AGRICULTURAL UNIVERSITY - PLOVDIV

FACULTY OF AGRICULTURE

DEPARTMENT OF PLANT PHYSIOLOGY, BIOCHEMISTRY AND GENETICS

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Analysis of genes responsible for capsaicin synthesis in pepper (genus *Capsicum*)

ABSTRACT

of the dissertation for the degree of Doctor of Education and Science in the scientific specialty "Genetics", professional field 6.1.

Scientific supervisor:

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Field trials and research were conducted in the period 2018-2021 in the Training and Experimental field of Agricultural University, Plovdiv and in the Laboratory of Molecular Biology of the Department of PPBG.

The dissertation contains 130 typewritten pages, 47 figures, 11 tables. The list of cited literature includes 266 sources in Latin letters.

The dissertation was discussed and proposed for defense at an extended council of the Department of Plant Physiology, Biochemistry and Genetics at the Faculty of Agronomy of the Agricultural University - Plovdiv.

The official defense of the dissertation will take place on..... from ..... hours of the meeting of the Specialized Scientific Jury at the Agricultural University, Plovdiv, with members:

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INTRODUCTION

Pepper (g. *Capsicum*) is one of the most cultivated vegetable crops both in Bulgaria and worldwide. Its mass cultivation is due to its nutritional value, high adaptability, its productivity and the content of substances beneficial to human health.

Capsaicinoids are such secondary metabolites used as a component in dietary supplements and pharmaceuticals. It has industrial uses for pest control and as an aerosol, part of police equipment.

Biosynthesis of capsaicinoids is a unique ability of species of the genus Capsicum. They are responsible for the pungent taste (spiciness) in the fruit. The capsaicinoid capsaicin is synthesized in the largest amount (about 69%), which, because of its high content, has become the namesake of all its analogues.

Analyzing structural genes in the capsaicin biosynthetic pathway helps to elucidate the genetic basis of pungency and its loss. Pungency is both a qualitative and quantitative characteristic. Its extent depends on the concentration of capsaicinoids that accumulate and is inherited quantitatively, whereas the ability of a fruit to be pungent or non-pungent is controlled by dominant and recessive alleles of several genes. Identification of mutant alleles in genes with qualitative effects on capsaicin synthesis and their manipulation enables screening and production of fruits with specific taste qualities, either pungent or sweet.

Analyzing and controlling the genes responsible for capsaicin production would help to increase diversity and identify pepper forms with different contents or lack of the alkaloid. In order to prioritize the cultivation of hot or sweet pepper, it is important to study the biodiversity of Bulgarian pepper accessions. Assessment of genetic relatedness and variation is critical for effective management and improvement of cultivated plants.

LITERATURE REVIEW

Contains the following sections:

- Origin and distribution of pepper (genus Capsicum)
- Economic importance of pepper
- Classification of pepper (genus Capsicum)
- Ecological role of capsaicin
- Structure and biosynthesis of capsaicin
- Genetic control of biosynthesis
- Physical factors affecting capsaicin synthesis
- Molecular genetic analysis methods
- Induction of mutations

PURPOSE AND TASKS OF THE RESEARCH

The aim of the present study was to determine the allelic status of Bulgarian pepper accessions with respect to loci determining capsaicin levels and to identify the presence of known and/or novel loci modifying capsaicin synthesis in them.

To achieve this goal, the following main tasks were planned:

1. Determination of the presence of *Pun1* gene alleles (*pun1-1*, *pun1-2* and/or *pun1-3*) in the parental pepper genotypes.
2. Conducting directed crosses between the selected parental forms.
3. Tracing the inheritance in F_1 and F_2.
4. Study for the presence of additional loci with effects on capsaicin synthesis.
5. Investigate the relationship between genetic affiliation and capsaicin biosynthesis in individual parental forms (*C. annuum* L. - var. Plovdivska kapiya, *C. chinense* Jacq. - type Habanero, *C. annuum* L. – var. Familiya, *C. frutescens* L., *C. annuum* L. – var. IZK Delicates), F_1 and F_2 of selected crosses

MATERIALS AND METHODS

1. Plant material

1. 1. Starting plant material - pepper seeds: varieties Plovdivska kapiya, Familiya and IZK Delicates of the species *Capsicum annuum* L.; *Capsicum chinense* Jacq. - type Habanero and *Capsicum frutescens* L.

1.2. Growing of plant material

1.2.1. Production of seedlings

1.2.2. Field cultivation of plant material

1.2.2.1. Agrometeorological characteristics

1.2.2.2. Field cultivation of the source plant material

1.2.2.3. Field cultivation of F_1 hybrids

1.2.2.4. Field cultivation of F_2

2. Experimental methods

2.1. Cross-pollination

2.1.1. Cross-pollination scheme

- ♀ *C. annuum* L. – var. Plovdivska kapiya x ♂ *C. chinense* Jacq. – type Habanero
- ♀ *C. annuum* L. – var. Plovdivska kapiya x ♂ *C. frutescens* L.
- ♀ *C. annuum* L. – var. Familiya x ♂ *C. chinense* Jacq. – - type Habanero
- ♀ *C. annuum* L. – var. Familiya x ♂ *C. frutescens* L.
- ♀ *C. annuum* L. – var. IZK Delicates x ♂ *C. chinense* Jacq. – type Habanero
- ♀ *C. annuum* L. – var. IZK Delicates x ♂ *C. frutescens* L.

2.1.2. Selective breeding

2.2. Seed production

2.2.1. F_1 seed production

2.2.2. Seed production for parental lines

2.2.3. F_2 seed production

2.3. Organoleptic analysis of fruits of pepper parent forms, F_1 and F_2 from selected crosses

3. Laboratory analyses

3.1. DNA extraction from the parental forms, F_1 and F_2 - OmegaBio-Tek's E.Z.N.A. Plant DNA Extraction Kit was used for DNA isolation.

3.2. Visualization of isolated genomic DNA - by electrophoretic separation of the products in 1% agarose gel stained with EtBr and photographed under UV light.

3.3. Determination of the allelic status of the *Pun1* gene

3.3.1 Primer sequences:

- pun1-1 fwd 1 – TCCTCATGCATCTCTTGCAG
- pun1-1 fwd2 – GCTCCACGGAAAAGACTCAT
- pun1-1 rev – CAAATGGCAGTTTCCCTTCTCTCATT
- pun12 fwd – TTCCCATATAGCCCACTTGC
- pun1-2 rev – GATGGAACTAAAGCTGTTGTCGTATG
- pun1-3 fwd – GTAGTTTTTCGGAAATGAAAAGTACT
- pun1-3 rev 1 – CACGCCTTGCCCAGCTTTGTAATCTT
- pun1-3 rev 2 – TCATGTCCATTCGGCCAAACAGTG

3.3.2. PCR reactions to distinguish *Pun1* alleles

3.3.2.1 The PCR reaction to distinguish the *pun1-1* allele

3.3.2.2. PCR reaction to distinguish the *pun1-2* allele

3.3.2.3. PCR reaction to distinguish the *pun1-3* allele

3.4. Amplification of a fragment of *Pun3*

3.4.1. Primers sequences of *Pun3*-CAPS marker:

- *Pun-3*-CAPS_F ATGGTGAGAACACCTTGCTAC
- *Pun-3*_R CTCCCTCTTCTTCAGGCCAATCTA

3.4.2. PCR reaction for amplification of a fragment of the Pun3 gene

3.5 ISSR analysis

3.5.1. Primers:

- ISSR P7 AC(8)YG
- ISSR P14 AG(8)YT
- ISSR P11 GA(8)YC
- ISSR P8 AC(8)G
- ISSR PE6 AC(8)CTG

3.5.2. ISSR PCR reactions

3.6. Analysis of PCR products

3.6.1. Preparation of electrophoresis buffer (TBE 0.5x)

3.6.2. Preparation of agarose gel
3.6.3. Electrophoretic separation of molecules
3.6.4. Visualization of products
3.7. Purification of DNA fragments (part of *Pun3*) from agarose gel - by ISOLATE II PCR and Gel Kit (BIOLINE)
3.8. Sequencing of amplified fragments - by Sanger method, by capillary electrophoresis of fluorescently labeled DNA fragments at Bio Basic Inc. laboratory in Markham, Ontario, Canada.

