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DEVELOPMENT OF A BLUNT-END CLONING STRATEGY TO CONFIRM THE *Rvi2* GENE IN APPLE VARIETIES CULTIVATED IN ROMANIA

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Abstract

The aim of this study was to construct a bacterial artificial chromosome (BAC) library to confirm the presence and structure of the Rvi2 gene, which provides resistance to apple scab caused by Venturia inaequalis, in several apple cultivars developed in Romania. This approach is intended to aid in detailed genetic analysis to improve apple breeding programs. The clones were sequenced and the results were compared with the sequence of the Rvi2 gene registered in the "National Center for Biotechnology Information" database (locus "AY626824"). BLAST analysis confirmed the presence of the Rvi2 gene in all 20 varieties: 'Alex', 'Bistrițean', 'Cezar', 'Ciprian', 'Dany', 'Discoprim', 'Delicios de Voinesti', 'Estival', 'Ionaprim', 'Luca', 'Pomona', 'Redix', 'Remar',', 'Romus 3', 'Romus 4', 'Romus 5', 'Salva', 'Starkprim', 'Voinea' and 'Voincel'. Query identity ranged from 94.6% to 100% for the forward primer and from 95.39% to 100% for the reverse primer, with query coverage ranging from 85% to 92% for the forward primer and 80% to 93% for the reverse primer. The results confirm the presence of the Rvi2 gene, demonstrating high sequence identity and coverage.

Key words*: breeding, competent cells, bacterial artificial chromosome, BLAST, resistance, Rvi2.*

INTRODUCTION

The SCAR amplification technique is technically simple and easy to perform. The main limitation is that the sequence data in the RAPD polymorphic fragment are necessary to design SCAR primers. This requirement of prior knowledge of sequence information hinders using the SCAR technique, as the costs for developing primers are high. Compared to RAPD primers, SCAR primers are longer, and the low reproducibility constraints associated with RAPD analysis are overcome in SCAR. The reason for improved reproducibility is that the PCR reaction is less sensitive to reaction conditions (Cheng et al., 2016; Abdin et al., 2012). In addition, being a molecular marker-based only on PCR, without restriction enzymes, a smaller amount of DNA is required for SCAR analysis compared to RFLP and AFLP techniques, where the concentration of DNA should not be less than 300 ng/µl. Due to their low acquisition cost and high reproducibility, SCAR markers have proven to be a valuable tool and a practical way to screen numerous samples simultaneously (Li et al., 2012; Kiran et al., 2010). $OPL19_{433}$ SCAR was developed by cloning the 550 bp fragment from the RAPD marker $OPL19_{550}$, located near the *Rvi2* gene, at a distance of 2.5 cM (Gardiner et al., 1999; Bus et al., 2000). The SCAR marker was also mapped at 1 cM (Bus et al., 2005b close to *Rvi2* (*Vh2*) in differential host 2 ('TSR34T15'). Still, it is also close to the *Rvi8* gene (*Vh8*) in differential host 8 (*Malus sieversii* W193B), being considered very useful for identifying varieties carrying the Vr gene (*Rvi2* or *Rvi8*), as demonstrated by Bus et al. (2005a). The identified sequences of the 433 bp fragments confirmed that the two genes belong to the same locus; in other words, they have homologous loci. The OPL19 marker has been used successfully in many research studies. Molecular studies in Greece on 20 domestic apple

cultivars cultivated in various regions led to identifying the *Rvi8* gene for nine varieties out of 20 (Karapetsi et al., 2020). Khankishiyeva (2020) used the OPL19 marker in a molecular screening for five, respectively eight apple varieties selected from the fruit gardens of the Fruit and Tea Growing Research Institute of Azerbaijan and twenty donors reference apple varieties from Julius Kühn-Institut (JKI) Dresden, Germany. Nurtaza et al. (2022) selected specimens of the species *M. niedzwetzkyana*, as follows: 17 specimens from the collection of the Astana Botanical Garden, three specimens from the Talgar Pomological Garden and three specimens from the Tscherkesay Canyon. Following the study, the OPL19 marker amplified the 433 bp fragment on all 23 samples. Using the OPB18 marker, Nurtaza et al. (2022) distinguished between the *Rvi2* gene and the *Rvi8* gene, only five species carrying the *Rvi8* gene, the rest having the *Rvi2* gene. The usefulness of the OPL19 marker can be seen in other research studies: Urbanovich and Kazlovskaya, 2008; Lyzhin and Saveleva, 2018; Dar et al., 2020; Höfer et al., 2021; Uzun et al., 2023; Iancu et al., 2022; 2023a; 2023b). Building a cDNA library is a complicated and laborious technology that involves a series of enzymatic procedures. After removing 3' and 5' heads, vector ligation is possible by treating cDNA with a blunt enzyme or T4 DNA Polymerase. The recombinant construct (cDNA-vector) is introduced into a host (*Escherichia coli*) to clone and build the BAC library (Cseke et al., 2011). The genomic region of interest is identified by sequencing clones and analyzing sequencing results (Bandara, 2016). Plasmid DNA was first isolated by Radloff et al. (1967), using a method that is based on differentiated absorption of ethidium bromide. Alkaline lysis Birnboim and Doly (1979) and boiling lysis Holmes and Quigley (1981) have become the most classical methods used in molecular biology. The aim of this work is to build a bacterial artificial chromosome (BAC) library to confirm the presence and structure of the *Rvi2* gene, known to confer resistance to the apple root rot (*Venturia inaequalis*), in certain apple cultivars developed in Romania. This approach will facilitate detailed genetic analysis and marker-assisted selection to improve apple breeding programs.

