# Conservation of *Malus Niedzwetzkyana* Dieck Ex Koehne Genotypes From Kazakhstan Resistant to Scab and Fire Blight Diseases

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# Abstract

Southeast Kazakhstan is part of the center of origin and the natural habitat of wild apple trees. Wild apples and their hybrids are involved in domestic apple breeding as a source of resistance to biotic and abiotic stress. *Malus niedzwetzkyana* is a valuable endemic apple species included in the Red Book of Endangered Species of Kazakhstan. The present study evaluated 23 *M. niedzwetzkyana* genotypes from different populations for their resistance to scab and fire blight diseases using SCAR markers. We determined that all 23 genotypes contained a 430 bp resistance allele for marker *OPB19*, and 5 of them also had a 799 bp resistance allele identified by marker *OPB18* for scab resistance. For fire blight, seven genotypes contained a 375 bp resistance allele and one genotype had a 397 bp resistance allele for the QTL *FBF7* locus identified by markers *AE10-375* and *GE-8019*, respectively. Eight genotypes with double resistance to scab and fire blight were selected for further *in vitro* amplification to develop a genetic pool for biodiversity preservation, apple breeding, and wild apple population revival, among other purposes. All *in vitro* regenerants were analyzed for the presence of apple chlorotic leaf spot virus, apple mosaic virus, apple stem grooving virus, apple stem pitting virus, and tomato ringspot virus. For each of the eight genotypes with double resistance, genetic profiles were developed based on 12 SSR markers. These genetic passports allow us to maintain the purity of the genetic pool and improve MAS in apple breeding.


# Introduction

Southeast Kazakhstan is part of the center of origin and the natural habitat of wild apple trees. In nature, wild apple trees can form forests situated in large spaces. The fruit forests of Kazakhstan contain plant material important in the prospect of botany, geography, and genetics. Wild apples and their hybrids have been used in domestic apple breeding as a source of resistance to biotic and abiotic stress [1, 2].

*Malus niedzwetzkyana* Dieck ex Koehne is among the valuable wild apple species. *Malus niedzwetzkyana* is an endemic species included in the Red Book of Kazakhstan and the International Red List of Endangered Species [3, 4]. The natural habitat includes areas of Kazakhstan (Karatau and Zailijskei Alatau), Kyrgyzstan (Jalal-Abad region), and western China (Xinjiang region) [1, 2, 5, 6].

*Malus niedzwetzkyana* is one of the components of the mixed fruit and nut forests in Central Asia. This species is an important genetic resource for apple breeding and the development of new advantageous varieties [7, 8]. The particular value of *M. niedzwetzkyana* is related to its genetic predisposition to scab and fire blight resistance [9]. The tree is also useful as an ornamental plant because of the coloring of its flowers, fruits, leaves, and trunk, which are pink because of the presence of anthocyanins. These compounds have antioxidative, anti-carcinogenic, and anti-inflammatory properties [10]. The fruits are also rich in biologically active compounds, such as polyphenols, flavonoids, and polysaccharides. The content of flavonoids available in apples is higher than in other fruits; according to previous studies, flavonoids from apples have a positive effect on aging processes, cognitive decline, weight control, and the health of respiratory, cardiovascular, and digestive systems [11].

The improvement of domestic apple productivity and the conservation of global biodiversity rely on the existence of resistant genotypes capable of defense against rapidly evolving pathogens. Higher plants evolve much slower than pathogens, especially perennial tree plants. Therefore, there is a continuous search for new resistance loci for marker-assisted selection (MAS). Scab and fire blight are among the most dangerous and economically significant pathogens of apple. Scab is the most common apple disease worldwide, affecting leaves, flowers, and fruits. The causal agent is hemibiotrophic fungus *Venturia inaequalis*; it can be found in places with industrial-scale apple production in a broad geographical range [12]. A higher level of resistance to the pathogen in domestic apple can be achieved using methods of targeted selection involving crosses with the resistant genotypes. Wild apples are the origin of potential resistance genes, so it is important to maintain and study the genetic pools of *M. siversii* and *M. niedzwetzkyana*. To date, 17 genes associated with resistance against scab are known, including *Rvi2*, *Rvi4*, *Rvi5*, *Rvi6*, *Rvi11*, *Rvi13*, and *Rvi1* [13].

Another widespread apple disease is fire blight of fruit crops, caused by the gram-negative bacterium *Erwinia amylovora*. The infection starts on the flowers or vegetative parts and leads to necrosis of the tissues. The resistance of apple to fire blight is a trait with dominant inheritance, associated with the *Alt* gene [14, 15]). In addition, five loci associated with quantitative resistance (QTL) to the pathogen are known. Among them, QTL *FBF7* has the strongest association with fire blight resistance; resistant variants are characterized by two SCAR markers, *AE10-375* and *GE-8019*. This QTL explained about 40% of the observed phenotypic variation [16].

The present work demonstrates the results of molecular genetic analysis of *M. niedzwetzkyana* genotypes growing in the Republic of Kazakhstan to determine resistance to scab and fire blight. Genotypes bearing resistance alleles against both scab and fire blight were introduced into a culture for the further selection of resistant varieties and the conservation of biodiversity.

