DEVELOPMENT OF TEST PROCEDURES AND THE SEARCH FOR OPTIMAL POSITIONS OF THE PRIMERS PLANTING USING THE PROGRAM PRIMERQUEST FOR IDENTIFICATION OF PLANT OBJECTS

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Abstract: Identification has similarities and differences with other kinds of assessment activity: quality assessment, control management and certification. The final result of identification is verification of compliance or detection of falsification. Common features are tests for definition of actual values. This paper studies the design of universal primers for type identification of fruit raw material (strawberry, gooseberry, cherry, raspberry, banana, wild rose, kiwi). To further verify the specificity of primers, sequencing of fragments is produced, which are read by each from the primer pairs. For this purpose, 8 polymerase chain reactions (PCR-reactions) are initiated, one from each primer pair corresponding to one type of raw material. A single alignment matrix for each of the studied objects is created as a result. Re-verification of each matrix is conducted for the presence of read errors or other disputed single-nucleotide substitutions. It is stated that the alignment matrices of the nucleotide sequences of raspberry, strawberry (fragaria viridis), gooseberry, wild rose, cherry, banana and kiwi are aligned on all sides and the protruding "bases" do not disturb the future work of programmes for the primers design. Universal non-intersecting primers are chosen to identify the fruit raw material under studying. As a result of the use of various software packages and of the database GenBank NCBI, we managed to find a suitable DNA zone for each of the tested samples of fruit raw material at the level of generic differentiation for further development on its basis of the universal primers. It is zone 18S rDNA. All the found sequences have both the conservative part for planting a pair of primers, and the variable one for reliable identification of species or for phylogenetic analysis. As part of the study, all samples of fruit raw material have been identified.

Keywords: Fruit raw material, identification, PCR, matrix, primers

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INTRODUCTION

Identification as an activity has its own structure which includes objectives and tasks, objects and subjects, means and methods [1].

Identification has similarities and differences with other kinds of assessment activity: quality assessment, control management and certification. Common features are tests for definition of actual values and compliance test with the requirements of regulatory documents. The differences lie in the list of criteria; in the subjects which determine the assessment activity; in the final result. The final result of identification is verification of compliance or detection of falsification [2, 3].

The term "identification" is interpreted differently. The analysis of the regulatory documents showed that the term "identification" has the following definitions [13].

Identification is the procedure by which compliances of the products, submitted to certification, are established with the requirements for this type of products, set by the regulatory documents (Sertifikatsiya pischevykh produktov i prodovol'stvennogo syr'ya v RF [Certification of foodstuffs and food raw material in the Russian Federation], 1996).

As criteria of identification the indicators, meeting the following requirements, should be selected:
– typicalness for a particular type, name or homogeneous product group;
– objectiveness and comparability;
– ability to test;
– difficulty of falsification.

The greatest significance has the typicalness which can be characterized by complex or, less often, individual indicators that complement each other and have a varying degree of accuracy [4, 5].

The objective of this paper is the study of universal primers design for type identification of fruit raw material. The tasks of this paper include the selection of universal primers and the identification of such fruits and berries like cherry, strawberry, raspberry, gooseberry, wild rose, banana and kiwi.

STUDY OBJECTS AND METHODS

Alignments were visualized in the programme GeneDoc.

Matrices were aligned from the two sides of alignments. Presence of protruding "bases" may disturb the future programme work [15].

In accordance with the objective and the tasks of the present paper, the study objects were: *Rubus idaeus* (raspberry, the grade “Nagrada”), *Fragaria vesca* (remontant wild strawberry, the grade “Berdskaya rannyaya”), *Ríbes úva-críspa* (garden gooseberry, the grade “Kooperator”), *Prunus fruticosa* (ground cherry, the grade “Altayskaya lastochka”), *Rosa majalis Herrm* (cinnamon rose), *Actinidia deliciosa* (kiwi delicatessen), *Músa paradisiaca* (banana of "extra" grade).