MATERIALS AND METHODS

Biological material studied

The 20 genotypes were created at Research Station for Fruit Growing Voinești ('Cezar', 'Ciprian', 'Discoprim', 'Delicios de Voinesti', 'Luca', 'Pomona', 'Redix', 'Remar', 'Voinea', 'Voinicel'), Research Station for Fruit Growing Bistrița ('Alex', 'Bistrițean', 'Dany', 'Ionaprim', 'Salva', 'Starkprim'), Research Station for Fruit Growing Cluj ('Estival') and registered by the Research Institute for Fruit Growing Pitesti ('Romus 3', 'Romus 4', 'Romus 5').

Chemical material

The fragment of interest was inserted in the vector using the "CloneJET PCR Cloning" kit from Thermo Scientific, the vector "pJET1.2" being of blunt type. Also included in this kit are two enzymes, "DNA Blunting Enzyme" and "T4 DNA Ligase", but also the buffer for the reactions to be initiated, "2X Reaction Buffer". The competent cells "DH10B Competently Cells" attached to the cloning kit were used for transformation. Also, the SOC medium was included in the competent cell kit. The LB medium (Luria Bertani) was prepared in the laboratory, having the following ingredients at 1 liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar and ampicillin 1 ml (100 mg/ml). The ampicillin stock solution was prepared by dissolving 5 g of ampicillin in 50 ml of ultra-pure water. The "Alkaline lysis method" was used to isolate plasmid DNA. This method involves preparing three solutions: alkaline solution I (glucose 50 mM, Tris-HCl 25 mM, EDTA 10 mM) at pH 8.0, Alkaline Lysis Solution II (0.2 N NaOH, 1% (g/v) SDS) and Solution III (potassium acetate 5 M and glacial acetic acid 2M). The Stock solutions were prepared at a volume of 100 ml in ultra-pure water.

PCR amplification

Has been worked in a reaction volume of 15 µl, including the following components in the final concentration: 11.5 µl "MyTaq™ Red Mix" from Meridian Bioscience; 0.1 µl for each of the F and R primers (0.6 µM in the final volume of reaction), 3 µl DNA (75 ng / µl) and 0.3 µl ultrapure water, under the following reaction conditions: initial denaturation at 95°C for 2.45

min.; 40 cycles of 1 min. at 94°C, 1 min. at 58°C and 2 min. at 72°C; final extension of 10 min. to 72°C.

Excise the fragment of interest

The amplified DNA fragment was excised from the gel using Sigma Aldrich's horizontal transilluminator, "MyView Compact UV Transilluminator", model 3100-E, producer Accuris Instruments, USA (Figure 1 and Figure 2).

Figure 1. Horizontal Transilluminator for excision of fragments of interest from agarose gel

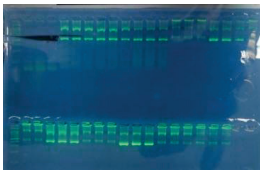


Figure 2. Excision of the fragment of interest from agarose gel

Purification of the excised fragment

It was made using the Geneaid "Gel/PCR DNA Fragments Extraction Kit", according to the manufacturer's instructions.

Blunting protocol

Using the DNA Blunting Enzyme included in the cloning kit, pipetting on the ice at the laminar flow hood, easy vortexing and incubation at 70^0C for five min., sticky ends have been converted to blunt ends. At the end of incubation, the samples were moved directly to the ice. The reaction mixture was: 2X Reaction Buffer (10 µl), excised and purified fragment (25 ng/µl), DNA Blunting Enzyme (1 µl), ultrapure water (5 µl), the total volume being 18 µl.

Blunt ligation reactions. Plasmid construction

The DNA fragment was ligated to the vector by adding the cloning vector and the binding enzyme over the components of the blunt reaction, also on ice. The mixture was vortexed and incubated at room temperature for five minutes. The volume of one reaction was 20 µl: 2x Reaction Buffer (10 µl), pJET 1.2/blunt Cloning Vector (1 µl), T4 DNA Ligase (1 µl), purified PCR product (1 µl), and 7 µl ultrapure water.

Transformation of competent cells

Initially stored at -80^0C, the competent cells were thawed slowly on wet ice. After thawing, each competent cell tube was aliquoted in volumes of 50 µL into Eppendorf tubes. Over the aliquot, 5 µl plasmid DNA volumes were added for each sample. After easy mixing, the plasmid DNA and competent cells were incubated on ice for 30 minutes. To relax the wall of competent cells, the cells received a brief heat shock for 30 seconds at 42^0C and were placed immediately on the ice for 2 minutes without mixing or shaking the tubes. In the end, it was pipetted 250 µl SOC medium over each sample and then incubated (at 37°C) for an hour, in a slightly vertical position, in a Thermoshake Incubator Shaker (225 rpm), producer Gerhardt, Germany (Figure 3).

Figure 3. The thermoshaker for incubation of transformed cells

Incubate bacterial culture on LB medium

The resulting samples from the transformation stage were diffused on sterile Petri plates and then incubated overnight at 37^0C. Only colonies containing the plasmid DNA of interest will grow, the colonies without plasmid being inhibited by the antibiotic (Figure 4).

Growing transformed cells overnight

To obtain a good plasmid DNA concentration, 10 colonies were picked from every petri plate and put in the liquid LB environment with antibiotic, then incubated (at 37°C) overnight in flat bottom glass balloons, in a volume of 100 ml (1 colony per 10 ml). A thermoshaker was used for incubation (Figure 5).