# Materials And Methods

Plant material

Leaves for molecular genetic analysis were sampled from 17 trees from four populations in Nur-Sultan city (collection of the Astana Botanical Garden; herbarium inventory number 536/20–552/20), three specimens of *M. niedzwetzkyana* were collected in the pomological garden (Talgar town; part of the Fruit and Vegetable Research Institute, Almaty, Kazakhstan) and three specimens from the wild population from the Tscherkesay canyon (near Tekeli town, Kazakhstan). The location of the populations: Nur-Sultan population 1—51°09'08.5"N, 71°24'59.2"E, population 2—51°07'43.4"N, 71°24'51.0"E, population 3—51°09'10.6"N, 71°25'50.3"E, population 4—51°06'23.2"N, 71°25'00.1"E; pomological garden—43°17'02.5"N, 77°11'35.4"E; and Tscherkesay canyon (Dzhungar Alatau)—44°47'34.6"N, 78°55'06.3"E.

Amplification of SCAR markers

DNA was extracted from leaves using a modified CTAB protocol [17]. The quantity and quality of extracted DNA were analyzed using a spectrophotometer (NanoDrop1000, Thermo Fisher Scientific, USA). In the work, two markers associated with scab resistance, *OPB18* [18] and *OPL19* [19], and two markers associated with fire blight resistance, *AE10-375* and *GE-8019* [16], were used (Table 1). Identification of markers' location on the genome of *Malus domestica*, cultivar 'Golden Delicious' (assembly ASM211411v1) was performed by Primer BLAST (NCBI).

Table 1
Marker, primer (F, forward and R, reverse) sequences, and PCR cycling for the amplification of SCAR markers

| Gen, locus | Marker | Primer sequence (5'–3') | PCR cycling |
|---|---|---|---|
| *Rvi8(Vh8)* | OPB18 | CCACAGCAGTCATTGGGA-F<br>CCACAGCAGTGCATAAAC-R | 1x 95$^0$C – 3 min, 20x (95$^0$ C – 60s; 60s at 65–55°C (touchdown annealing temperature dropping 0.5°C per cycle); 72$^0$C – 90s), 20x (95$^0$ C – 60s; 55°C – 60s; 72$^0$C – 90s), 1x 72$^0$ C 10 min |
| *Rvi2(Vh2)* | OPL19 | ACCTGCACTACAATCTTCACTAATC-F<br>GACTCGTTTCCACTGAGGATATTTG-R | 1x 95$^0$C – 3 min, 40x (94$^0$ C -60 s; 55$^0$ C – 60 s; 72$^0$ C – 90 s), 1x 72$^0$ C – 10 min. |
| F7 QTL | AE10-375 | CTGAAGCGCACGTTCTCC-F<br>CTGAAGCGCATCATTTCTGATAG-R | 1x 95$^0$C – 3 min, 35x (95$^0$ C -40 s; 60$^0$ C – 40 s; 72$^0$ C – 60 s), 1x 72$^0$ C – 10 min. |
| F7 QTL | GE-8019 | TTGAGACCGATTTTCGTGTG-F<br>TCTCTCCCAGAGCTTCATTGT-R | 1x 95$^0$C – 3 min, 35x (95$^0$ C -40 s; 60$^0$ C – 40 s; 72$^0$ C – 60 s), 1x 72$^0$ C – 10 min. |

For each DNA sample, 60 ng DNA was amplified in a 25 µL reaction mix containing 1× Taq buffer (750 мМ Tris HCl, pH 8.8, 200 мМ $(NH_4)2SO_4$, 0.1% Tween 20), 2.5 мМ $MgCl_2$, 0.2 мМ dNTPs, 0.2 мМ of each of the respective primers, and 1 unit Tag polymerase (Thermo Scientific, USA). The PCR cycling conditions for every marker are described in Table 1. Amplification results were analyzed using electrophoresis in 1.5% agarose gel in TAE buffer.

In vitro cultivation of M. niedzwetzkyana genotypes

Microclones of *M. niedzwetzkyana* were propagated in the Laboratory of Plant Biotechnology and Selection of the National Center of Biotechnology. The previously developed protocol for microclonal propagation was applied [20]. The axillary buds from one-year-old shoots were used as the source material. For each genotype, we used no less than 20 buds from different branches of the plant. Aseptic

treatment of the buds was performed for 4 minutes using 12% hydrogen peroxide solution, resulting in up to 80% viable sterile explants for introduction into *in vitro* culture. The *M. niedzwetzkyana* explants were cultivated on the QL medium with the addition of 0.5 mg/L 6-BAP and 1.5 mg/L kinetine; the main shoots were formed on day 50. For the multiplication of additional shoots, the explants were cultivated on QL medium with 0.5 mg/L 6-BAP and 0.01 mg/L IBA for 50 days. The shoots were rooted in QL medium (half of the concentration) with the addition of 10 g/L sucrose and 1.5. mg/L IBA. Thus, the *M. niedzwetzkyana* genotypes bearing resistance alleles against scab and fire blight were introduced into a culture and propagated as microclones.