4. Methods of data processing

4.1. Statistical processing of the organoleptic analysis and PCR reaction data to determine the allelic status of *Pun1* in F_2 plants of the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero - by $\chi 2$ statistic.
4.2 Statistical processing of the ISSR analysis data - constructing hierarchical clusters with the statistical package "SPSS for Windows".
4.3. Bioinformatic analysis
4.3.1. *Pun3* coding region analysis based on the information obtained from NCBI (National Center for Biotechnology Information).
4.3.2. Comparison of obtained sequences with reference genomes - in Sol Genomics Network, using BLAST (basic local alignment search tool).

RESULTS AND DISCUSSION

1. Detecting the presence of *Pun1* gene alleles in the parental pepper forms.

The biosynthesis of capsaicin is complex and requires intermediates from two different metabolic pathways that finally combine, with the condensation reaction being controlled by an acyltransferase encoded by the *Pun1* gene. It is the main gene that qualitatively controls this process. Mutations in *Pun1* lead to loss of pungency in the fruit. Research on allelic composition in hot and sweet peppers has continued over a century. Advances in biotechnology now make it possible to analyze genes in detail and identify recessive alleles using molecular markers and direct sequencing. Mutations in the *Pun1* locus are the most common, preferred and used in sweet pepper breeding because of their qualitative inheritance.

To determine the allelic status of the *Pun1* gene (*pun1-1*, *pun1-2*, and/or *pun1-3*) in the parental pepper forms, a series of DNA analyses were performed. Allele-specific PCR primers known from the literature to detect the functional nucleotide polymorphisms of different mutant alleles of *Pun1* were used in the reactions. Genomic DNA from the three Bulgarian non-pungency pepper cultivars (*C. annuum* L. – var. Plovdivska kapiya, var. Familiya and var. IZK Delicates) and the two hot pepper cultivars (*C. chinense* Jacq. - type Habanero and *C. frutescens* L.) was analyzed.

The DNA extracted was of similar quality (no degraded fragments) and in approximately equal amounts (in most cases between 300 and 500 ng of genomic DNA). When the quantity or quality of DNA obtained was considered unsatisfactory, extraction was performed again to satisfy the specified parameters (Fig. 1).

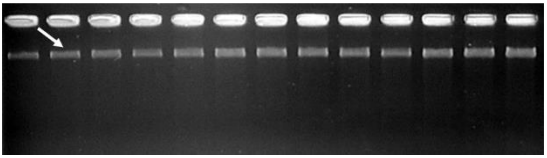


Figure 1. Results of genomic DNA extraction with OmegaBio-Tek's E.Z.N.A. Plant DNA Extraction Kit: Arrow - visualized products in samples used in the study.

1.1. Analysis for the presence of the *pun1-1* allele

The *pun1-1* allele is known to be responsible for the lack of pungency in *C. annuum* L. as a result of a 2.5 kb deletion encompassing the promoter and most of the first exon. A 3-primer PCR marker was used to distinguish reliably the *pun1-1* allele from other *Pun1* alleles. By positioning the first forward primer (*pun1-1*fwd1) in the promoter region and the reverse primer (*pun1-1*rev) in the second exon, amplification occurs only in the absence of this mutation. Since in this case the primers span a large portion of the gene, the product that is amplified is 1064 bp in size. The second forward primer (*pun1-1*fwd2) binds upstream of the mutated region. In combination with the reverse primer (*pun1-1*rev), a product is amplified only when the *pun1-1* mutation is present. The product is 746 bp in size. In order to amplify products only from the active gene, the reverse primer was designed in a region containing significant sequence differences from the *catf2* pseudogene (Fig. 2).

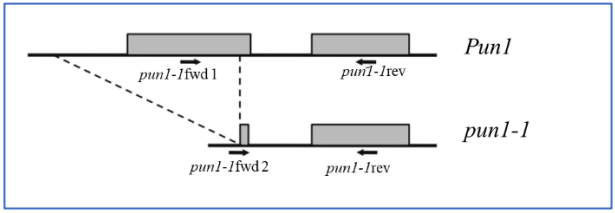


Figure 2. Allele-specific marker to distinguish the *pun1-1* allele binding regions of the *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev primers.

Genomic DNA from five plants each of the five initial accessions was subjected to PCR amplification to detect the presence of the *pun1-1* recessive allele. The results were visualized and photographed under UV light (Fig. 3 and Fig. 4). In sweet Bulgarian pepper cultivars of the species *C. annuum* L., 746 bp products were amplified. According to literature data, fragments of this size correspond to the presence of the recessive *pun1-1*. Each of the sweet pepper samples produced only one fragment indicating the absence of other known alleles. Therefore, it can be concluded that in the sweet peppers examined, the *pun1-1* allele is in a homozygous state (*pun1-1*/*pun1-1*). The lack of capsaicin synthesis and pungency, respectively, is due to the homozygous state of this mutation.

In *C. chinense* Jacq. - type Habanero and *C. frutescens* L., 1064 bp products were amplified. The results indicate the absence of *pun1-1*. In these samples, it is possible that both the *Pun1* dominant allele and another of its recessive alleles (*pun1-2* or *pun1-3*) are present.

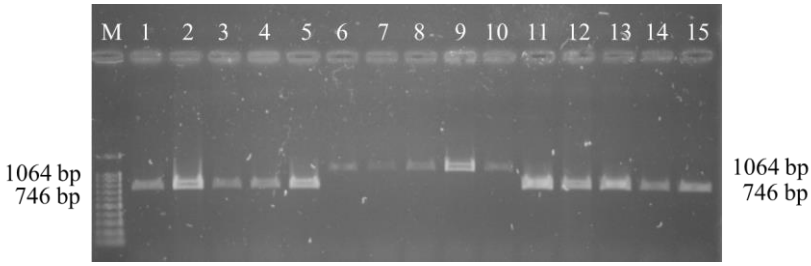


Figure 3. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev to detect the presence of the recessive *pun1-1* in the parental forms: M - 100 bp. molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.-No. 304225), start 1 - 5 – non-pungent pepper *C. annuum* L. – var. Plovdivska kapiya - product size 746 bp.; start 6 - 10 - pungent pepper *C. chinense* Jacq. - type Habanero - product size 1064 bp.; start 11 - 15 – non-pungent pepper *C. annuum* L. - variety Familiya - product size 746 bp.

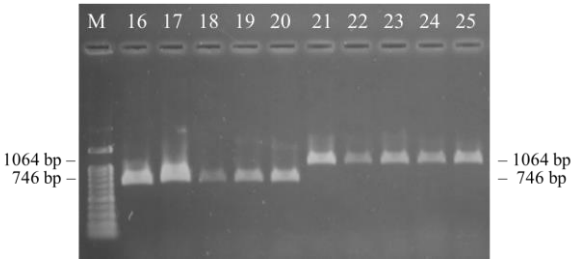


Figure 4. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev to detect the presence of the recessive *pun1-1* in the parental forms: M - 100 bp molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.-No. 304225), start 16 - 20 – non-pungent pepper *C. annuum* L. – var. IZK Delicates - product size 746 bp; start 21 - 25 - pungent pepper *C. frutescens* L. - product size 1064 bp.

1.2. Analysis for the presence of the pun1-2 allele

The marker used to detect the presence of the recessive *pun1-2* detected the 4 bp deletion characteristic of this allele. The two primers were designed to flank this deletion in exon 1. In the presence of the mutation, a fragment of 392 bp is amplified, and in its absence, a fragment 4 bp larger, 396 bp, is amplified. Products of 396 bp are amplified both in the presence of the *Pun1* dominant allele and in the presence of the *Pun1-3* recessive allele. In the presence of the recessive *pun1-1*, no product is amplified because its large deletion includes the amplified region (Fig. 5).

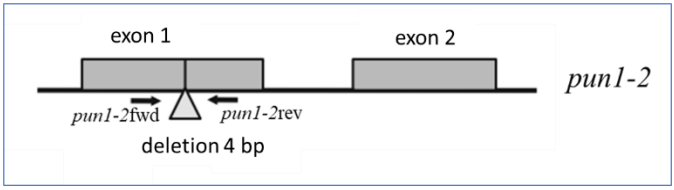


Figure 5. Allele-specific marker to distinguish the *pun1-2* allele-binding regions of the *pun1-2*fwd and *pun1-2*rev primers.

To detect the presence of the *pun1-2* mutant allele, the PCR reaction was performed with total samples of the five extracted DNAs and the five

primers. After the PCR reaction with the primers *pun1-2*fwd and *pun1-2*rev, products were amplified only in the pungent pepper *C. chinense* Jacq. - type Habanero and *C. frutescens* L. (Fig. 6).