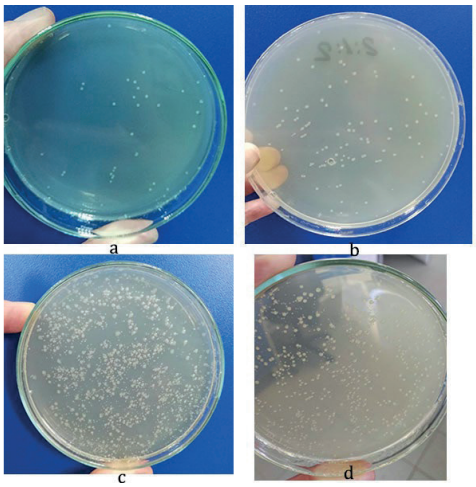


Figure 4. *E. coli* colonies containing plasmid DNA: a. 40 colonies; b. 100 colonies; c. > 500 colonies; d > 300 colonies

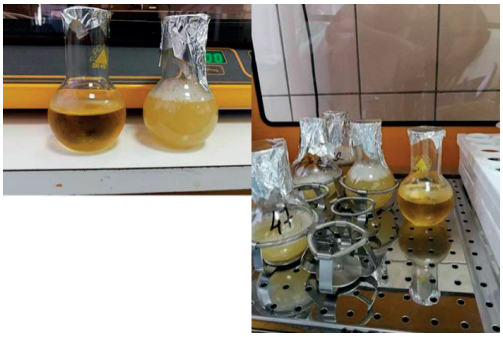


Figure 5. Growing *Escherichia coli* in liquid LB medium

Isolation of Plasmid DNA by Alkaline Lysis

Plasmids were purified using the classic alkaline lysis protocol (Birnboim and Doly, 1979; Sambrook et al., 1989). The working method involved the following steps:

Precipitation of crop grown overnight*:* 1 ml of *E. coli* culture, grown overnight, was pipetted into a 2 ml tube. After centrifuging the cell culture at 12,000 rpm for 5 minutes and discarding the supernatant, the cells were pelleted to remove them from the growth medium. This stage was repeated five times (Figure 6).

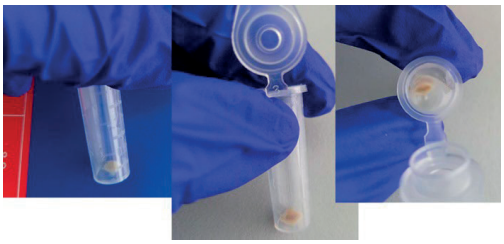


Figure 6. Bacterial pellet sedimentation

Resuspension bacterial cells: the bacterial pellet was resuspended in 350 µl alkaline solution I. At this stage, 10 µl de RNase A were also added. The mixture was left to incubate for 5 minutes at room temperature, with a slight inverse of the tube from time to time to eliminate bacteria through hyperlytic osmosis and release total DNA and other contents (Figure 7).

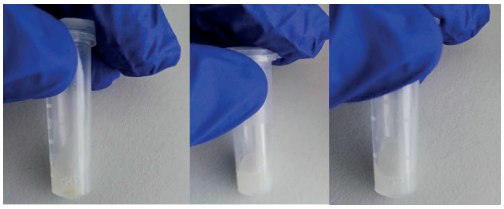


Figure 7. Bacterial pellet resuspension

Bacterial lysis*:* to denature the plasmid, chromosomal DNA and proteins, it was added, over alkaline solution I, the alkaline lysis solution II (400 µl), followed by tube inversion 6 times and placed on ice for 10 minutes.

Neutralization. Selective renaturation of plasmid DNA*:* selective renaturation of plasmid DNA with its resuspension was done by adding the acid III solution (500 µl) over alkaline solutions I and II used in previous stages. This was followed by incubation on ice for 10 minutes and centrifugation at 12.000 rpm for 30 minutes. The chromosomal DNA and proteins were denatured and precipitated by centrifugation, and the plasmid DNA, due to its smaller size, was renatured, diffusing in the supernatant (Figure 8).

Figure 8. Selective renaturation of plasmid DNA

Precipitation, purification and elution of plasmid DNA: plasmid DNA, recovered from the supernatant, was isolated and purified following the instructions recommended by "ISOLATE II Plant DNA Kit" from Meridian Bioscience, USA.

Evaluation of the results

Fragments amplified by PCR reaction and plasmid DNA were loaded into 3% agarose gel and migrated using a horizontal electrophoresis system, from Thistle Scientific, UK. The gel was read with the "Essential V6" imaging platform, producer Uvitec Cambridge, UK. Plasmid DNA sequencing was outsourced to Macrogen Europe, USA. The query in the NCBI database was done using the BLAST program, which will search equal-length segments. When aligned to one another, without gaps, they have maximal aggregate scores. For Query Identity % (the number of exact matches in the alignment relative to the query length) and Query Coverage % (the alignment length, including mismatches, relative to the query) was used blastn algorithm. This algorithm compares a nucleotide query sequence with nucleotide sequence database (Altschul, 2005) and was described by Altschul et al. (1990). For interpretation of its results, BLAST provides three related pieces of information in the form of the raw scores, bit scores and E-values. BLAST uses statistical theory to produce a bit score and expect value (E-value) for each alignment pair (query to hit) (Madden, 2002; Gibas and Jambeck, 2001). In the case of blastn, the scores are assigned to a simple matching matrix /non-matches for nucleotides, using mathematical formulas: $S= \sum(match\ score) - \sum(\text{gap penalties})$ for raw score and $S= \frac{\lambda S - \ln(k)}{ln2}$ for bit score (Gertz, 2005). For a single distinct alignment, BLAST programs convert scores to "E-values" using the equation: $E=Kmn\,e^{-\lambda S}$, where K and λ are Karlin-Altschul parameters, m effective length of the query sequence, and n the length of the database to which it is compared (Karlin and Altschul, 1990; Altschul, 2005). E-value indicates the likelihood that the match is due to chance, and a lower value means the match is significant. An E-value greater than 1 indicates that the query sequence has been aligned to a sequence in the database to which it is not related. Biological significance is typically represented for E-values less than 0.1 or 0.05 (Madden, 2002; Pertsemlidis and Fondon, 2001). The dendrogram was constructed using Minitab 18, which calculates the genetic distance based on the Euclidean matrix.