Detection of apple viruses in plant regenerants

*In vitro* regenerants were tested for the presence of 5 apple viruses before further mass reproduction and genetic pool formation. Detection was performed for apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), and tomato ringspot virus (ToRSV). These apple viruses are broadly distributed in orchards and gardens in Kazakhstan [21]. Analysis was performed for every regenerant of each genotype. The number of regenerants varied from 20 to 40. Detection was based on the RT-PCR method using specific primers developed previously by the authors [21] for each virus (Table 2). Amplification conditions and analyses were described in detail by [21].

Table 2
Sequences of primers for detection of apple viruses (Gritsenko et al. 2020)

| Virus | Forward primer sequence (5′–3′) | Reverse primer sequence (5′–3′) |
|---|---|---|
| ACLSV | TAGGTGAGAGGCTCTATTCACATCTTGA | GCAATTGGAATATCCCCTTCTGCGA |
| ASPV | TCACGGAGGTAATTATCAGGACGG | GCTGTGCAAGCAGGAGCACCAGGT |
| ASGV | AGCGAGGCGCCACCGGGTAGGAGT | GAGTTCTGCCTGGAAGTGGCAGCA |
| ApMV | AAGGTCCGAATCCGATGGACCGAAA | GCGGCGAAATTCGTCTTAAACTCCA |
| ToRSV | TTTTTTGTGGGCATTCCATGATGTG | CGGCCACTCAAACCTCCAGTCATC |

SSR profiling of in vitro virus-free propagated genotypes

Twelve SSR markers, namely GD12, GD147, CH01h10, CH01h01, CH04c07, Hi02c07, CH01f03b, CH02d08, CH02c11, CH04e05, CH01f02, and CH02c09, were used ([22–25]. These markers are widely used in genotyping apple genetic resources and are suggested by the European Cooperative Programme for Plant Genetic Resources (ECPGR). CH04e05, CH02c11, CH02c09, CH02d08, CH04c07, CH01h01, Hi02c07, and CH01h10 are highlighted as priority group 1 of the ECPGR marker set, whereas CH01f02, CH01f03b, GD12, and GD147 belong to priority group 2. The primer sequences, fluorescent dye, and multiplex group are described in Table 3. Amplification of each SSR marker was conducted in a 15 µl reaction mix containing 1x DreamTaq buffer (Thermo Scientific, USA), 0.2 мM dNTPs, 0.2 мM of each of

the respective primers for each SSR marker, and 1 unit DreamTaq polymerase (Thermo Scientific, USA). The program of amplification for each multiplex group was as follows: 94°C for 3 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing for 90 s at 60°C with a 1°C decrease in temperature each cycle, and elongation at 72°C for 60 s. The second step of 30 cycles was denaturation at 94°C for 30 s, followed by annealing at 50°C for 90 s, and further elongation at 72°C for 60 s. Final elongation continued for 10 min at 72°C. PCR was performed using a Mastercycler Pro S thermocycler (Eppendorf, Hamburg, Germany). Fragment analysis was conducted using Applied Biosystems 3500 (Thermo Scientific, USA). SSR genotyping data were analyzed using GeneMapper™ Software 6. The resulting genotyping data, the profiles of 8 genotypes, were analyzed in GeneAlex 6.5. The following parameters were determined: number of different alleles, number of effective alleles, Shannon's information index, observed heterozygosity, expected heterozygosity, unbiased expected heterozygosity, and fixation index.

Table 3
Sequences of primers (F- forward, R-reverse), fluorescent dye, linkage group and multiplex group for SSR genotyping

| Marker | Primer sequence (5'–3'), | Fluorescent dye | Linkage group | Multiplex group |
|---|---|---|---|---|
| GD12 | F-TTGAGGTGTTTCTCCCATTGGA<br>R-CTAACGAAGCCGCCATTTCTTT | TAMRA | 3 | III |
| GD147 | F- TCCCGCCATTTCTCTGC<br>R- GTTTAAACCGCTGCTGCTGAAC | ATTO565 | 13 | III |
| CH01h10 | F-TGCAAAGATAGGTAGATATATGCCA<br>R- AGGAGGGATTGTTTGTGCAC | HEX | 8 | II |
| CH01h01 | F-GAAAGACTTGCAGTGGGAGC<br>R- GGAGTGGGTTTGAGAAGGTT | TAMRA | 17 | II |
| CH04c07 | F- GGCCTTCCATGTCTCAGAAG<br>R- CCTCATGCCCTCCACTAACA | 6-FAM | 14 | II |
| Hi02c07 | F- AGAGCTACGGGGATCCAAAT<br>R-GTTTAAGCATCCCGATTGAAAGG | ATTO565 | 1 | II |
| CH01f03b | F- GAGAAGCAAATGCAAAAC CC<br>R- CTCCCCGGCTCCTATTCTAC | HEX | 9 | III |
| CH02d08 | F- TCCAAAATGGCGTACCTCTC<br>R-GCAGACACTCACTCACTATCTCTC | HEX | 11 | I |
| CH02c11 | F- TGAAGGCAATCACTCTGTGC<br>R- TTCCGAGAATCCTCTTCGAC | TAMRA | 10 | I |
| CH04e05 | F- AGGCTAACAGAAATGTGGTTTG<br>R- ATGGCTCCTATTGCCATCAT | 6-FAM | 7 | I |
| CH01f02 | F-ACCACATTAGAGCAGTTGAGG<br>R- CTGGTTTGTTTTCCTCCAGC | 6-FAM | 12 | III |
| CH02c09 | F-TTATGTACCAACTTTGCTAACCTC<br>R-AGAAGCAGCAGAGGAGGATG | ATTO565 | 15 | I |