Primers were selected with the use of the programme PrimerQuest (http://eu.idtdna.com/Primerquest/Home/Index). Computer processing and sequences alignment were performed in the programmes ClustalW and GeneDoc, the construction of phylogenetic trees was performed in the programme Mega 6 [6, 7].

To further verify the specificity of primers, sequencing of fragments was produced, which are read by each from the primer pairs [14, 17]. For this purpose, 8 polymerase chain reactions (PCR-reactions) were initiated, one from each primer pair corresponding to one type of raw material [8, 9]. The obtained PCR-products were re-precipitated by ethanol in the presence of ammonium acetate, dried and then sequenced according to Sanger using the device ABI Prism 3500xl. The sequencer output data - chromatograms - were converted into nucleotide sequence and then, using the BLAST algorithm, were compared to the NCBI sequences, present in GenBank [10, 11].

RESULTS AND DISCUSSION

At this research stage previously conducted alignments were visualized and corrected in the program GeneDoc [12]. Thus, a single alignment matrix for each of the studied objects was created (Fig. 1–7). Re-verification of each matrix was conducted for the presence of read errors or other disputed single-nucleotide substitutions.

Matrices were aligned from the two sides of alignments. Presence of protruding "bases" may disturb the future programme work.

The analysis of the figure shows that the alignment matrices of the nucleotide sequences of raspberry, strawberry (fragaria viridis), gooseberry, wild rose, cherry, banana and kiwi are aligned on all sides and the protruding "bases" do not disturb the future work of programmes for the primers design.

Rectangular alignment matrices for each of the studied objects are presented in the figures.

Then, each matrix was loaded to the program PrimerQuest for sequences algorithmic analysis and search for optimal positions of the primers planting.

In the settings it was always stated that the maximum size of the amplicon, read by a pair of primers, should not exceed 300 b.p. An optimal pair of primers was selected from the ones, offered by the programme (Fig. 8). The following parameters were taken into consideration: primer length, annealing temperature, amplicon location.

Analyzing Fig. 8, optimal primers were selected.

Primers for the studied types of fruit raw material with the recommended parameters for PCR (visualization of the programme PrimerQuest) are indicated in Figures 9–15.

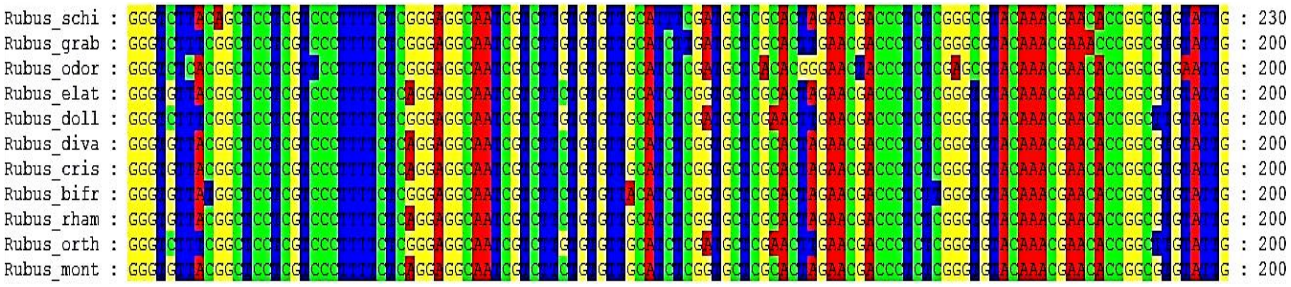

Fig. 1. Part of alignment matrix of *Rubus idaeus* nucleotide sequences.