RESULTS AND DISCUSSIONS

The OPL19 marker amplified the corresponding 433 bp fragment of the dominant allele *Vr* gene (*Rvi2* or *Rvi8*) in all 20 genotypes (Figure 9). The integrity and presence of plasmid DNA was checked on the agarose gel (Figure 10). Blastn used as a search algorithm in our query, compares the nucleotide query sequence (sequences of the 20 samples) with sequence of the *Rvi2* gene, the result being displayed in the alignment block. The sequence associated with the gene of interest is recorded in the "National Center for Biotechnology Information" database, the locus name being under the name "AY626824" (Figure 11). These results confirm the presence of the *Rvi2* gene in all 20 analyzed genotypes, demonstrating the efficiency of the OPL19 marker in identifying this gene. Amplification of the 433 bp fragment, verification of plasmid DNA integrity and blastn analysis with the reference sequence from the NCBI database (locus AY626824) support the validity of the methodology used. These findings are relevant to marker-assisted selection in apple breeding programs. Each alignment block consists of three lines: the query sequence, the matching sequence, and the subject sequence; these alignments are sorted by "score", "E-value", "percent identity", "query start position" and "subject start position". The matching sequence is represented by bases that match, indicated by vertical lines, and non-matched bases, marked by empty spaces. The blastn algorithm results for the 20 clones showed a matching percentage, ranging from [94.6% to 100%] for the forward primer and

[95.39% to 100%] for the reverse primer. The percentage of the cover between the query sequence and the subject sequence was in the range [85-92%] for the forward primer and [80-93%] for the reverse primer (Table 1). A large query cover (90-100%) indicates that the alignment covers almost the entire query sequence, while a small percentage means that only a small part of the sequence aligns. If the E-value of the query is = 0, it does not matter if the query coverage is <100%. These results indicate a high degree of similarity between the query sequences and the reference *Rvi2* gene, confirming the reliability of the blastn analysis in identifying and validating the presence of the target gene in the studied apple genotypes. The highest percentage of identity was for the variety 'Pomona' (forward and reverse primer), and the most significant cover was for the variety 'Salva' (reverse primer) (Figure 12 and Figure 13). The results: 2e-155 ($2x10^{-155}$), 2e-176 ($2x10^{-176}$), 5e-158 ($5x10^{-158}$) and 7e-163 ($7x10^{-163}$) for E-value are negligible, and the likely that the match is due to chance is zero. Using the Minitab 18 program, the dendrogram was made, both for sequencing with the forward primer and for sequencing with the reverse primer, taking into account the values from the blastn analysis (percent identity, query coverage, max score and total score). These findings confirm the strong genetic similarity between the studied apple varieties and the *Rvi2* gene, with 'Pomona' showing the highest identity and 'Salva' the most significant coverage. The extremely low E-values indicate that the matches are highly significant and not due to chance. The dendrogram analysis further supports the genetic relationships among the genotypes, reinforcing the accuracy of the blastn results. The dendrogram analysis identified 19 clusters and two main groups, A and B, which reflect the genetic diversity among the apple cultivars (Figure 14 and Figure 15). As shown in Tables 2 and 3, the highest similarity between the query sequences and the reference sequence was found in the cultivars 'Remar' and 'Starkprim' for the forward primer, and 'Dany' and 'Jonaprim' for the reverse primer. In contrast, the lowest similarity was observed in the cultivars 'Alex' and 'Cezar' for both primers. These results help to better understand the genetic relationships among the studied genotypes, which is valuable for future breeding programs.