# Results And Discussion

Natural forests of wild fruit plants are among the most valuable plant communities in terms of their uniqueness, genetic potential, and scientific and practical significance [26]. Wild plants are the originators of all cultivated plants and remain an important source of genetic material for improving the quality of crops for the food supply [27]. Therefore, the conservation and rational use of wild plants will always be important since the success of breeding depends on a wide choice of potential source material [28]. For example, the breeding of the Golden Delicious apple variety involving wild apple trees made it possible to intensify world apple production more than 2 times over 25 years [26]. Despite the high value of wild fruit plants, their areas of habitat have been subjected to catastrophic reduction, a decrease in species and genetic diversity, and degradation of natural forests over the past decades. These problems threaten biodiversity and cause damage to ecosystems. The Central Asian region is one of the world's largest centers for the growth of wild apple and apricot. Apple, apricot, and walnut forests are unique to Kazakhstan [26]. The species that form these communities are classified as endangered and require protection (https://www.bgci.org/our-work/projects-and-case-studies/global-trees-campaign/). However, in the country, up to 70% of apple forests have been reduced over the past 40 years. The revival of wild apple forests is the main priority in the conservation of the country's wild flora, which is achieved by a fundamental comprehensive study of the genetic basis of plants. Previously, three genotypes of *M. niedzwetzkyana* from the Krutoye tract (Kazakhstan) were sampled and subjected to molecular genetic analysis. When assessed with the use of 16 microsatellite markers, these genotypes were grouped in clusters among *M. sieversii* genotypes [29]. To date, no wide-scale studies of *M. niedzwetzkyana* have been carried out either in Kazakhstan or other countries, and the percentage of *M. niedzwetzkyana* trees in the wild population is also unknown. The potential of *M. niedzwetzkyana* as a genetic source for apple breeding in Kazakhstan has not yet been investigated.

Currently, wild apple specimens are known to be the main source of resistance genes against biological stress. For example, scab resistance genes *Vh2*, *Vh4*, and *Vr* were inherited from wild apple species [12, 13, 30]. These genes can be identified using *OBP18* and *OPB19* SCAR markers. *OBP18* and *OPB19* markers are located on chromosome 2. We identified the exact location of these markers using *Malus domestica* genome, cultivar 'Golden Delicious' (assembly ASM211411v1) (Fig. 1). The position of *OBP18* ranges from 31968883 to 31968254 b.p. Marker *OPL19* are located between 31391752 and 31390613 b.p. The *OPB18* marker was developed for the analysis of the *Vr* gene and can be present by 628 and/or 799 bp alleles. The fragment of 799 bp determines the dominant allele of resistance to scab in apple genotypes, whereas the 628 bp fragment indicates the susceptible genotype in homozygote form. It was also shown that *M. sieversii*, another species of wild apple, predominantly bears the 799 bp allele. *OPL19* confirms the presence or absence of resistant allele of genes $Vh_2$ and $Vh_8$; resistance is indicated by a product of 430 bp in length. The studied *M. niedzwetzkyana* genotypes from Nut-Sultan city and 3-P from the pomological garden had scab resistance alleles, only in the case of *OPL19*, whereas the individuals from the wild population of Tscherkesay canyon and 1-P and 2-P genotypes from the pomological garden represented resistance alleles for both markers (Fig. 1). Another important disease of apple and pear trees

is fire blight, which is widely distributed in Kazakhstan. *Erwinia amylovora*, a bacterial pathogen that causes fire blight, annually infects hectares of apple trees in the south and southwestern region of Kazakhstan [31]. Multiple treatments during the growing season with copper-containing chemicals lead to enormous soil pollution. One of the promising and effective methods to prevent the spread of the pathogen is the cultivation of resistant cultivars. The primary source of resistance loci for apple breeding is wild apples, including *M. niedzwetzkyana*. The SCAR markers *AE10-375* and *GE-8019* are widely used for the identification of variants of QTL *FBF7* associated with fire blight resistance. We identified the location of marker *AE10-375* on chromosome 7 of *Malus domestica* genome, cultivar 'Golden Delicious' (assembly ASM211411v1), which ranges from 31500788 to 31500436 b.p. (Fig. 1). The identification of marker *GE-8019* was not successful and Blast primer analysis did not reveal any fragments of targeted size flanked by forward and reverse primers on the chromosome 7. But we counted three fragments in size of less than 1000 b.p. in the genome. Fragment in size of 418 b.p. and 138 b.p flanked by only reverse primer were identified on chromosome 10 and 1, respectively. Reverse primer is completely complementary to its positions in the genome in case of fragment 418 b.p. and has one mismatch in formation of 138 b.p fragment. Also, 430 b.p. fragment flanked by forward and reverse primers was identified on chromosome 11 with 9 mismatches.

