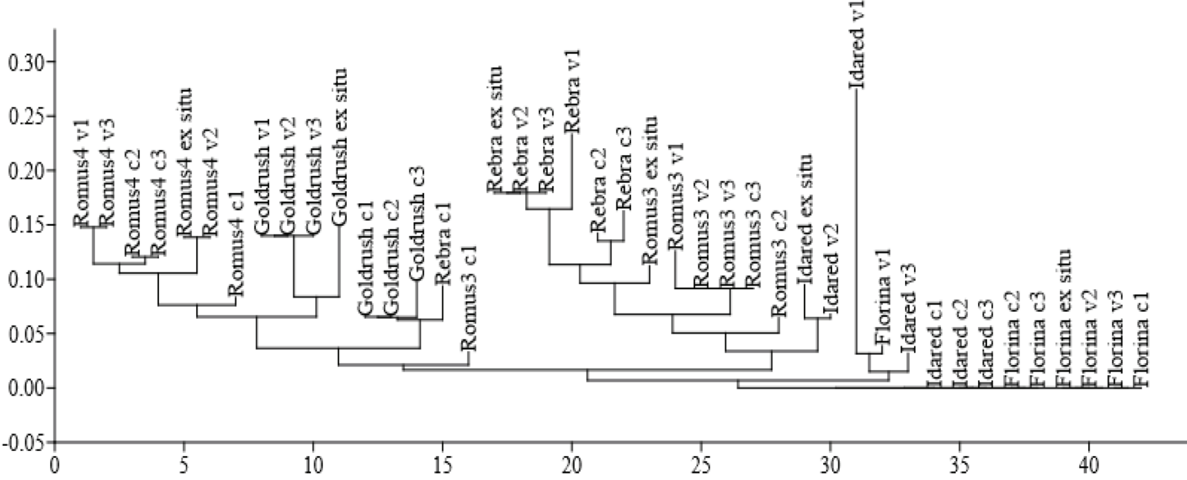

Figure 4. Neighbor-joining tree based on Dice coefficient calculated by SSR markers of apple genotypes originating from three conservation strategies: field collection (ex situ), in vitro collection (v1, v2, v3), and cryocollection (c1, c2, c3).

management of a germplasm collection of vegetatively propagated species requires periodic evaluation of the genetic composition of the preserved material (Martín et al., 2013). Maintenance of true-to-type clonal fidelity is an essential factor to be monitored during conservation of vegetatively propagated species. With an increased number of plants obtained by clonal propagation, the analysis of somaclonal variation or genetic fidelity requires efficient screening methods (Druart, 2003). SSR markers have a number of positive features that make them superior to other molecular markers in the genetic characterization of individuals, such as their multiallelic nature, codominant inheritance, high abundance, reproducibility, transferability over genotypes, and extensive genome coverage (Schlötterer, 2004). Previous studies assessed the genetic stability in *Malus* shoots recovered after cryopreservation using various techniques including cytological examination (Hao et al., 2001), AFLP (Hao et al., 2001), and ISSR (Li et al., 2014, 2015; Liu et al., 2008). It was shown that the number of alleles obtained by amplification with the 11th SSR primer was 1–3, with an average of 1.6. The set of microsatellite markers used showed a low level of polymorphism among the studied genotypes (Table 6), which is in agreement with similar studies (Guilford et al., 1997; Silfverberg-Dilworth et al., 2006), but it was lower than the amount reported in other studies for apple inbred lines and hybrids (Zhang et al., 2007; Gharghani et al., 2009; Farrokhi et al., 2011; Sikorskaite et al., 2012; Pérez-Romero et al., 2015; Ganopoulos et al., 2017). The lower value obtained in our study may be due to the use of agarose gel electrophoresis for the screening of SSR markers compared to polyacrylamide gel electrophoresis or automated analysis that would be able to resolve allelic variation at a finer scale than gel electrophoresis analysis. Nevertheless, PCR and agarose gel electrophoresis are valuable methods for rapid screening of germplasm and genetic stability after conservation. Most primers generated 1 or 2 characteristic alleles in all cultivars and the PIC value dropped dramatically (GD100, GD142, GD96, CH04AE07, and CH04g10 primers) (Table 6). The highest PIC value (0.89) was obtained in the case of the GD162 marker, when 3 polymorphic alleles were obtained. The markers showing higher PIC values such as GD162, CH05e03, CH03g07, CH05d11, and CH05c02 could be used in genetic diversity or stability studies in apple and for cultivar discrimination. The use of more than one DNA amplification technique to amplify different regions of the genome may provide a better strategy for observing genetic variation than a single method (Wang et al., 2014). SSR markers are preferable to other marker systems because once primer sequences flanking the SSRs are available, this technique is less labor-intensive and time-consuming than AFLP and ISSR. The RAPD methodology is easier than SSR due to the use of universal primers that randomly amplify DNA, but the sensitivity of the RAPD assay to the reaction conditions is very high and leads to reproducibility problems. Thus, for assessment of genetic stability of preserved plants, SSR and ISSR markers are the most valuable tools that can identify high levels of polymorphism (Goulão and Oliveira, 2001). In our study, we used SSR primers that usually show 1–3 alleles/locus in order to more easily identify the differences between ex situ and preserved plants. For cultivar discrimination it is more useful to use markers such as SSR and ISSR that show high levels of polymorphism. Several authors suggested finding a minimal set of 7–11 markers that are most valuable for such purpose (Roja et al., 2008;

Bouhadida et al., 2011; Sikorskaite et al., 2012; Pérez-Romero et al., 2015; Ganopoulos et al., 2017).

In conclusion, the morphological features of plants regenerated following cryopreservation were similar to those of noncryopreserved plants. At the same time, no major genetic differences between plants from the field collection, micropropagated plants, or plants recovered after cryopreservation were detected as shown by the Dice coefficient, thereby demonstrating that cryopreservation by encapsulation-dehydration is a practical method for long-term conservation of apple genotypes.

Acknowledgments
This study was supported by the research project PNII 51-067/2007 and partially by the core program PN2019-2022 - BIODIVERS 3, BIOSERV 25N/2019 and the project 22PFE/2018 financed by the Romanian Ministry of Research and Innovation.

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