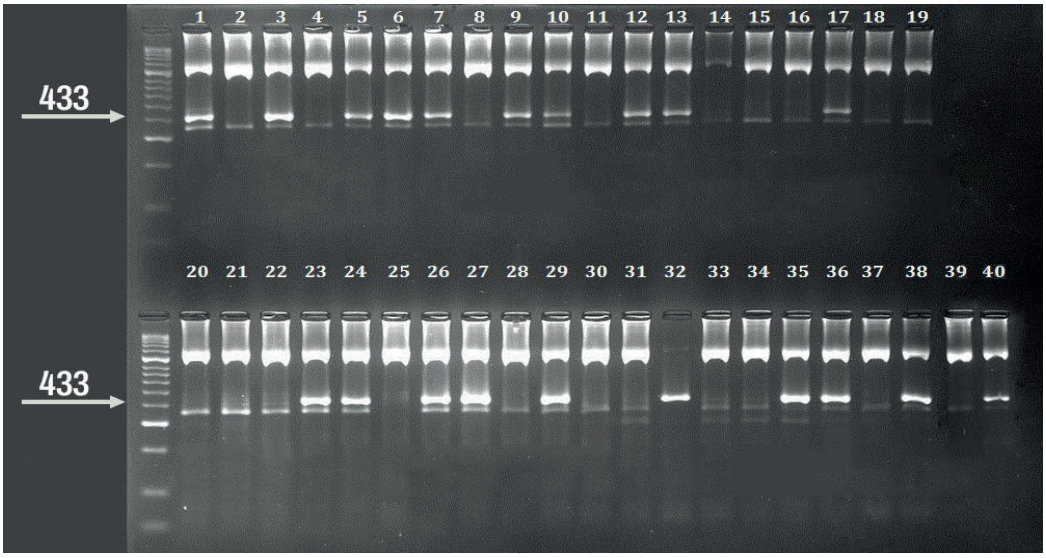


Figure 9. Electrophoretic profile obtained with OPL19 markers, the amplified fragment size being 433 bp.
1. 'Estival', 2. 'Rebra', 3. 'Bistriţean', 4. 'Aura', 5. 'Romus 3', 6. 'Dany', 7. 'Romus 5', 8. 'Productiv de Cluj', 9. 'Luca', 10. 'Ciprian', 11. 'Irisem', 12. 'Slava', 13. 'Ionaprim', 14. 'Rustic', 15. 'Precoce de Ardeal', 16. 'Iris', 17. 'Starkprim', 18. 'Auriu de Cluj', 19. 'Generos', 20. 'Colonade', 21.'Nicol', 22. 'Colmar', 23. 'Delicios de Voineşti', 24. 'Romus 4', 25. 'Remus', 26. Redix'', 27. 'Alex', 28. 'Doina', 29. 'Voinicel', 30. 'Inedit', 31. 'Dacian', 32. 'Voinea', 33. 'Valery', 34. 'Real', 35. 'Discoprim', 36. 'Cezar', 37. 'Frumos de Voineşti', 38. 'Pomona', 39. 'Revidar', 40. 'Remar'

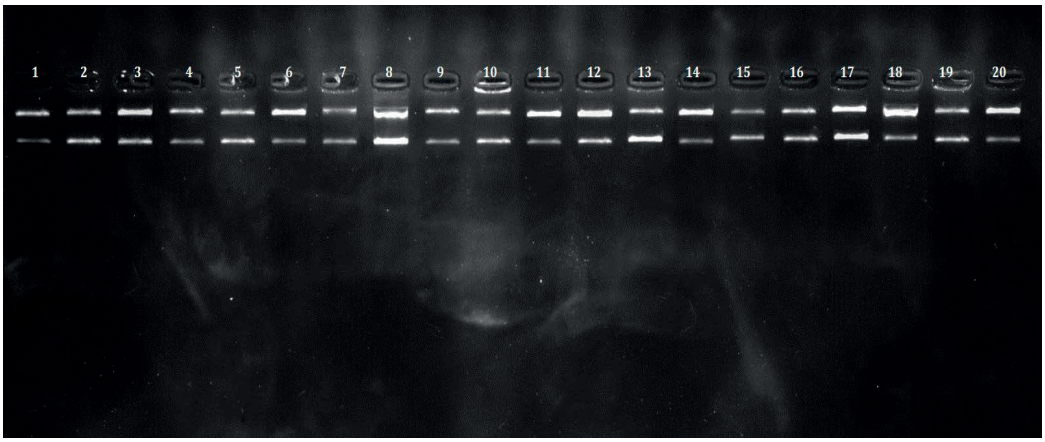
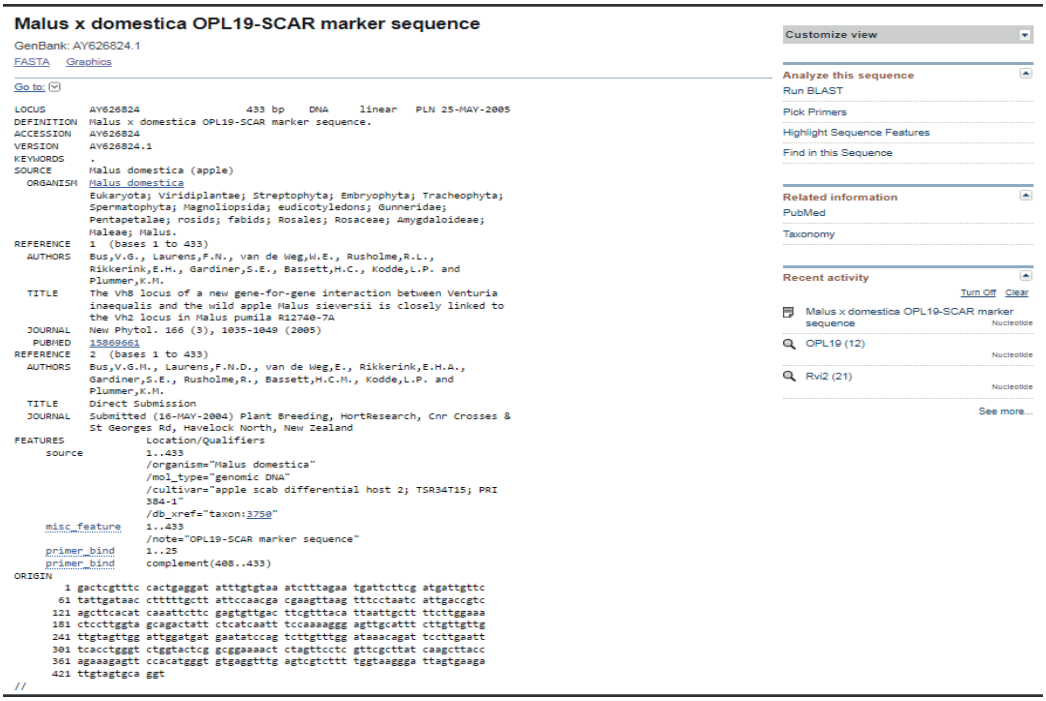