The dominant alleles of lengths 375 bp and 397 bp of markers *AE10-375* and *GE-8019*, respectively, indicate the resistant genotype of the apple tree. Only seven genotypes bearing the resistance allele for *AE10-375* were identified in this study. The resistance allele for *GE-8019* was found only in the 3-W apple genotype from the wild population of Tscherkesay canyon (Fig. 1). The presence of resistance alleles for both markers had been previously revealed in the genotypes with high resistance to the pathogen compared with the genotypes bearing only the resistance allele for one of two markers [16]. In the present study, no specimens bearing resistance alleles for both markers were found (Table 4). In this investigation, we identified three genotypes from the wild population of Tscherkesay with resistance alleles to scab for both markers, *OPB18* and *OPL19*, together with resistance alleles to fire blight for *AE10-375* or *GE-8019*. We also identified five genotypes from Nur-Sultan city bearing resistance alleles for markers *OPL19* and *AE10-375*. The specimens from the pomological garden only had resistance alleles to scab for markers *OPB18* and/or *OPL19*. *Malus niedzwetzkyana* genotypes with resistance alleles for both scab and fire blight were introduced into a culture. The microclones of the selected specimens were propagated and adapted to the conditions *ex vitro* (Fig. 2). The process and methods of *in vitro* propagation of *Malus niedzwetzkyana* were comprehensively described in our previous work, and we followed all conditions [20]. We adjusted salt and sucrose concentrations for rooting of *M. niedzwetzkyana* shoots. Before genetic pool formation and further in vitro amplification, all plant regenerants were tested for the presence of ACLSV, ASPV, ASGV, ToRSV, and ApMV viruses. These apple viruses are the most common in Kazakhstan and cause significant economic damage to the agricultural sector [21]. The accumulation and spread of viruses throughout the whole plant significantly affects the productivity and survival of apple trees. ACLSV was detected in 3 of 30 regenerants belonging to genotype 1-W and in 1 of 35 regenerants belonging to genotype 10. ACLSV is the important latent virus of apple trees with infection rates of 80–100% in many commercial apple cultivars and leads to yield losses

up to 30–40% [32, 33]. Virus transmission caused by grafting, pruning, or propagation of materials and nematodes represents a major threat to the fruit industry. The remaining ASPV, ASGV, ToRSV, and ApMV viruses were not detected. Infected plant regenerants were removed. We did not find any studies on the detection of viral pathogens in wild apple populations. We detected ACLSV for the first time in the *M. niedzwetzkyana* apple tree from a wild population. In our opinion, ACLSV infection was transmitted to a wild population of apples from cultivated apple tree gardens in mountain areas that developed during the Soviet Union period. During that period, grafting of cultivated apple scions onto wild apple rootstocks was a widespread method. Nevertheless, investigation of the evolution of the ACLSV isolate detected in *M. niedzwetzkyana* from a wild population is of particular interest and will be achieved in future work. The virus was also found in genotype 10 from the population of Nur-Sultan city. Nur-Sultan genotypes are located within the gardens of the city, near or together with cultivated apple trees. Therefore, the infection from neighboring gardens or trees has a high probability. Since the regenerants are considered stock for MAS and for the formation of orchards with the aim of reviving the wild apple trees, obtaining virus-free material is an important task. Thereby, *in vitro* virus-free collection and planting material of eight *M. niedzwetzkyana* genotypes were obtained. Further, we developed genetic profiles for these double-resistant genotypes using 12 SSR markers (Fig. 3). We analyzed SSR markers approved by the ECPGR. These markers are widely used by the international community for the certification of varieties and genotypes and make it possible to unify the process of genetic identification. In the results of SSR profiling of 8 *M. niedzwetzkyana* genotypes, we identified 5 different alleles for markers CH01f02 and GD12 and 3 alleles for markers CH01h01, CH04c07, CH02d08, and CH04e05. For the remaining markers, the number of detected alleles was 4 (Fig. 3). All 12 SSR markers showed high levels of polymorphism for 8 genotypes. The Tscherkesay canyon genotypes are noticeably different from Nur-Sultan and pomological garden samples and include unique alleles for markers CH02c11 (239 bp), CH02c09 (254 bp), GD12 (160 bp), and GD147 (121 bp). Additionally, the 3-W genotype was unique for markers CH01h10 (116 bp), Hi02c07 (120 bp), CH04c07 (120 bp), CH01f03b (142 bp), CH02c11 (221 bp), CH01f02 (178 bp; 186 bp), CH04e05 (220 bp), and GD147 (143 bp). Marker CH01h01 indicated a unique allele of 117 bp for the 2-W and 3-W genotypes. The 151 bp allele of marker Hi02c07 was identified in the 1-W and 2-W genotypes, whereas the 166 bp allele of marker CH01f02 was detected only in 1-W. The CH02d08 marker was not distinguished by unique alleles. Of particular interest is the 3-W genotype, which differs significantly in its genetic profile in comparison with other samples, including 2 genotypes from Tscherkesay canyon. This genotype has unique alleles in 8 SSR markers. The collection of *M. niedzwetzkyana* samples from Tscherkesay canyon was performed with a significant distance between the trees; the trees were at a distance of at least 30 meters from each other. Additionally, according to the results of the resistance analysis, only 3-W genotype resistance to fire blight was associated with the GE8019 marker; 2 other genotypes were positive for the AE10-375 marker. Tscherkesay canyon genotypes are a perspective genetic pool for breeding new cultivars bearing loci associated with resistance to scab and fire blight. The results of the performed work helped to preserve important genetic material of the endangered *M. niedzwetzkyana* species and would assist its further use in apple breeding, conservation, and revival of wild apple populations.