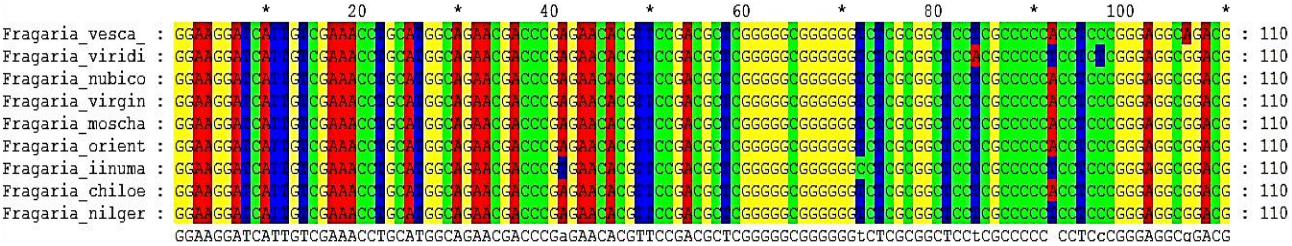

Fig. 2. Part of alignment matrix of *Fragaria vesca* nucleotide sequences.

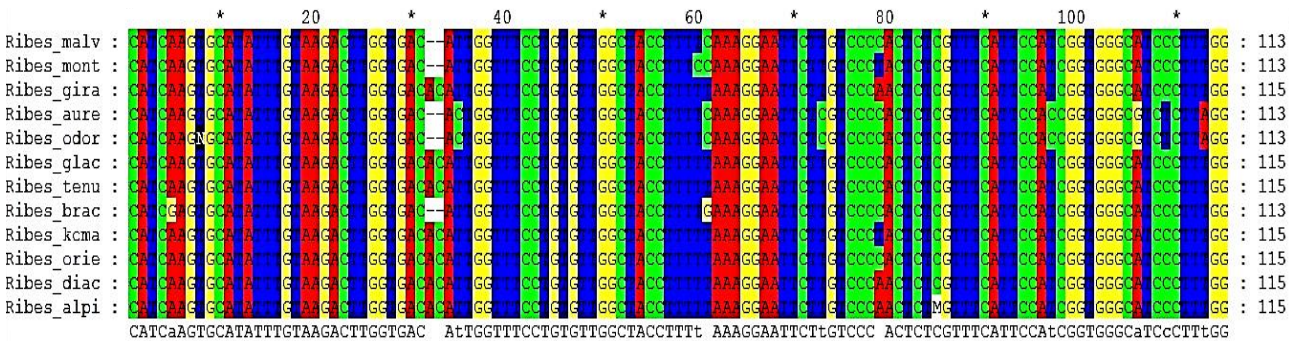

Fig. 3. Part of alignment matrix of *Ríbes úva-críspa* nucleotide sequences.

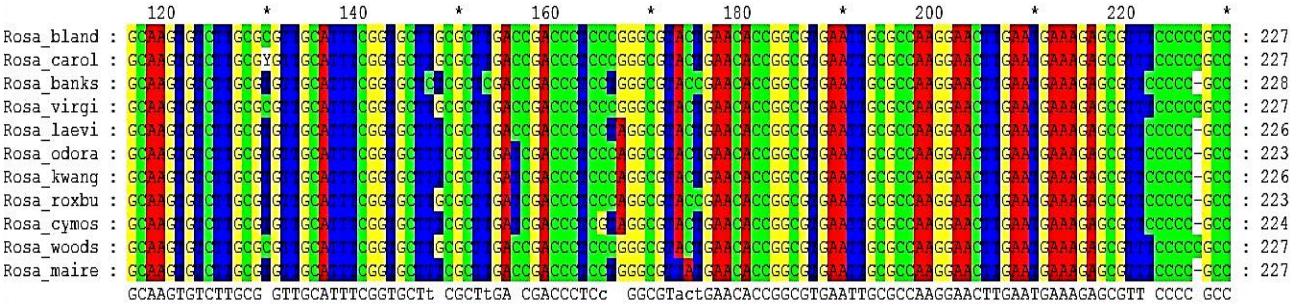

Fig. 4. Part of alignment matrix of *Rosa majalis Herrm* nucleotide sequences.