Figure 10. Electrophoretic profile for plasmid ADN
1. 'Alex', 2. 'Bistriţean', 3. 'Cezar', 4. 'Ciprian', 5. 'Dany', 6. 'Discoprim', 7. 'Delicios de Voineşti', 8. 'Estival', 9. 'Jonaprim', 10. 'Luca', 11. 'Pomona', 12. 'Redix', 13. 'Remar', 14. 'Romus 3', 15. 'Romus 4', 16. 'Romus 5', 17. 'Salva', 18. 'Starkprim', 19. 'Voinea', 20. 'Voinicel'

Table 1. Blastn analysis results for forward and reverse primer

Cultivar	Percent identity		Query coverage		Max score		Total score		E-value	
	Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse
Alex	97.46%	96.78%	90%	91%	645	643	645	643	0.0	0.0
Bistriţean	96.49%	97.74%	91%	91%	649	664	649	664	0.0	0.0
Cezar	97.97%	97.13%	91%	80%	673	558	673	558	0.0	7e-163
Ciprian	97.98%	97.20%	90%	89%	666	645	666	645	0.0	0.0
Dany	96.74%	98.23%	91%	91%	649	673	649	673	0.0	0.0
Discoprim	98.45%	97.26%	89%	91%	663	654	663	654	0.0	0.0
Delicios de Voineşti	96.98%	95.39%	90%	80%	643	533	643	533	0.0	2e-155
Estival	97.26%	97.68%	92%	89%	665	649	665	649	0.0	0.0
Jonaprim	96.94%	97.78%	89%	92%	638	673	638	673	0.0	0.0
Luca	98.65%	97.97%	85%	90%	642	660	642	660	0.0	0.0
Pomona	100%	100%	86%	81%	673	686	699	726	0.0	0.0
Redix	94.6%	98.68%	90%	87%	604	656	604	656	2e-176	0.0
Remar	99.23%	97.69%	90%	89%	689	650	689	650	0.0	0.0
Romus 3	97.38%	96.98%	87%	91%	639	646	639	646	0.0	0.0
Romus 4	97.16%	89.71%	88%	89%	637	662	637	662	0.0	0.0
Romus 5	97.69%	98.26%	88%	92%	652	682	652	682	0.0	0.0
Salva	95.75%	97.49%	91%	93%	627	659	627	659	0.0	0.0
Starkprim	99.23%	99.23%	90%	90%	689	689	689	689	0.0	0.0
Voinea	97.93%	95.69%	88%	80%	651	543	651	543	0.0	5e-158
Voinicel	98.24%	98.24%	91%	91%	678	679	678	679	0.0	0.0



Malus x domestica OPL19-SCAR marker sequence

GenBank: AY626824.1

FASTA Graphics

Go to:

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VERSION     AY626824.1
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REFERENCE   1  (bases 1 to 433)
  AUTHORS   Bus,V.G., Laurens,F.N., van de Weg,W.E., Rusholme,R.L.,
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            Plummer,K.M.
  TITLE     The Vh8 locus of a new gene-for-gene interaction between Venturia
            inaequalis and the wild apple Malus sieversii is closely linked to
            the Vh2 locus in Malus pumila R12740-7A
  JOURNAL   New Phytol. 166 (3), 1035-1049 (2005)
   PUBMED   15869661
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Customize view

Analyze this sequence
Run BLAST
Pick Primers
Highlight Sequence Features
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Malus x domestica OPL19-SCAR marker sequence — Nucleotide
OPL19 (12) — Nucleotide
Rvi2 (21) — Nucleotide
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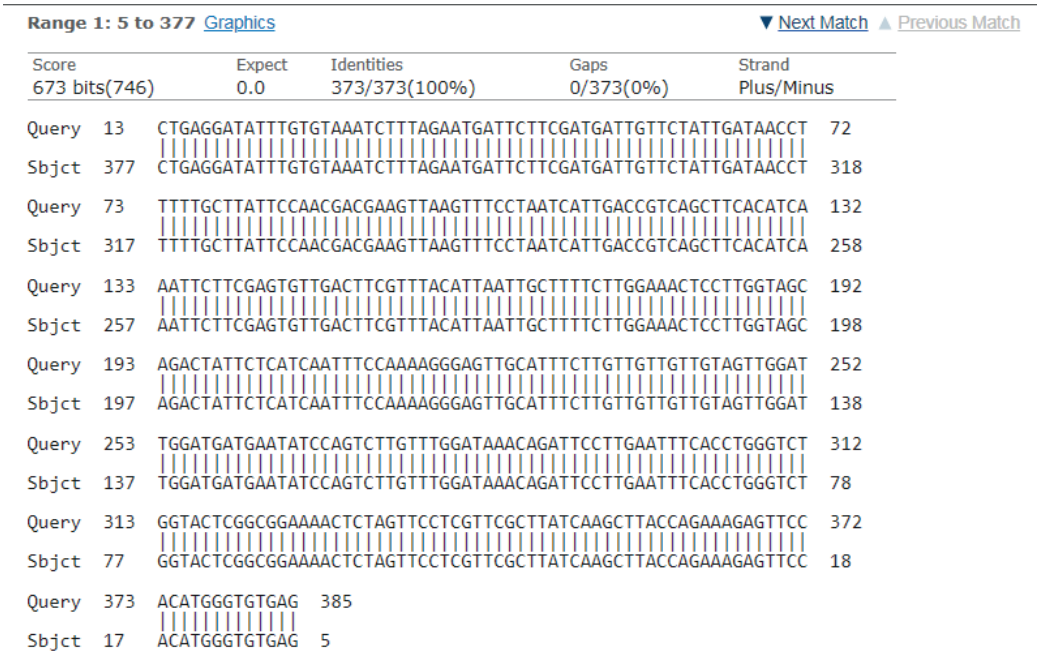
Figure 11. The *Rvi2* gene recorded in the National Center for Biotechnology Information database