Table 4
Genetic analysis of *M. niedzwetzkyana* genotypes using SCAR markers

| Genotype | Marker | | | |
|---|---|---|---|---|
| | Scab | | Fire blight | |
| | *OPB18* | *OPL19* | *AE10-375* | *GE8019* |
| 1 | - | + | - | - |
| 2 | - | + | + | - |
| 3 | - | + | + | - |
| 4 | - | + | - | - |
| 5 | - | + | - | - |
| 6 | - | + | - | - |
| 7 | - | + | - | - |
| 8 | - | + | - | - |
| 9 | - | + | + | - |
| 10 | - | + | + | - |
| 11 | - | + | - | - |
| 12 | - | + | + | - |
| 13 | - | + | - | - |
| 14 | - | + | - | - |
| 15 | - | + | - | - |
| 16 | - | + | - | - |
| 17 | - | + | - | - |
| 1-P | + | + | - | - |
| 2-P | + | + | - | - |
| 3-P | - | + | - | - |
| 1-W | + | + | + | - |
| 2-W | + | + | + | - |
| 3-W | + | + | - | + |

# Conclusion

In the present work, 23 *M. niedzwetzkyana* genotypes of the different populations from Kazakhstan were analyzed for resistance to scab and fire blight diseases using genetic SCAR markers. Resistance alleles to scab were evaluated using *OPB18* and *OPL19* markers. *OPB18* revealed five resistant *M. niedzwetzkyana* genotypes, including specimens from the pomological garden and the wild population. All 23 studied apple genotypes had a 430 bp resistance allele for marker *OPL19*. Resistance to fire blight was evaluated using SCAR markers *AE10-375* and *GE-8019*; seven resistance genotypes from Nur-Sultan and Tscherkesay wild populations were identified by marker *AE10-375* and only one from Tscherkesay by *GE-8019*. No specimens resistant to fire blight were found among the genotypes from the pomological garden. The genotypes resistant to scab bearing both markers *OBP18* and *OPL19* consist of only 22% of the investigated samples, and no genotypes resistant to fire blight by both markers *AE10-375* and *GE-8019* were identified. All three genotypes from the Tscherkesay wild population contain scab-resistance alleles for both markers *OPB18* and *OPL19* and fire blight-resistance alleles for *AE10-375* or *GE-8019*. Five genotypes from Nur-Sultan contain loci for resistance to scab and fire blight, according to analysis by markers *OPL19* and *AE10-375*, respectively. Eight genotypes with double resistance to scab and fire blight were selected for further *in vitro* amplification to develop a genetic pool for biodiversity preservation, apple breeding, and wild apple population revival, among others. All *in vitro* regenerants were analyzed for the presence of ACLSV, ASPV, ASGV, ToRSV, and ApMV. Four regenerants of the 1-W and 10 genotypes were infected by ACLSV and excluded from cultivation and investigation. For each of the eight genotypes with double resistance, genetic profiles were developed based on 12 SSR markers. These genetic passports allow us to maintain the purity of the genetic pool and improve MAS in apple breeding.

# Declarations


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### Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

### Author Contributions

Nurtaza A., Dyussembekova D. - Methodology and manuscript draft writing

Pozharskiy A.- Data collection and computer analysis

Nizamdinova G., Taskuzhina A - Formal analysis and investigation.

Dolgikh S. Kakimzhanova A. – Botanical identification of plant material; discussion of results and reviewing final manuscript.

Gritsenko D. – Conceptualization, manuscript writing and reviewing

All authors read and approved the final manuscript

### Ethical statement

The study involving plants/plant material from wild population was conducted in accordance with relevant institutional, national, and international guidelines and legislation. Collection of plant material of *Malus niedzwetzkyana* for the research purposes was conducted in accordance with the Decision of council of the Eurasian Economic Commission of January 26, 2018 No. 15. About approval of Rules of proper practice of cultivation, collection, processing and storage of initial raw materials of plant origin.

### Availability of data

SSR genotyping data on M. niedzwetzkyana genotypes from Kazakhstan can be accessed from the Open Science Foundation website using following link: https://osf.io/dz5gf