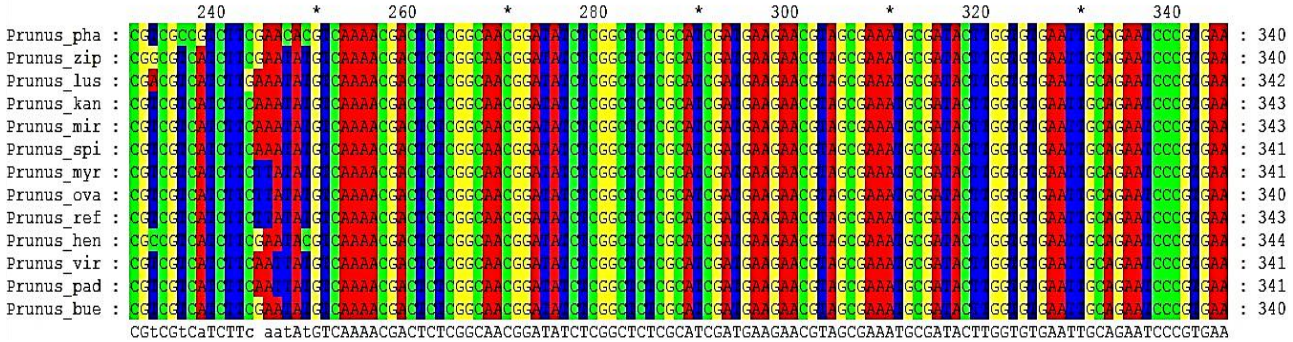

Fig. 5. Part of alignment matrix of *Prunus fruticosa* nucleotide sequences.

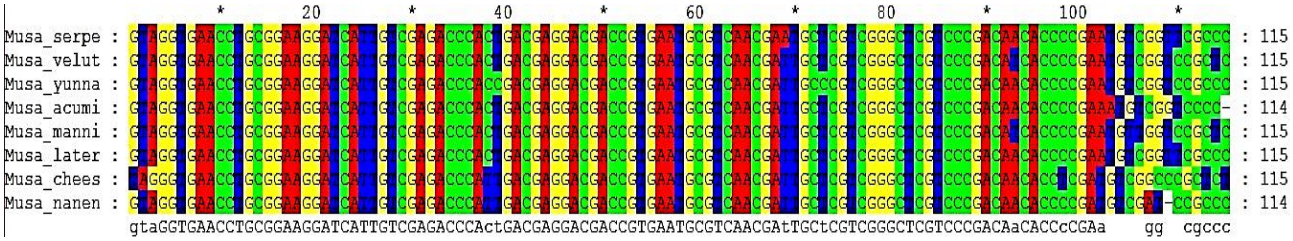

Fig. 6. Part of alignment matrix of *Músa paradisiaca* nucleotide sequences.

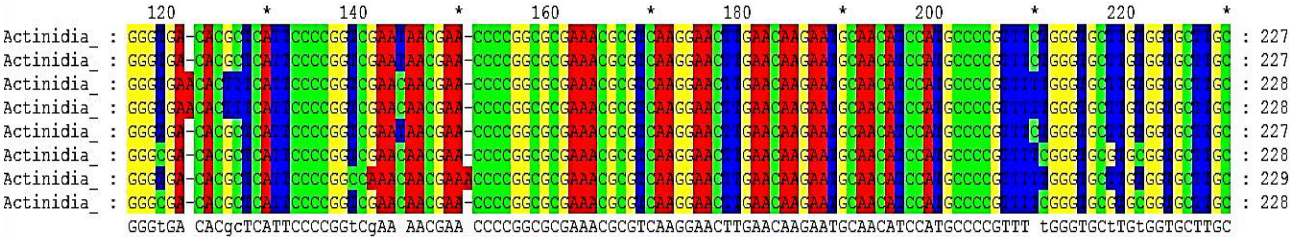

Fig. 7. Part of alignment matrix of *Actinidia deliciosa* nucleotide sequences.