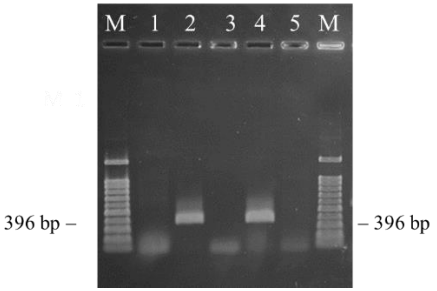


Figure 6. PCR reaction with primers: *pun1-2*fwd and *pun1-2*rev to detect the presence of the recessive *pun1-2* in the parental forms: M - 100 bp molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.-No. 304225), start 1 – non-pungent pepper *C. annuum* L. – var. Plovdivska kapiya - no product; start 2 - pungent pepper *C. chinense* Jacq. - type Habanero - product size 396 bp.; start 3 - non-pungent pepper *C. annuum* L. – var. Familiya - no product; start 4 - pungent pepper *C. frutescens* L. - product size 396 bp.; start 5 - non-pungent pepper *C. annuum* L. – var. IZK Delicates - no product.

It was difficult to determine the exact size of the fragments at start 2 and 4 (392 bp or 396 bp). The presence of at least one dominant *Pun1* allele is mandatory in pungent peppers. It is possible that the two products (392 bp and 396 bp in size) overlap, which does not preclude the presence of both *Pun1* and *pun1-2* alleles together. According to literature data (Wyatt et al, 2012), bands of size 396 bp are an indicator of the presence of the wild-type *Pun1* allele or the recessive *pun1-3* allele. In hot peppers, the *pun1-3* allele also could be present in a heterozygous state. At start 1, 3, and 5 in sweet Bulgarian pepper varieties of the species *C. annuum* L. no fragments were observed. These results further confirm that the absence of pungency in these pepper plants is due to the homozygous state of the *pun1-1* allele (*pun1-1*/*pun1-1*).

1.3. Analysis for the presence of the pun1-3 allele

When the *pun1-3* allele is present in *C. frutescens* L. a large deletion in the second exon results in the loss of 70 amino acids in the *Pun1* protein. The allele is neither transcribed nor translated. To detect the presence of *pun1-3*, a 3-primer PCR marker system was used.

Primer *pun1-3*fwd binds to the intronic region between exon 1 and exon 2. Primer *pun1-3*rev1 binds within exon 2 in the presence of the *Pun1* wild-type allele. Use of this primer pair results in amplification of a 586 bp product. Primer *pun1-3*rev2 binds to the genomic sequence near the *pun1-3* mutation site. In combination, primers *pun1-3*fwd and *pun1-3*rev2 produce a product of size1033 bp in the presence of the *pun1-3* recessive allele (Fig. 7).

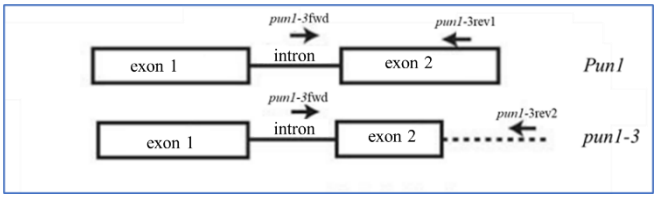


Figure 7. Allele-specific marker to distinguish the *pun1-3* allele - primer binding regions *pun1-3*fwd, *pun1-3rev1* and *pun1-3rev2*

In the reaction to detect the *pun1-3* recessive allele, bands of size 586 bp were amplified using the primers *pun1-3*fwd, *pun1-3*rev1, and *pun1-3*rev2 in all 25 samples. According to the literature, this indicates the presence of the *Pun1*, *pun1-1* or *pun1-2* alleles, and the absence of a band of size 1033 bp indicates the absence of the *pun1-3* allele (Fig. 8, Fig. 9).

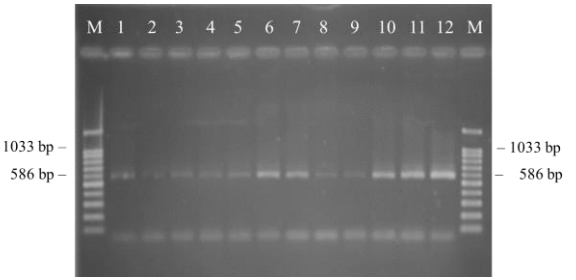


Figure 8. PCR reaction with primers: *pun1-3*fwd, and, *pun1-3*rev1 and *pun1-3*rev2: M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.-No. 304225); start 1-5 - pungent pepper *C. annuum* L. – var. Plovdivska kapiya; start 6 - 10 - pungent pepper *C. chinense* Jacq. - type Habanero; starts 11 and 12 non-pungent pepper *C. annuum* L. - var. Familiya.

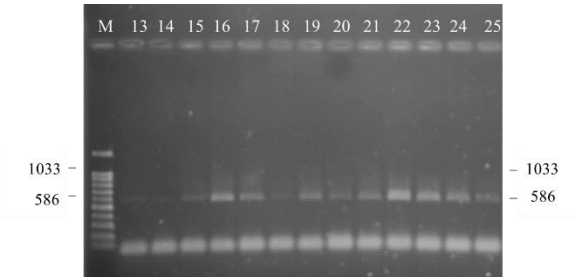


Figure 9. PCR reaction with primers: *pun1-3*fwd, *pun1-3*rev1 and *pun1-3*rev2: M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat. No. 304225); start 13 - 15 – non-pungent pepper *C. annuum* L. – var. Familiya; start 16 - 20 - pungent pepper *C. chinense* Jacq. - type Habanero; start 21 - 25 non-pungent pepper *C. annuum* L. – var. IZK Delicates.

Analyses conducted to determine the *Pun1* allelic status showed that in Bulgarian cultivars Plovdivska Kapiya, Familiya and IZK Delicates the loss of pungency is due to the presence of the most widespread mutant allele - *pun1-1*. In *C. chinense* Jacq. - type Habanero and *C. frutescens* L. it is

possible that the dominant *Pun1* allele is homozygous (*Pun1*/*Pun1*) or present in combination with the recessive *pun1-2* (*Pun1*/ *pun1-2*).

2. Directed crosses between selected parental forms.

2.1. Selection of parental forms

Among the cultivated peppers, special attention is paid to the most widespread and economically important species *C. annuum* L., which includes both pungent (hot pepper) and non-pungent (sweet pepper) forms. The cultivars selected and grown in Bulgaria belong to this species, most of them with sweet fruits. Detailed characterization of its phenotypic and genetic diversity is therefore of key importance. In this regard, three sweet pepper varieties (*C. annuum* L. - variety Plovdivska kapiya, variety Familiya and variety IZK Delicates) were selected as starting plant material. In order to follow the inheritance of genes and their mutant alleles responsible for the lack of pungency in F_1 and F_2, two pepper species with the alternative expression of the trait (hot *C. chinense* Jacq. - type Habanero and *C. frutescens* L.) were also selected as parental forms.

2.2. Accounting for hybridization

After 260 simple crosses, 102 fruits were obtained by controlled pollination. The hybridization efficiency of each cross was calculated (Table 1). Hybridization was most efficient in cross *C. annuum* L. - var. Plovdivska kapiya x *C. frutescens* L. - 57.5%, and the lowest percentage of efficiency in the cross *C. annuum* L. - var. Familiya x *C. chinense* Jacq. - type Habanero - 15%. This result shows that the hybridization method used is acceptably efficient. The number of developed fruits and the seeds in them were sufficient for the study. Seeds from all developed fruits were used to produce F_1 generation.

Table 1 - Results from the hybridization

Crosses	Number of crosses performed	Number of developed fruits	Efficiency in %
C. annuum L. – var. Plovdivska kapiya x *C. chinense Jacq.* – type Habanero	40	8	20
C. annuum L. – var. Plovdivska kapiya x *C. frutescens L.*	40	23	57,5
C. annuum L. – var. Familiya x *C. chinense Jacq.* – type Habanero	40	6	15
C. annuum L. – var. Familiya x *C. frutescens L.*	50	26	52
C. annuum L. – var. IZK Delicates x *C. chinense Jacq.* – type Habanero	40	11	27,5
C. annuum L. – var. IZK Delicates x *C. frutescens L.*	50	28	56

2.3. Phenotypic inheritance in F_1.

The fruit phenotype of the F_1 generation plants was followed to confirm successful hybridization. Qualitative traits such as fruit color and shape were easily determined visually and served as an estimate of the outcome of the crosses. Twenty-five F_1 plants from each of the six crosses were grown. The fruits of the plants from each of the crosses were of the same phenotype. This homogeneity in the expression of quality traits indicates the hybrid nature of the plants obtained (Fig. 10).