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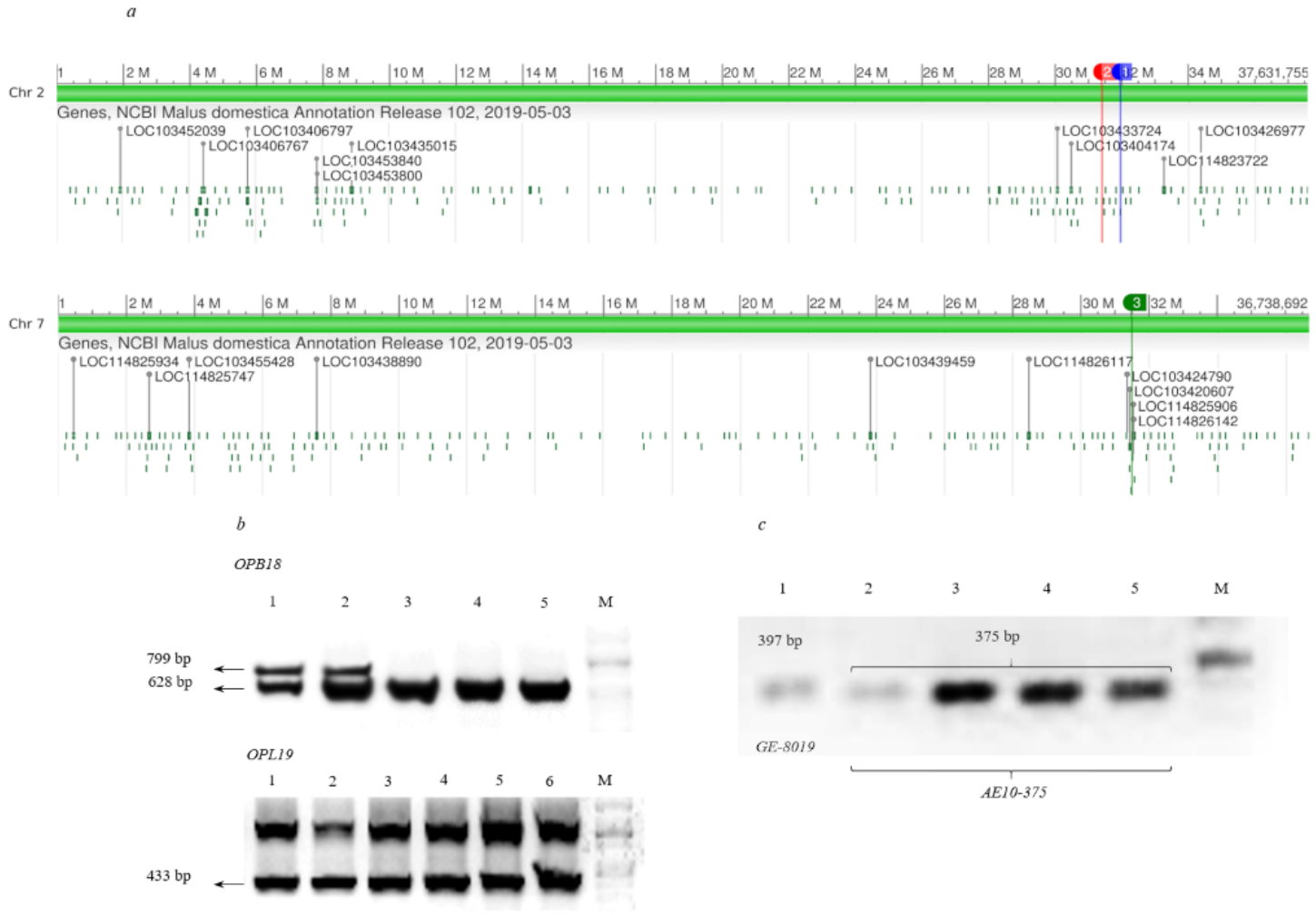
# Figures




## Figure 1

The location of *OPB18* (blue 1), *OPL19* (red 2) and *AE10-375* (green 3) markers on chromosome 2 and 7, respectively (**a**). Analysis of *M. niedzwetzkyana* genotypes using **b** scab markers *OPB18*, *OPL19* and **c** fire blight markers *AE10-375* and *GE-8019*. OPB18 marker: 1- 1-W and 2- 3-W genotypes of wild population; 3- 1 genotype and 4- 2 genotype of Nur-Sultan; 5 - 3-P genotype of Pomological garden. OPL19 marker: 1- 1-W, 2- 2-W, and 3- 3-W genotypes of wild population; 4- 2-P genotype of Pomological garden; 4- 1 genotype, 5- 2 genotype, 6 – 3 genotype of Nur-Sultan. *AE10-375* and *GE-8019* marker: 1- 3-W genotype and 2- 2-W genotype of wild population; 3- 3 genotype, 4- 9 genotype, and 5 - 12 genotype of Nur-Sultan. See also supplementary file 1 for the original full-size images.