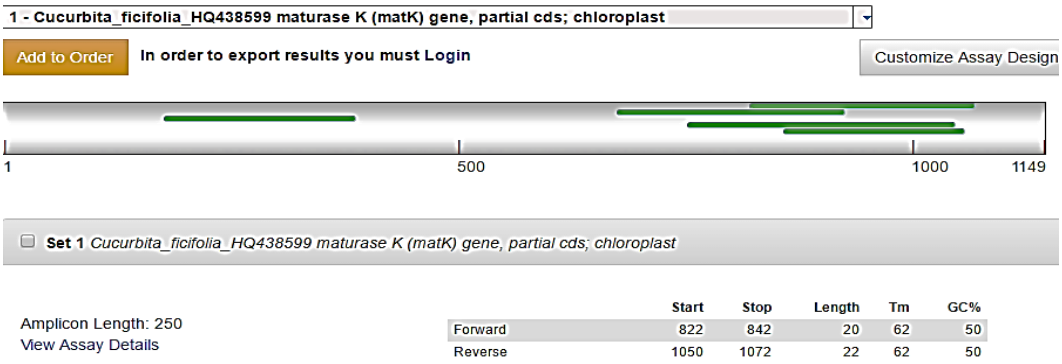

Fig. 8. Selection of an optimal primer pair in the programme PrimerQuest.

Parameter Set: General PCR (Primers only)
Sequence Name:
Amplicon Length: 271

		Start	Stop	Length	Tm	GC%
Forward	CGATGAAGAACGTAGCGAAATG (Sense)	354	376	22	62	45.5
Reverse	CGATAGGCAACAGAGGTTTGA (AntiSense)	604	625	21	62	47.6

Base	Sequence
1	TTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGAGAACATGTTTCA
101	ACGCTTGGGGGCGAAGGGTCTTACAGCTCCTCGTCCCTTTTCTCGGGAGGCAATCGTCTTGTGTGTTGCATTTCGATGCTCGCACTAGAACGACCCTCTC
201	GGGCGTACAAACGAACACCGGCGTGTATTGCGCCAAGGAACTTGAATGAAAGAGCGTTTCCCCGTCGTCCCGGAAACGGTGTGCGTACGGTTGGTTACGT
301	CATCTTCAATATGTCTAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAAT
401	CCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGCCTGCCTGGGCGTCACACGTCGTTGCCCCCCCAACCCCC
501	TCGGGAGTTGGGCGGGACGGATGATGGCCTCCCGTGTGCTCCGTCATGCGGTTGGCATAAAAAACAAGTCCTCGGCGACTAACGCCACGACAATCGGTGG
601	TTGTCAAACCTCTGTTGCCTATCGTGTGCGCGTGTCGAACGAGGGCTCAATGAACCATGCTGCATTGATTCGTCGATGCTTTCAACGCGACCCCAGGTCA
701	GGCGGGGGTTACCC

Note. Hereinafter: Green - direct, Red - reverse

Fig. 9. Universal primers for *Rubus idaeus* identification.

Parameter Set: General PCR (Primers only)
Sequence Name:
Amplicon Length: 276

		Start	Stop	Length	Tm	GC%
Forward	CCGTGAACCATCGAGTCTTT (Sense)	351	371	20	62	50
Reverse	GCTTACCGACGCGCTTTA (AntiSense)	609	627	18	62	55.6