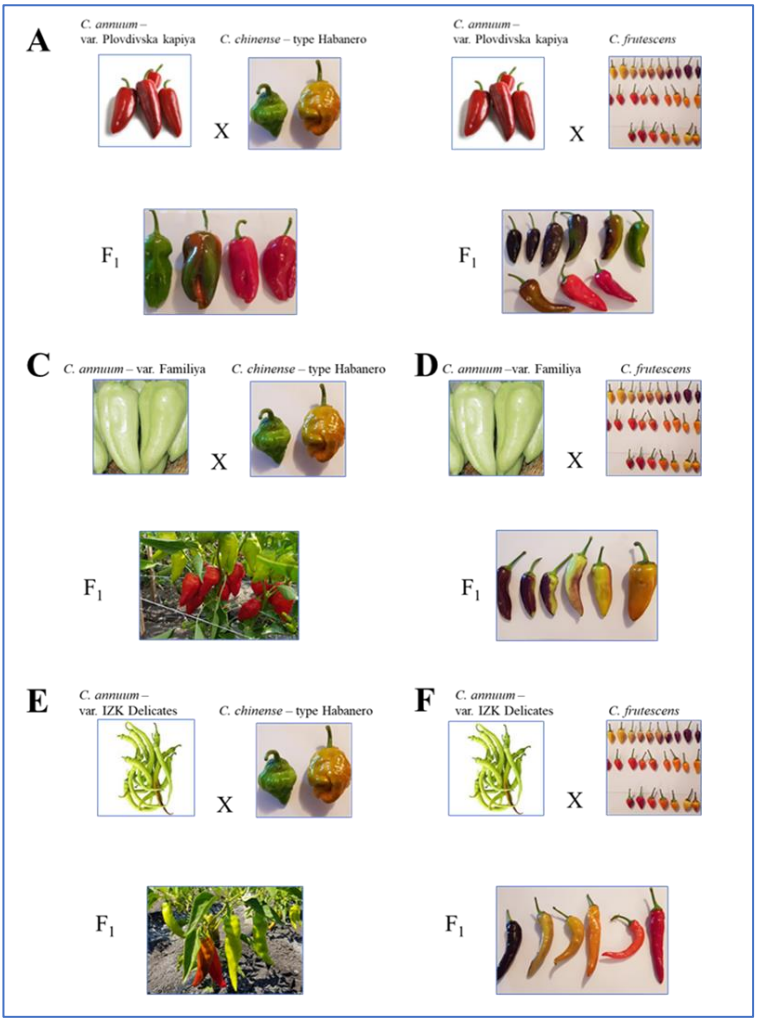


Figure 10. Fruit shape and color of F_1 plants from the crosses - **A.** *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero; **B.** *C. annuum* L. – var. Plovdivska kapiya x *C. frutescens* L.; **C.** *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero; **D.** *C. annuum* L. – var. Familiya x *C. frutescens* L.; **E.** *C. annuum* L. – var. IZK Delicates x *C. chinense* Jacq. - type Habanero; **F.** *C. annuum* L. – var. IZK Delicates x *C. frutescens* L.

2.4. Hybrid populations

After carrying out controlled self-pollination, the F_1 plants produced 69 fruits (Tab. 2).

Table 2. Results after F_1 self-pollination

F_1 crosses	**Number of developed fruits (self-pollinated)**
C. annuum L. - var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero	11
C. annuum L. - var. Plovdivska kapiya x *C. frutescens* L.	8
C. annuum L. - var. Familiya x *C. chinense* Jacq. - type Habanero	8
C. annuum L. - var. Familiya x *C. frutescens* L.	10
C. annuum L. - var. IZK Delicates x *C. chinense* Jacq. – type Habanero	12
C. annuum L. - var. IZK Delicates x *C. frutescens* L.	20

As a result, a sufficient quantity of seed has been produced to be able to grow plants of all crosses. However, for the analysis of genes responsible for capsaicin biosynthesis in F_2, the hybrid populations *C. annuum* L. x *C. chinense* Jacq. - type Habanero, *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero and *C. annuum* L. – var. IZK Delicates x *C. frutescens* L.

3. Tracing the inheritance in F_1 and F_2.

3.1. Determination of the allelic composition of the *Pun1* gene in F_1

Since the genotype of the parental forms (*pun1-1*/*pun1-1* and *Pun1*/*Pun1*) was determined and the presence of *pun1-2* and *pun1-3* was not detected, the PCR reactions were performed with allele-specific primers to

detect the presence of *pun1-1*. Genomic DNA from five plants from each of the six crosses was used in these reactions. The results were visualized under UV light. Both 746 bp and 1064 bp products were amplified (Fig. 11 and Fig. 12).

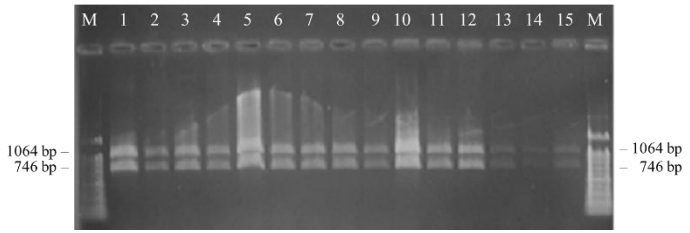


Figure 11. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev to determine the allelic state of *Pun1* at F_1 of the crosses: M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.-No. 304225); start 1 - 5 *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero; start 6 -10 - *C. annuum* L. – var. Plovdivska kappiya x *C. frutescens* L.; start 11-15 - *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero.

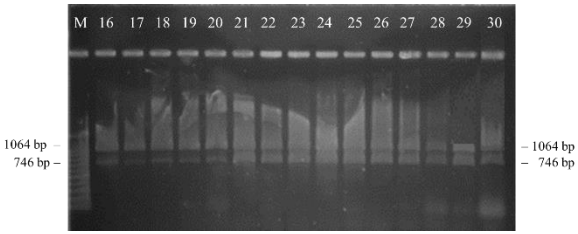
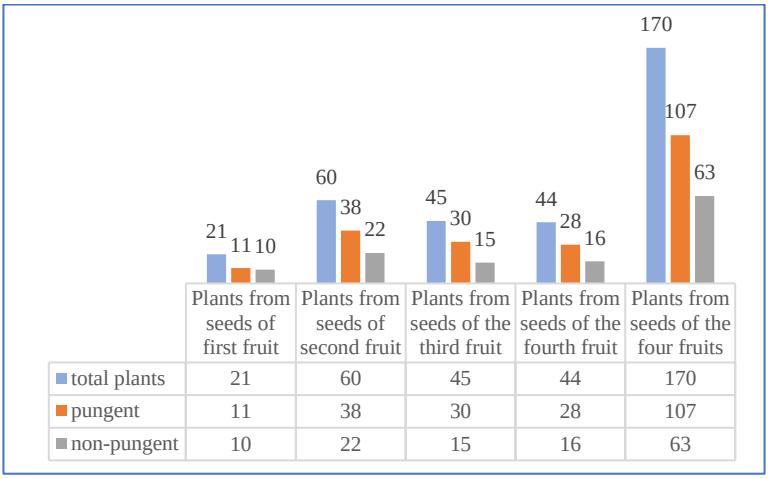


Figure 12. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev to determine the allelic state of *Pun1* at F_1 of the crosses: M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.-No. 304225); start 16 - 20 - *C. annuum* L. – var. Familiya x *C. frutescens* L.; start 20 - 25 - *C. annuum* L. - variety IZK Delicates x *C. chinense* Jacq. - type Habanero; start 26 - 30 - *C. annuum* L. - variety IZK Delicates x *C. frutescens* L.

In all lanes, products of size 746 bp and 1064 bp were amplified. According to literature data, a fragment of size 746 bp indicates the presence of the *pun1-1* allele and a fragment of size 1064 bp the presence of the *Pun1* allele. These results indicate that the crosses were carried out successfully and the hybrid plants are heterozygous with the *Pun1*/*pun1-1* genotype.