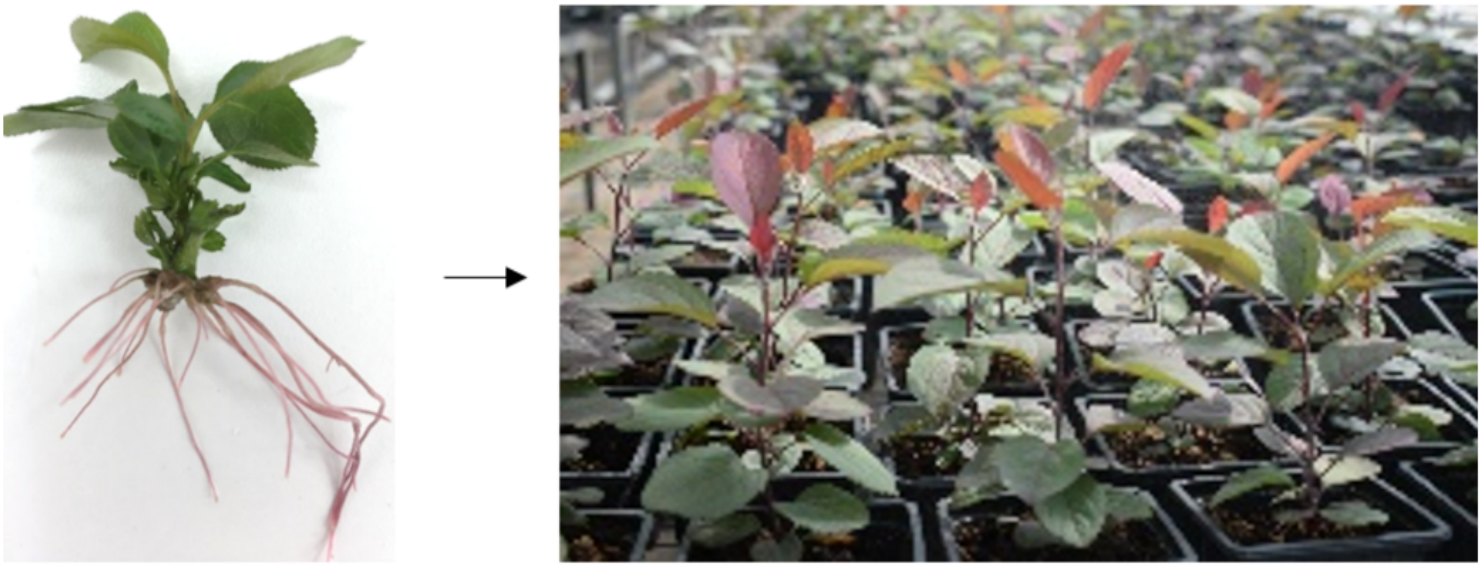



**Figure 2**

*In vitro* cultivation of *M. niedzwetzkyana* genotypes for conservation

SSR markers, fragment size

| Genotype | CH01h10 | CH01h01 | CH04c07 | Hi02c07 | CH01f03b | CH02d08 | CH02c11 | CH04e05 | CH01f02 | CH02c09 | GD12 | GD147 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 94 96 | 111 113 | 108 108 | 112 114 | 154 154 | 210 210 | 213 215 | 200 200 | 168 168 | 248 248 | 150 152 | 141 149 |
| 3 | 94 94 | 111 111 | 108 108 | 112 114 | 154 156 | 210 210 | 213 213 | 200 200 | 168 188 | 240 242 | 150 152 | 141 141 |
| 9 | 96 98 | 111 113 | 108 110 | 112 114 | 154 156 | 210 210 | 213 215 | 206 206 | 168 188 | 240 248 | 152 154 | 141 141 |
| 10 | 94 96 | 113 113 | 108 110 | 112 112 | 156 176 | 210 212 | 215 215 | 200 206 | 188 188 | 240 242 | 154 158 | 141 149 |
| 12 | 96 96 | 111 113 | 108 108 | 112 114 | 156 156 | 210 216 | 213 213 | 206 206 | 168 168 | 242 242 | 152 158 | 141 149 |
| 1-W | 96 98 | 113 113 | 108 108 | 114 151 | 154 176 | 210 210 | 213 239 | 200 206 | 166 166 | 240 254 | 158 160 | 121 149 |
| 2-W | 96 98 | 113 117 | 108 110 | 114 151 | 154 176 | 210 210 | 215 239 | 200 206 | 188 188 | 240 254 | 154 160 | 121 149 |
| 3-W | 98 116 | 117 117 | 108 120 | 114 120 | 142 176 | 210 216 | 221 239 | 200 220 | 178 186 | 242 254 | 154 160 | 121 143 |

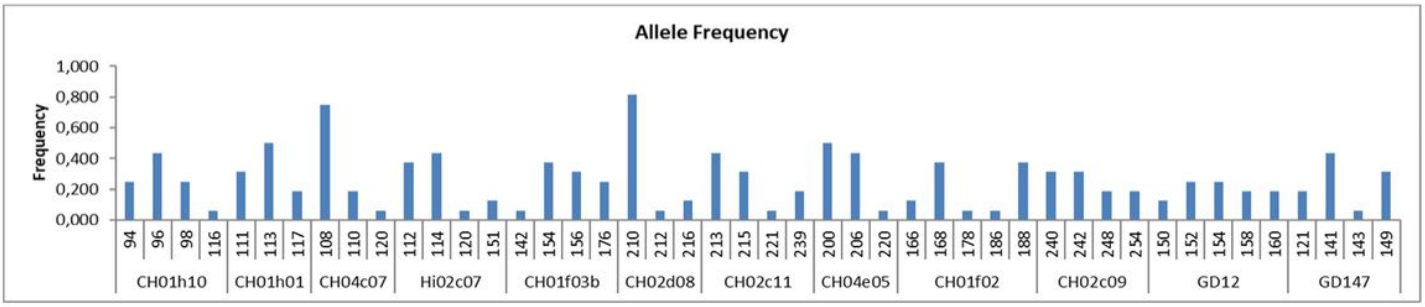



**Figure 3**

SSR profiling of *in vitro* propagated genotypes with double resistance to scab and fire blight

# Supplementary Files

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