Base	Sequence
1	GGAAGGATCATTGTCGAAACCTGCATGGCAGAACGACCCGAGAACACGTTCCGACGCTCGGGGGCGGGGGGTCTCGCGGCTCCTCGCCCCCTCCTCCCGG
101	GAGGCGGACGTCTCGCGCGTCGCGCTCGGCGCTTCCGCCTGGCCGACCCTTCCGGGCGTACCGAACACCGGCGTGAATTGCGCCAAGGAACTTGAATGAA
201	AGAGCGTTCCCCCGCCGTCCCGGAGACGGAGACCGCGCGGGTGGTTCGTCGTCTTCAGTATGTCTAAACGACTCTCGGCAACGGATATCTCGGCTCTCGC
301	ATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCGTTAGGC
401	CGAGGGCACGTCTGCCTGGGCGTCACACGTCGTTGCCCCCCCGACCCCTTCGGGGGCCGGACGGGACGGATGATGGCCTTCCCGTGTGCCCCGTCACGCG
501	GTTGGCATAAATACCGAGTCCTCGGCGACCGGCGCCGCGACAATCGGTGGTTGTGAAACCTCGGTGCCTTGTCGCGTGCGTGAGTCGATCGCGGGACTTC
601	CTTAACCTTAAAGCGCGTCGGTAAGCCGACGCTTTCAACGCGACCCCAGGTCAGGCGGGTTACCCGCTGAATTTAA

Fig. 10. Universal primers for *Fragaria vesca* identification.

Parameter Set: General PCR (Primers only)
Sequence Name:
Amplicon Length: 269

		Start	Stop	Length	Tm	GC%
Forward	CGTCGTCTCATATGTCCATCAA (Sense)	14	36	22	62	45.5
Reverse	GGAGCAATAAAGCACCACATAC (AntiSense)	261	283	22	62	45.5

Base	Sequence
1	CTGTTGTCGCGTCCGTCGTCTCATATGTCCATCAAGTGCATATTTACAAGACTTGGTGACATTGGTTTCCTGTGTTGGCTACCTTTTCAAAGGAATTCTC
101	GTCCCAACTCTCGTTTCATTCCATCGGTGGGCATCCCTCTGTGGATTGTCTTGGTGGACCTTCAAGTGTTTCTCGTGTGCCATTCACGCTACATTTTWAT
201	GCGGCGATGACATGGTCCACGGGTTGCTACTCGTAATCTCGGATTCGGAACATGTTGTGGGTATGTGGTGCTTTATTGCTCCATCTGCCCAAGCAGAGCT
301	TCTGTTGCTCGGCAAGAACGACAGTCGTGCTCGTGTTGACCTCTCCGGCATGCATGTGCTCGGTTTGGCTCATGCGACGCCCGACTTCGCAAAGGAATGC
401	TACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTGT

Fig. 11. Universal primers for *Ríbes úva-críspa* identification.

Parameter Set: General PCR (Primers only)
Sequence Name:
Amplicon Length: 227

		Start	Stop	Length	Tm	GC%
Forward	GTTTCCTGTGTTGGCTACCT (Sense)	65	85	20	62	50
Reverse	TGGGCAGATGGAGCAATAAA (AntiSense)	272	292	20	62	45

Base	Sequence
1	CTGTTGTCGCGTGCGTCGTCTCATATGTCCATCAAGTGCATATTTGTAAGACTTGGTGACATTGGTTTCCTGTGTTGGCTACCTTTTCAAAGGAATTCTT
101	GTCCCCACTCTCGTTTCATTCCATCGGTGGGCATCCCTTTGGGGATTGTCTTGGTGGACCTTCAAGTGTTTCCTGTGTGCCATTCACGCTACATTTTAAT
201	GCGGCGATGACATGGTCCACGGGTTGCTACTCGTAATCTCGGATTCGGAATATGTTGTGGGTATGTGGTGCTTTATTGCTCCATCTGCCCAAGCAGAGCT
301	TCTGTTGCTCGGCAAGAACGACAGTCGTGCTCGTGTTGACCTCTCCGGCATGCATATGCTCGGTGTGGCTCATGCGACGCCCGACTTCGAAAAGGAATGC
401	TACCTGGTTGATCCTGCCAGTAGTCATA

Fig. 12. Universal primers for *Rosa majalis Herrm* identification.