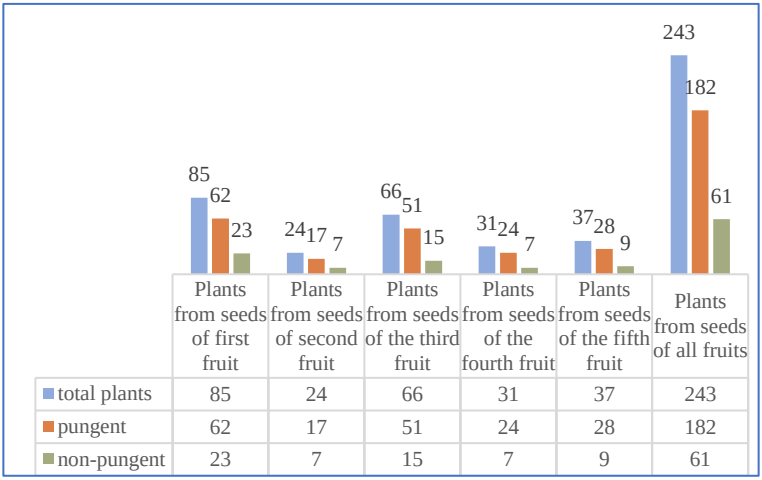
3.2. Organoleptic analysis.

The presence and absence of capsaicin in the fruits of the parental forms, F_1 and F_2 of the crosses was analyzed organoleptically. In the parental forms, the presence of capsaicin was detected in the fruits of all plants and *C. chinense* Jacq. - type Habanero and *C. frutescens* L. (scutellina was perceived) and the absence of *C. annuum* L. in the fruits of all plants – var. Plovdivska kapiya, var. Familiya and var. IZK Delicates (no pungency was perceived). All fruits of F_1 plants (from crosses *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero; *C. annuum* L. – var. Plovdivska kapiya x *C. frutescens* L.; *C. annuum* L. - variety Familiya x *C. chinense* Jacq. - type Habanero; *C. annuum* L. – var. Familiya x *C. frutescens* L.; *C. annuum* L. – var. IZK Delicates x *C. chinense* Jacq. - type Habanero and *C. annuum* L. – var. IZK Delicates x *C. frutescens* L.) were found to contain capsaicin (pungency is perceived). In F_2 plants of the three crosses (*C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero, *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero and *C. annuum* L. – var. IZK Delicates x *C. frutescens* L.) segregation in the expression of the fruit pungency trait was observed. The data from the organoleptic analysis performed were used to compare the results obtained from the experiment (Fig. 13, Fig. 14 and Fig. 15) and the predictions of the chosen hypothesis.



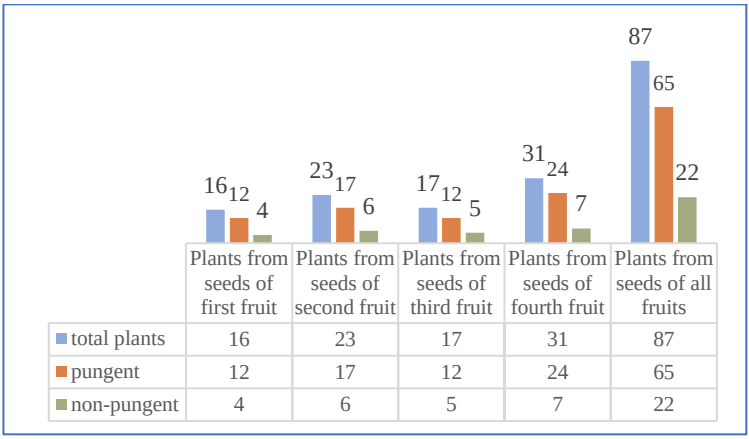
	Plants from seeds of first fruit	Plants from seeds of second fruit	Plants from seeds of the third fruit	Plants from seeds of the fourth fruit	Plants from seeds of the four fruits
total plants	21	60	45	44	170
pungent	11	38	30	28	107
non-pungent	10	22	15	16	63

Figure 13. Results of organoleptic analysis of F_2 plants of the cross C. *annuum* L. - variety Plovdivska kapiya x *C. chinense* Jacq. - type Habanero: Number of plants with non-pungent and pungent fruits.



	Plants from seeds of first fruit	Plants from seeds of second fruit	Plants from seeds of the third fruit	Plants from seeds of the fourth fruit	Plants from seeds of the fifth fruit	Plants from seeds of all fruits
total plants	85	24	66	31	37	243
pungent	62	17	51	24	28	182
non-pungent	23	7	15	7	9	61

Figure 14. Results of organoleptic analysis of F_2 plants of the cross *C. annuum* L. - cultivar Familiya x *C. chinense* Jacq. - type Habanero: Number of plants with non-pungent and pungent fruits.



	Plants from seeds of first fruit	Plants from seeds of second fruit	Plants from seeds of third fruit	Plants from seeds of fourth fruit	Plants from seeds of all fruits
total plants	16	23	17	31	87
pungent	12	17	12	24	65
non-pungent	4	6	5	7	22

Figure 15. Results of organoleptic analysis of F_2 plants of the cross *C. annuum* L. - variety IZK Delicacy x *C. frutescens* L.: Number of plants with sweet and spicy fruits.

3. 3. Statistical processing of organoleptic analysis data

The hypothesis assumed in the present study is that capsaicin biosynthesis in the selected species and cultivars is controlled by a single *Pun1* gene. The genotype of the parental forms (pungent pepper homozygous dominant *Pun1*/*Pun1* and non-pungent pepper homozygous recessive *pun1-1*/*pun1-1*) was established. By organoleptic analysis, the fruits of all F_1 plants were determined to be pungent. Their genotype was also determined to be *Pun1*/*pun1-1*. In F_2, ¾ of the plants were expected to have pungent fruits and ¼ non-pungent fruits.

After comparing the data obtained in the experiment with the expected results, variations within a very wide range were observed. The $\chi 2$ statistic was used to assess the variance. The data obtained deviates significantly from the theoretical data, if the $\chi 2$ exceeds the specified critical value at the 5%

confidence level of the assumption. The determination of this critical value was based on the frequency probability distribution. Critical values for different numbers of classes are calculated depending on the degrees of freedom of the equation.

The degree of freedom of the equation is equal to (n-1), where *n* is the number of phenotypic classes considered (in this case there are two - plants with pungent and with sweet fruits). The χ^2 value was compared at the degree of freedom: n-1 = 2-1=1.

3.3.1 Statistical processing of organoleptic analysis data of fruits from F_2 plants *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero

In the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero, a segregation in the expression of the fruit pungency trait was observed. The data from the organoleptic analysis performed were used to compare the results obtained from the experiment and the predictions of the chosen hypothesis (fruit pungency is controlled by a single *Pun1* gene). Under this hypothesis it was expected that ¾ of the plants would have pungent fruit and ¼ would have sweet fruit. The data obtained showed that out of 243 plants analyzed, 183 plants had pungent fruits and 60 plants had sweet fruits. In order to compare the data obtained in the experiment to the theoretical expectations and to assess the bias, the χ^2 statistic was also calculated here (Table 3). The calculated value of χ^2 = 0.021, with a degree of freedom =1 is well below the critical value of 3.841.

Table 3. Value of the χ^2 under the hypothesis "segregation of the fruit pungency trait expression in a 3:1 ratio" in F_2 of the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero

parameters	**pungent**	**non-pungent**	$\sum$
p (observed)	183	60	243
q (expected)	182	61	243
$\frac{(p-q)^2}{q}$	0,005	0,016	χ^2 = 0,021

We can assume that the experimental data in this cross are consistent with the predictions of the chosen hypothesis. The segregation of the fruit pungency trait is in the expected 3:1 ratio.

3.3.2. Statistical processing of organoleptic analysis data of fruits from F_2 plants *C. annuum* L. – var. IZK Delicates x *C. frutescens* L.

The data obtained in F_2 plants of the cross *C. annuum* L. – var. IZK Delicates x *C. frutescens* L. showed that of the 87 plants analysed, 65 had spicy fruits and 22 had sweet fruits. Here, the reported results are in full agreement with the expected ones and the value of $\chi^2 = 0$ (Table 4).

Table 4. Value of the χ^2 index under the hypothesis "segregation of the fruit pungency trait expression in a 3:1 ratio" in F_2 of the cross *C. annuum* L. – var. IZK Delicates x *C. frutescens* L.

parameters	**pungent**	**non-pungent**	$\sum$
p (observed)	65	22	87
q (expected)	65	22	87
$\frac{(p-q)^2}{q}$	0	0	$\chi^2 = 0$

The segregation of fruit pungency trait expression was in the expected 3:1 ratio and the experimental data in this cross were also consistent with the predictions of the chosen hypothesis.