Range 1: 5 to 377 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
673 bits(746)	0.0	373/373(100%)	0/373(0%)	Plus/Minus

```
Query  13   CTGAGGATATTTGTGTAAATCTTTAGAATGATTCTTCGATGATTGTTCTATTGATAACCT  72
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  CTGAGGATATTTGTGTAAATCTTTAGAATGATTCTTCGATGATTGTTCTATTGATAACCT  318

Query  73   TTTTGCTTATTCCAACGACGAAGTTAAGTTTCCTAATCATTGACCGTCAGCTTCACATCA  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  TTTTGCTTATTCCAACGACGAAGTTAAGTTTCCTAATCATTGACCGTCAGCTTCACATCA  258

Query  133  AATTCTTCGAGTGTTGACTTCGTTTACATTAATTGCTTTTCTTGGAAACTCCTTGGTAGC  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  AATTCTTCGAGTGTTGACTTCGTTTACATTAATTGCTTTTCTTGGAAACTCCTTGGTAGC  198

Query  193  AGACTATTCTCATCAATTTCCAAAAGGGAGTTGCATTTCTTGTTGTTGTTGTAGTTGGAT  252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  AGACTATTCTCATCAATTTCCAAAAGGGAGTTGCATTTCTTGTTGTTGTTGTAGTTGGAT  138

Query  253  TGGATGATGAATATCCAGTCTTGTTTGGATAAACAGATTCCTTGAATTTCACCTGGGTCT  312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  TGGATGATGAATATCCAGTCTTGTTTGGATAAACAGATTCCTTGAATTTCACCTGGGTCT  78

Query  313  GGTACTCGGCGGAAAACTCTAGTTCCTCGTTCGCTTATCAAGCTTACCAGAAAGAGTTCC  372
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   GGTACTCGGCGGAAAACTCTAGTTCCTCGTTCGCTTATCAAGCTTACCAGAAAGAGTTCC  18

Query  373  ACATGGGTGTGAG  385
            |||||||||||||
Sbjct  17   ACATGGGTGTGAG  5
```

Figure 12. Alignment section from a BLAST report showing pair-wise sequence alignment between the query sequence for cultivar 'Pomona' and the sequence of the *Rvi2*

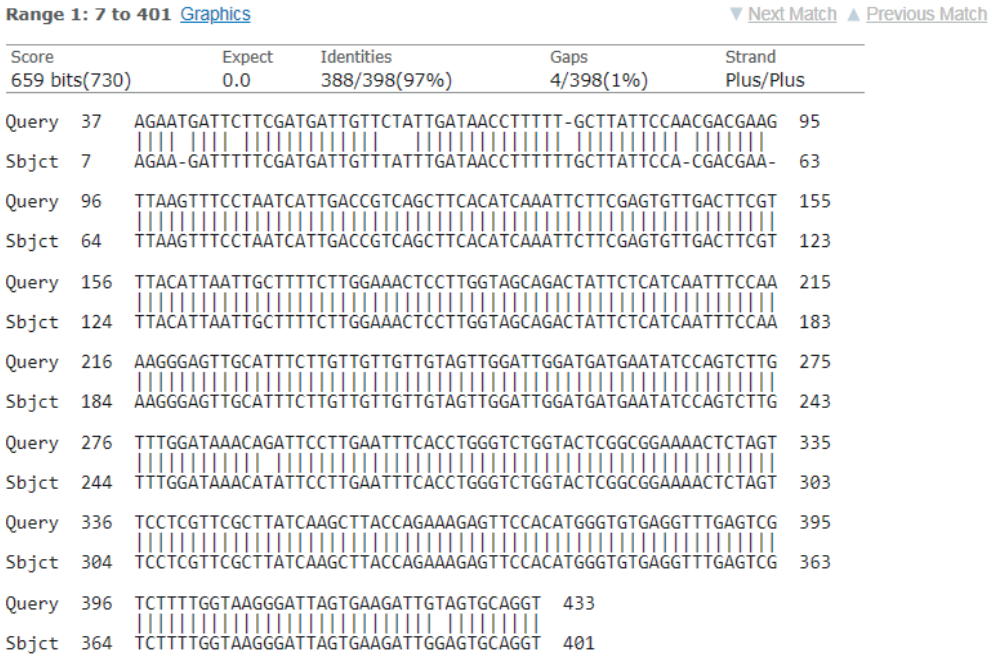


Figure 13. Alignment section from a BLAST report showing pair-wise sequence alignment between the query sequence for cultivar 'Salva' and the sequence of the *Rvi2*

Table 2. Amalgamation Steps

Step	Number of clusters	Similarity level	Distance level	Clusters joined		New cluster	Number of obs. in new cluster
1	19	100,000	0,000	13	18	13	2
2	18	99,998	0,002	2	5	2	2
3	17	98,824	1,414	16	19	16	2
4	16	98,823	1,414	9	15	9	2
5	15	98,823	1,414	4	8	4	2
6	14	98,823	1,415	7	10	7	2
7	13	97,647	2,828	9	14	9	3
8	12	96,471	4,243	4	6	4	3
9	11	96,470	4,243	2	16	2	4
10	10	96,470	4,243	1	7	1	3
11	9	94,118	7,071	3	20	3	2
12	8	90,588	11,314	1	9	1	6
13	7	84,304	18,868	11	13	11	3
14	6	82,353	21,213	1	2	1	10
15	5	82,353	21,213	3	4	3	5
16	4	72,941	32,527	12	17	12	2
17	3	68,918	37,363	3	11	3	8
18	2	43,529	67,882	1	12	1	12
19	1	0,000	120,208	1	3	1	20