Parameter Set: General PCR (Primers only)
Sequence Name:
Amplicon Length: 285

		Start	Stop	Length	Tm	GC%
Forward	CTTGGTGTGAATTGCAGAATCC (Sense)	362	384	22	62	45.5
Reverse	CATCTTTACTTCTAGCCCTCGAC (AntiSense)	624	647	23	62	47.8

Base	Sequence
1	ATTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACCTGCCCGGCAGAACGACCCGAGAACCAGTTTC
101	GGAACTGGGGGCGAGGGGTCTCGCGGCTCCTCCTCCCTTCGTCYCGGGAGGGTCGCGTCGCGTTCGCGCGGCCGGCCCTTCCGGGCGTACAAACGAACAC
201	CGGCGCGAATTGCGCCAAGGAACTTGAACGAGAGAGCGCGCCCCTGCGGCCCCGGAAACGGTGCGCGCGGGCGGCGTCGCCGTCTTCGAACACGTCAAAA
301	CGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCT
401	TTGAACGCAAGTTGCGCCCGAAGCCGTTAGGCCGAGGGCACGCCTGCCTGGGCGTCACGCGCCGTTGCCCCCCCGACCGATCCCTCGGGATCGCGGGGGG
501	CGGATGGTGGCCTCCCGTGCGCTCCGCCGCGCGGTTGGCATAAATACCAAGTCCCCGGCGACGCGCGCCACGACGATCGGTGGTTGCGAAACCTCGGTTG
601	CCCGTCGTGTGCGGCCGTCGCKCGTCGAGGGCTAGAAGTAAAGATGCTCGGCTCCGGCTCGGCTCTCAACGCGACCCCAGGTCAGGCGGGGTTACCCGCT
701	GAATTTA

Fig. 13. Universal primers for *Prunus fruticosa* identification.

Parameter Set: General PCR (Primers only)
Sequence Name:
Amplicon Length: 245

		Start	Stop	Length	Tm	GC%
Forward	GGAAGGATCATTGTCGAGACC (Sense)	15	36	21	62	52.4
Reverse	CGTTGCCGAGAGTCATACAA (AntiSense)	240	260	20	62	50

Base	Sequence
1	GTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCCACTGACGAGGACGACCGTGAATGCGTCAACGAATGCTCGTCGGGCTCGTCCCGACAACACCCCG
101	AATGTCGGTTCGCCCTCGGGCGGGACGATCGAGGGGATGAACTACCAACCCCGGCGCGGATAGCGCCAAGGAACACGAACATCGAAGTCGGAGGGCCTCG
201	CTGCATGCAGGAGGCTACAATTCCGACGGTGACACCCCATTGTATGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG
301	CGATACCTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAGGCCATCCGGCTAAGGGCACGCCTGCCTGGGCGTC
401	ACGCTTTCGACGCTTCGTCGTTGCCCCCTCGGGGGGTGGGGGCGAACGCGGAGGATGGCCCCCCGTGCCGGAAGGTGCGGTTGGCCGAAGATCGGGCCGT
501	CGGTGGTTGTCGAACACGACGCGTGGTGGATGCCTTGTGCGAGCCGTACGTCGTGCCTTCGGAACCCGGGCGAGGCCTCGAGGACCCAAGTCGTGGTGCG
601	AGTCGATGCCACGGACCGCGACCCCAGGTCAGGTGGGGCTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA

Fig. 14. Universal primers for *Músa paradisiaca* identification.