3.3.3. Statistical processing of organoleptic analysis data of fruits from F_2 plants *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq.- type Habanero

The calculations in F_2 of the cross *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero showed that the calculated value of χ^2 = 13,945 (Table 7). At degree of freedom = 1, this value significantly exceeds the critical value of 3.841, indicating either a problem with the experimental design/execution or the need for rejection of the hypothesis.

Table 5: Value of the χ^2 under the hypothesis "segregation of the fruit pungency trait expression in a 3:1 ratio" in F_2 of the cross *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero

parameters	**pungent**	**non-pungent**	$\sum$
p (observed)	107	63	170
q (expected)	128	42	170
$\frac{(p-q)^2}{q}$	3,445	10,5	χ^2 = 13,945

The data obtained from the organoleptic analysis of F_2 plants of the cross *C. annuum* L. - cultivar Plovdivska kapiya x *C. chinense* Jacq.- type Habanero showed that segregation of the fruit pungency trait was not in the expected ratio of 3:1.

Since the experimental data in this cross were not consistent with the predictions of the chosen hypothesis, another hypothesis had to be considered. The absence of capsaicinoids and lutein, respectively, in fruits is associated with the presence of mutations in the *Pun2*, *pAMT*, *CaKR*, and

Pun3 genes (Arce-Rodríguez and Ochoa-Alejo, 2019). Since these genes encode products functioning upstream of the capsaicin synthesis step (encoded by *Pun1*), the disrupted function of any of them would have an epistatic effect on *Pun1*. Given that *Pun3* encodes the transcription factor *CaMYB31*, which regulates the expression of *Pun1* and major capsaicin biosynthetic genes, it was assumed that evidence for its involvement in the F_2 population under consideration should be sought. *Pun3* is known to control pungency in non-pungent pepper species *C. annuum* L., through a recessive non-functional allele in the homozygous state (Han et al., 2019). Therefore, the next assumption made was that*Pun3* blocks through recessive epistasis *Pun1* expression in plants with non-pungent fruits from this population. Assuming that the pungent parent *C. chinensis* - type Habanero has genotype $P_1P_1P_3P_3$ and the sweet parent *C. annuum* L. - var. Plovdivska kapiya has either $p_1p_1p_3p_3$ or $p_1p_1P_3p_3$ genotype, then in F_1 only plants with spicy fruits should be obtained, with genotype $P_1p_1P_3p_3$ or $P_1p_1P_3P_3$. If the selfed flowers developed on a plant heterozygous for both genes (with the $P_1p_1P_3p_3$ genotype), the 9:7 ratio of plants with pungent to those with sweet fruits in F_2 is expected (Fig. 16).

Gametes	**P_1P_3**	**P_1p_3**	**p_1P_3**	**p_1p_3**
P_1P_3	$P_1P_1P_3P_3$ pungent	$P_1P_1P_3p_3$ pungent	$P_1p_1P_3P_3$ pungent	$P_1p_1P_3p_3$ pungent
P_1p_3	$P1P_1P_3p_3$ pungent	$P_1P_1p_3p_3$ non-pungent	$P_1p_1P_3p_3$ pungent	$P_1p_1p_3p_3$ non-pungent
p_1P_3	$P_1p_1P_3P_3$ pungent	$P_1p_1P_3p_3$ pungent	$p_1p_1P_3P_3$ non-pungent	$p_1p_1P_3p_3$ non-pungent
p_1p_3	$P_1p_1P_3p_3$ pungent	$P_1p_1p_3p_3$ non-pungent	$p_1p_1P_3p_3$ non-pungent	$p_1p_1p_3p_3$ non-pungent

Figure 16. Punnett square for self-pollination of a plant with genotype $P_1p_1P_3p_3$ results in the formation of 16 possible genotype combinations and an expected phenotypic segregation ratio of 9:7.

The results of the organoleptic analysis and the predictions of the chosen hypothesis (*Pun3* blocks by recessive epistasis the expression of *Pun1* and the segregation of pungency trait expression in a 9:7 ratio) were

compared using the χ^2 statistic. It was expected that 9/16 of the plants would have pungent fruit and 7/16 would have non-pungent fruit. The data obtained showed that out of 177 plants analyzed, 107 had pungent fruits and 63 had non-pungent fruits, while 96 pungent and 74 non-pungent fruits were expected. The calculated value of χ^2 = 2.895, with degree of freedom = 1 is below the critical value of 3.841 (Table 6).

parameters	**pungent**	**non-pungent**	∑
p (observed)	107	63	170
q (expected)	96	74	170
$\frac{(p-q)^2}{q}$	1,260	1,635	χ^2 = 2,895

Table 6: Value of χ^2 under the hypothesis " segregation of pungency trait expression in a 9:7 ratio" in F_2 of the cross *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero

According to the results obtained, we can assume that the experimental data in this cross are consistent with the predictions of the 9:7 segregation hypothesis. Based on the analysis carried out so far, one can see that the result of the cross conforms to the assumption that two genes are involved simultaneously in determining presence/absence of pungency in fruit.

3.4. Determination of the *Pun1* gene allelic composition at F_2

Given that *Pun1* is a major gene controlling pungency in pepper fruit, to clarify its role in the hybrid populations studied, its allelic status was determined in the F_2 of the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero and in F_2 plants of the cross *C. annuum* L. – var.

Plovdivskaya kapiya x *C. chinense* Jacq. - type Habanero, the fruits of which were organoleptically determined to be sweet.

3.4.1. Determination of the allelic composition of the *Pun1* gene at F_2 of the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero

Although the χ^2 criterion is an objective measure of the correctness of the chosen hypothesis, an analysis at the molecular level was also performed. The genotype of 175 F_2 plants of the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero was determined.

By a series of PCR reactions, the allelic composition of the *Pun1* gene was determined in each plant. PCR reactions were performed with the primers *pun1-1*fwd 1, *pun1-1*fwd 2, and *pun1-1*rev. A 746 bp amplified product in plants with sweet fruits indicated the homozygous state of the *pun1-1* allele (*pun1-1*/*pun1-1*). A fragment of 1064 bp corresponds to the homozygous state of the *Pun1* (*Pun1*/*Pun1*) wild-type allele. The presence of both fragments (746 bp and 1064 bp in size) indicates the presence of both alleles of the gene, i.e., *Pun1*/*pun1-1* genotype. Plants with pungent fruits showed *Pun1*/*Pun1* or *Pun1*/*pun1-1* genotype, and plants with sweet fruits showed *pun1-1*/*pun1-1* genotype. The results of the PCR reactions (Fig. 17) were in complete agreement with the results of the organoleptic analysis.

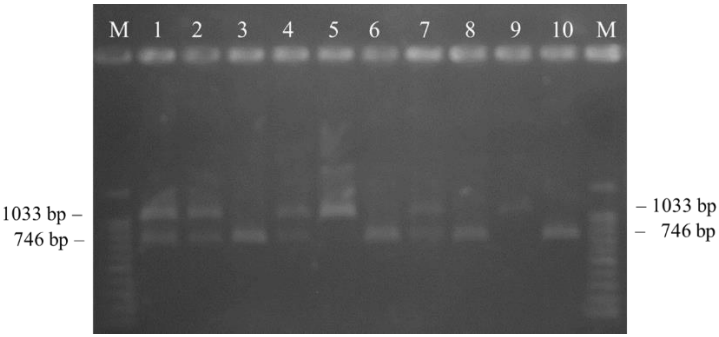


Figure 17. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2, and *pun1-1*rev to determine the *Pun1* allelic state in (1-10) F_2 plants of the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero: M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat. No. 304225); 1 - products of 1064 bp and 746 bp - *Pun1*/*pun1-1*; 2 - products of 1064 bp and 746 bp - *Pun1*/*pun1-1*; 3 - product of 746 bp - *pun1-1*/*pun1-1*; 4 - products of 1064 bp and 746 bp - *Pun1*/*pun1-1*; 5 - product 1064 bp - *Pun1*/*Pun1*; 6 – 746 bp - *pun1-1*/*pun1-1*; 7 - products 1064 bp and 746 bp - *Pun1*/*pun1-1*; 8 - product 746 bp - *pun1-1*/*pun1-1*; 9 - product 1064 bp - *Pun1*/*Pun1*; 10 - product 746 bp - *pun1-1*/*pun1-1*

Pun1 genotyped F_2 plants (*C. annuum* L. var. Familiya x *C. chinense* Jacq. - type Habanero) were divided into three groups: *Pun1*/*Pun1* group, *Pun1*/*pun1-1* group, and *Pun1-1*/*pun1-1* group (Table 7).