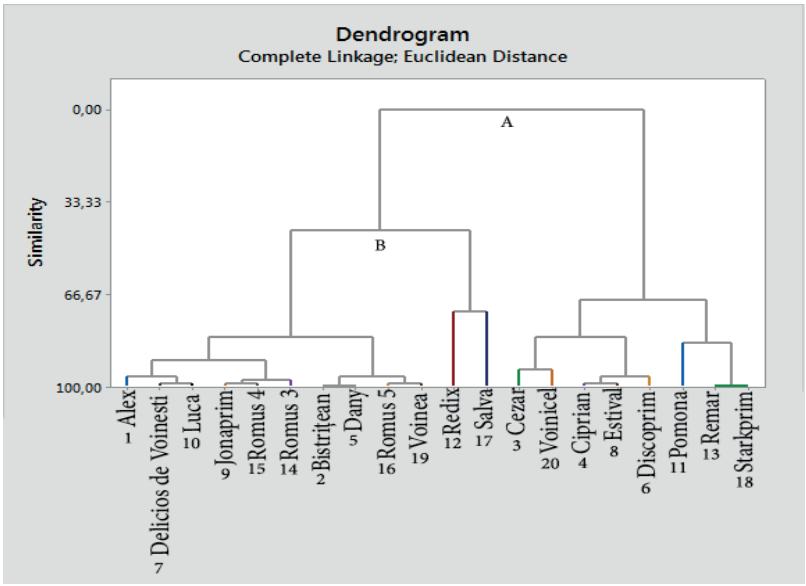


Figure 14. The level of similarity shown in relation to BLAST analysis for the forward primer

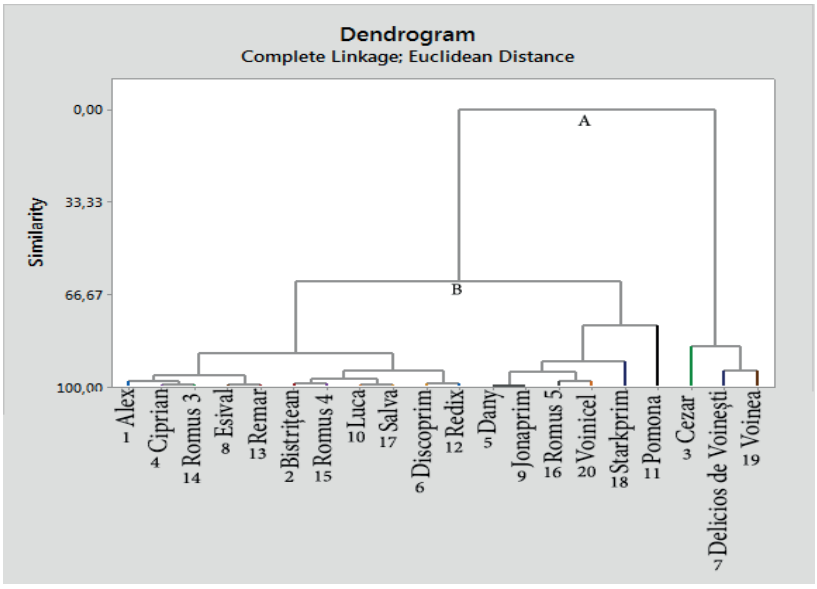


Figure 15. The level of similarity shown in relation to BLAST analysis for the reverse primer

Table 3. Amalgamation Steps

Step	Number of clusters	Similarity level	Distance level	Clusters joined		New cluster	Number of observation in new cluster
1	19	99,9955	0,011	5	9	5	2
2	18	99,4258	1,414	8	13	8	2
3	17	99,4257	1,414	4	14	4	2
4	16	99,4257	1,415	10	17	10	2
5	15	98,8515	2,829	6	12	6	2
6	14	98,8511	2,830	2	15	2	2
7	13	98,2774	4,243	1	4	1	3
8	12	98,2774	4,243	16	20	16	2
9	11	97,1289	7,071	2	10	2	4
10	10	95,9805	9,900	1	8	1	5
11	9	94,8321	12,728	5	16	5	4
12	8	94,2579	14,142	7	19	7	2
13	7	94,2579	14,142	2	6	2	6
14	6	90,8126	22,627	5	18	5	5
15	5	87,9416	29,698	1	2	1	11
16	4	85,6447	35,355	3	7	3	3
17	3	77,8426	54,571	5	11	5	6
18	2	62,0456	93,477	1	5	1	17
19	1	0,0000	246,288	1	3	1	20

CONCLUSIONS

In conclusion, the OPL19 marker has proven to be effective for molecular screening to highlight the *Vr* gene (*Rvi2* or *Rvi8*), confirming its utility in the genetic analysis of apple varieties. The results of the BLAST analysis identified the gene sequence in all 20 varieties and revealed intraspecific differences based on the matching sequence. The method developed in this study will be extended to other molecular screenings to build a database that will support the construction of genetic maps. Furthermore, in an extended study using the BLAST program, intraspecific differences can be identified by comparing the nucleotide sequences of the varieties, offering valuable insights for future research and breeding programs.

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