Parameter Set: General PCR (Primers only)
Sequence Name:
Amplicon Length: 283

		Start	Stop	Length	Tm	GC%
Forward	GACCCGCGAACTTGTCTAATA (Sense)	18	39	21	62	47.6
Reverse	GCATTTCGCTACGTTCTTCATC (AntiSense)	279	301	22	62	45.5

Base	Sequence
1	AACCTGCCTAGCAGAATGACCCGCGAACTTGTCTAATACTCTCGGGGAAGCGAAAGGTTGGTTTTTATGGCCTCCTTTTTCTTCCCTTTGCCGGGTGTGC
101	TCGTGTTGCCCTATGGGTGACACGCTCATTCCCCGGTCGAATAACGAACCCCGGCGCGAAACGCGTCAAGGAACTTGAACAAGAATGCAACATCCATGCC
201	CCGTTTCTGGGTGCTTGTGGTGCTTGCTCTATCATAAACGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC
301	GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCTGAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCA
401	CGCATTGTGTCGCCCACCCGACTCAAGCCTTGCCAAGGCCTGCGTGTGGGTGGGCGGATATTGGCCCCCCGTGCACATTAGTGAACGGTCGGCCTAAAAA
501	TGAGTCCTTGGCAATGGACGTCACAACAAGTGGTGGTTGACAAACCGTTGCGTCCTGTTGTGCTTGCCCCCATTGCTAATGGTTTACTTTTGACCCTAGT
601	GTGCCGTTGCCACGGCTTCGATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTTACAAGGATT
701	CCCTTAGTAACGGCGAGCGAACCGGGAATAGCCCAGCTTGAAAATCGGGCGATCTCGTCGTCCGAATTGTAGTCTGGAGAAA

Fig. 15. Universal primers for *Actinidia deliciosa* identification.

Table 1. Universal primers for PCR test-systems

Name of food raw material	Nucleotide pair primer length	Nucleotide pair amplicon length	Primers
Strawberry (fragaria vesca)	20 18	276	CCGTGAACCATCGAGTCTTT GCTTACCGACGCGCTTTA
Gooseberry	22 23	269	CGTCGTCTCATATGTCCATCAA GGAGCAATAAAGCACCACATAC
Cherry	22 23	285	CTTGGTGTGAATTGCAGAATCC CATCTTTACTTCTAGCCCTCGAC
Raspberry	22 21	271	CGATGAAGAACGTAGCGAAATG CGATAGGCAACAGAGGTTTGA
Banana	21 20	245	GGAAGGATCATTGTCGAGACC CGTTGCCGAGAGTCATACAA
Wild rose	20 21	227	GTTTCCTGTGTTGGCTACCT TGGGCAGATGGAGCAATAAA
Kiwi	21 22	283	GACCCGCGAACTTGTCTAATA GCATTTCGCTACGTTCTTCATC
Pumpkin	24 20	297	AGATACGCCACTTCTGATGAATAA GGATGCCCTAACACGTTACA

On the basis of Figures 9–15, universal non-intersecting primers were selected for determination by PCR method of fruit raw material (strawberry, gooseberry, cherry, raspberry, banana, kiwi). These primers are represented in Table 1.

Analyzing the tabular data, with the use of various software packages and of the database GenBank NCBI, we managed to find a suitable DNA zone for each of the tested samples of fruit raw material at the level of differentiation for further development on its basis of the universal primers. It is zone 18S rDNA. All the found sequences have both the conservative part for planting a pair of primers, and the variable one for reliable identification of species or for phylogenetic analysis.

Thus, as a result of study, a single alignment matrix for each of the studied objects of fruit raw material was created with the use of the programme GeneDoc, re-verification of each matrix is conducted for the presence of read errors or other disputed single-nucleotide substitutions [12, 16].

Sequences algorithmic analysis and search for optimal positions of the primers planting are conducted with the use of the programme PrimerQuest with indication in the settings of maximum amplicon size, read by each primer pair, which does not exceed 300 b.p.

Optimal pairs for each type of fruit raw material are selected from the ones, offered by the programme, taking into consideration the following: primer length, annealing temperature, amplicon location.

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