According to the chosen hypothesis (pungency/lack of pungency in fruits is controlled by a single *Pun1* gene), in F_2, ¼ of the plants are expected to be of *Pun1*/*Pun1* genotype, $^2/_4$ of the plants to be of *Pun1*/*pun1-1* genotype, and ¼ to be of *pun1-1*/*pun1-1* genotype.

Table 7. Genotyping results of F_2 plants of the cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero - numbers of plants with genotype *Pun1*/*Pun1*, *Pun1*/*pun1-1* and *pun1-1*/*pun1-1*

	Plants with genotype *Pun1/Pun1*	**Plants with genotype *Pun1/pun1-1***	**Plants with genotype *pun1-1/pun1-1***
Number of plant	5, 9, 15, 17, 28, 32, 38, 56, 57, 61, 62, 64, 66, 73, 79, 81, 83, 84 88, 89, 91, 93, 98, 104, 105, 110, 111, 114, 115, 116, 123, 1124, 132, 134, 138, 143, 150, 152, 155, 161, 167, 170	1, 2, 4, 7, 11, 12, 13, 14, 16, 21, 23, 24, 25, 26, 29, 30, 31, 33, 34, 35, 39, 40, 42, 47, 48, 49, 51, 52, 53, 54, 55, 58, 63, 65, 67, 69, 71 72, 75, 76, 77, 78, 80, 85, 86, 87, 90, 94, 97, 99, 100, 101, 103, 107 112, 117,119, 121, 122, 125, 126, 127, 129, 130, 131, 135, 136, 137, 140, 141, 142, 144, 145, 146, 147, 149, 151, 153, 154, 157, 159, 160, 162, 169, 171, 172, 173, 174	3, 6, 8, 10, 18, 19, 20, 22, 27, 36, 37, 41, 43, 44, 45, 46, 50, 59, 60, 68, 70, 74, 82 92, 95, 96, 102, 106, 108, 109, 113, 118, 120, 128, 133, 139, 148, 156, 158, 163, 164, 165, 166, 168,175

Legend: - plants from seeds of first fruit; - plants from seeds of second fruit; - plants from seeds of third fruit

The $\chi 2$ statistic was used to compare the data obtained in the PCR reactions with the expected results and to evaluate the bias. The value of the $\chi 2 = 0.123$, with a degree of freedom of - 2, is well below the critical value = 5.991 (Table 8).

Table 8: Value of χ^2 under the hypothesis "genotype segregation *Pun1*/*Pun1* to *Pun1*/*pun1-1* to *pun1-1*/*pun1-1* in a 1:2:1 ratio" in F_2 of the cross *C. annuum* L. – var. Familiya x *C. chinense* - type Habanero

parameters	***Pun1/Pun1***	***Pun1/pun1-1***	***pun1-1/pun1-1***	∑
p (observed)	42	88	45	175
q (expected)	44	87	44	175
$\frac{(p-q)^2}{q}$	0,09	0,011	0,022	χ^2 = 0,123

We can assume that the results from PCR reactions in the F_2 plants of cross *C. annuum* L. – var. Familiya x *C. chinense* Jacq. – type Habanero are consistent with the predictions of the chosen hypothesis. Based on the analyses carried out, it can be concluded that the absence of capsaicin and, respectively, of pungency in the fruits of the Bulgarian pepper var. Familiya is due to the transition to the homozygous state of the recessive *pun1-1*. The pungent parent *C. chinense* Jacq. - Habanero has the *Pun1*/ *Pun1* genotype.

3.4.3. Determination of *Pun1* gene allele composition in F_2 plants of the cross *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero

To analyze the effect of the recessive *pun1-1*, on the loss of pungency in F_2 peppers of the cross *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero, with organoleptically determined sweet fruits, they were genotyped by PCR reaction using a three-primer system (*pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev) for allele-specific marker. The allelic composition of the *Pun1* gene was determined on 68 F_2 plants (63 organoleptically determined as non-pungent and 5 as pungent for the control) (Fig. 18 and Fig. 19).

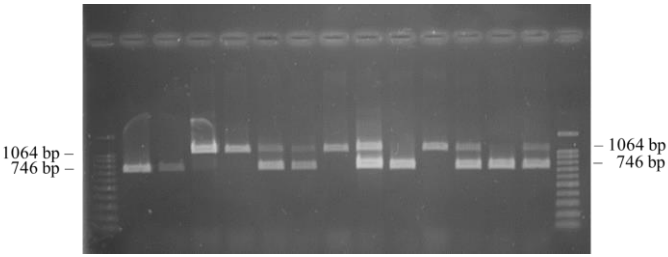


Figure 18. PCR reaction for determination of *Pun1* allelic status of F_2 plants (*C. annuum* – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero), with organoleptically determined non-pungent fruits: M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat. - No. 304225); Start 1, 2, 9 and 12 - product of size 746 bp, genotype *pun1-1*/*pun1-1*; Start 3, 5, 6, 8, 11, 13 - products of size 1064 bp and 746 bp, genotype *Pun1*/*pun1-1*; Start 4, 7 and 10 - products of size 1064 bp, genotype *Pun1*/ *Pun1*.

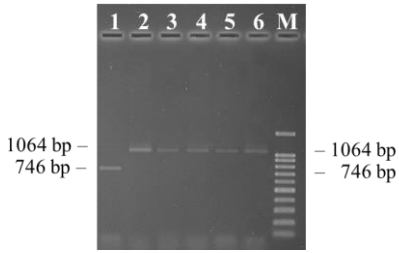


Figure 19. PCR reaction to determine the *Pun1* allelic status of F_2 plants (*C. annuum* L. - var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero) - Start 1: plant No. 169, with organoleptically determined non-pungent fruits, fragment size 746 bp, genotype *pun1-1*/*pun1-1*; Start 2-6 plants No. 1, 2, 3, 162 and 166 with organoleptically determined pungent fruits, fragment size 1064 bp, genotype *Pun1*/*Pun1*; M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.- № 304225).

Genetic analysis of the F_2 peppers identified as non-pungent from the studied cross showed that in addition to the expected *pun1-1*/*pun1-1* condition, plants with heterozygous (*Pun1*/*pun1-1*) and homozygous dominant (*Pun1*/*Pun1*) condition of the *Pun1* gene were also observed. These

results show once again that *pun1-1* is not the only factor determining the lack of pungency in them.

The analysis showed that the genotype of the plants used as control with organoleptically determined hot fruits was *Pun1*/*Pun1*. The result of this PCR reaction agrees with the results of the analyses performed so far.

4. Investigation for the presence of additional loci with effect on capsaicin synthesis.

Organoleptic analysis data for the segregation ratio between plants with pungent and non-pungent fruit in the F_2 cross *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero, were not consistent with the predictions of the chosen hypothesis (capsaicin biosynthesis is controlled solely by the *Pun1* gene). This necessitated analyzing for the presence of an additional genetic locus that may result in a loss of capsaicinoid production. A genetic analysis was performed to determine the relationship between the loss of pungency in the fruit of plants of this hybrid population and the involvement of another locus in the control of this trait.

To determine whether *Pun3* is the second gene that controls the lack of pungency in the hybrid population, mutations at this locus were screened for.

4.1. Amplification and isolation of a fragment of the *Pun3* gene

The 68 F_2 plants genotyped for *Pun1* (63 with organoleptically determined sweet fruits and 5 with pungent fruits as control) were subjected to a PCR reaction to amplify and isolate a fragment of the *Pun3* gene.

A *Pun3*-CAPS marker developed by Han et al. (2019) was used to amplify a portion of the *Pun3* gene. In this segment (that includes exons 1 and 2, the intron between them, and part of the intron between exon 2 and exon 3), the only known mutation in this locus that affects capsaicinoid production was identified and reported to date (Fig. 20).

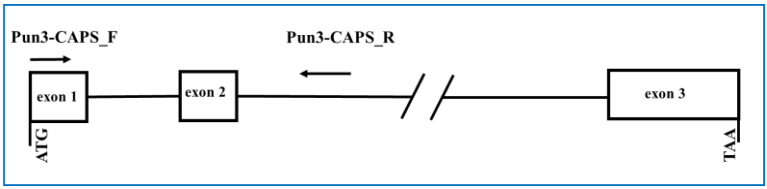


Figure 20. Allele-specific marker for *Pun3* allele discrimination - *Pun3*-CAPS_F and *Pun3*-CAPS_R primer binding regions - The *Pun3* cDNA sequence contains three exons of 133, 130 and 484 bp and two introns.

After the PCR reaction, fragments as large as 873 bp were amplified in all 63 samples from F_2 plants (Fig. 21). The fragments from agarose gel were purified and sequenced.

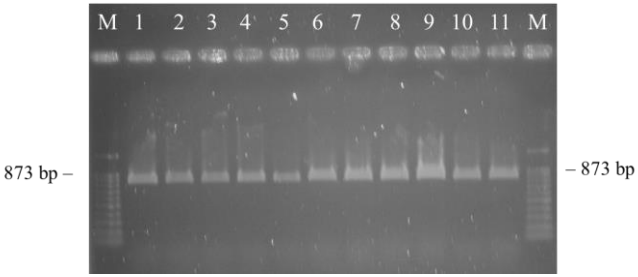


Figure 21. PCR reaction with primers: *Pun-3*-CAPS_F and *Pun-3*-CAPS_R : M - DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat.-No. 304225); Start 1-11 – F_2 (*C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. – type Habanero).

4.2. Sequencing of fragments (part of *Pun3*)

Fragments (part of *Pun3*) from plants in which the dominant *Pun1* allele was found to be present but organoleptically determined to have non-pungent fruits were sequenced. The largest fragment sequenced from such a sample was 851 bp (Table 9). To serve as controls, fragments from samples of plants with *Pun1*/*Pun1* and *Pun1*/*pun1-1* genotypes determined to be pungent and those with the *Pun1-1*/*pun1-1* allele detected and determined

organoleptically to be non-pungent were also sequenced. The size of the sequenced fragments of plants genotyped as *Pun1*/*Pun1* and *Pun1*/*pun1-1* identified as hot were 846 bp, the difference between these and the 851-bp fragment being that the last 5 bp were missing. The rest of the sequences are completely identical. In samples from plants identified as sweet, with the *pun1-1*/*pun1-1* genotype, the sequenced fragments were 840 bp in size. Here, in addition to the last 5 bp, 6 bp of the beginning of the 851-bp sequence are missing. The rest of the sequences are again completely identical. Since all samples amplified fragments of the same size after the PCR reaction, the sequence differences could be attributed to errors in fragment purification or in the sequencing itself.

Table 9. Nucleotide sequence of a fragment (part of *Pun3*) from a plant in which the *Pun1* dominant allele was found to be present but organoleptically determined to be non-pungent - 851 bp.

AGGGGAAGAAGAAGAAGGGGACATGGGACTCCTGAAGAAGATAGGAAGTTAA
CAGCATATATTGCAAAATATGGCTCATGGAACTGGCGCCAACTTCCCAAGTATG
CTGGTGAGAATTTCTGTAACTAATTAAGGTTTTAAATTGGTTTGCTGTTAATATT
CATTCATAAAATAATAATTACTCCTATTTTATGTTTGTATTTCACTCTAGTTCAT
GAGATTACGAAAATATGAAATTTAGATATTTAAATCCAGTAAGCTTAAATCAG
ATATGTGAGTAATGTATTAAAATATATGTTTGGTGAATATCTTGTTGTTTTAAAT
ATTTCATGTGAGATGTCGAATTTAAAGAATTAATAAATATAGGAAATGACATTC
TTCTAACAGACTAAAAAAATTAGACACATATATTGAAACGAAAGTACTTCTAT
ATTAGTATCATATTCCATTATTGGAGAGTTCCAATGAAATAATTAACGGAATGT
TGTTTTTTGGTGAATTAGGACTAGCAAGGTGTGGAAAGAGCTGCAGACTTCGAT
GGATGAATCACTTACGGCCAAATGTTAAAAGAGGGAATTATACCAAAGAAGAA
GATGAAATCATCTTGAACCTCCATGCTCAACTTGGAAATAGGTACGTACTCTTC
TTCCACATGTCCGATTTACCTGCTAATAGCACTGGTCAATGCTAGTTGAATTGA
GGTGTATTCAAAATTTGAATTTAATGAATTTAACTGTTAATATTTTTAAGATTGA
ACTCATTGAATCTAATATTTGCTAGGAGTCTAGAAAAATTAGTTCATATGAAAT
CATAGACAAAACGTTAGATTGGCCTCTGAAGAAGAGGGAGAA

Chromatograms from sequencing show no baseline noise, and the peaks are sufficiently distinct (Fig. 22).

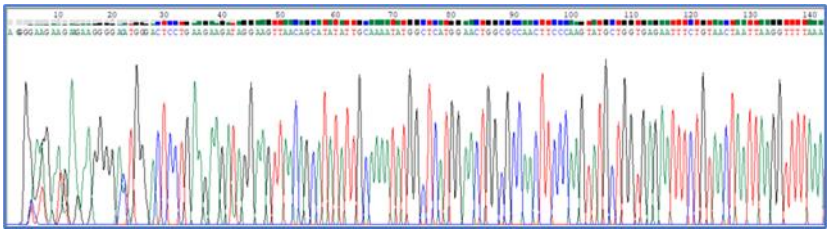
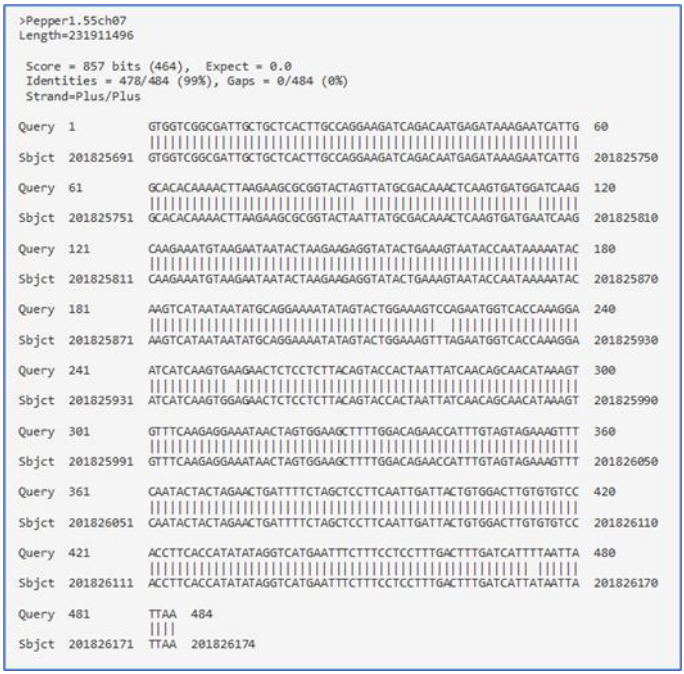


Figure 22. Chromatogram of sequenced fragment (851 bp) - part of the fragment (142 nucleotides)

4.3. Location and nucleotide sequence of *Pun3*

A check at NCBI (National Center for Biotechnology Information) showed that information is available only for the coding regions of *Pun3* (*Capsicum annuum* L. R2R3-MYB transcription factor 31 (MYB31), mRNA, GenBank: MF062086.1). The available sequence was divided into three parts according to the size of the exons known from the literature (133 bp, 130 bp and 484 bp). Using BLAST in SolGenomics (https://solgenomics.net/), the exact location of *Pun3* (exons and introns in between) on chromosome 7 was found in the three reference genomes (Zunla-version 2.0, UCD10X-version 1.0, and CM334-version 1.55). The sequences (exon 1 and exon 2) of the coding region matched 100% in all three reference genomes. BLAST of the exon 3 sequence showed 99% sequence identity to chromosome 7 of the reference genomes. There were 6 nucleotide substitutions, at the same positions, between the exon 3 sequence and the three reference genomes (Fig. 23).



```
>Pepper1.55ch07
Length=231911496

 Score = 857 bits (464),  Expect = 0.0
 Identities = 478/484 (99%), Gaps = 0/484 (0%)
 Strand=Plus/Plus

Query  1          GTGGTCGGCGATTGCTGCTCACTTGCCAGGAAGATCAGACAATGAGATAAAGAATCATTG  60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201825691  GTGGTCGGCGATTGCTGCTCACTTGCCAGGAAGATCAGACAATGAGATAAAGAATCATTG  201825750

Query  61         GCACACAAAACTTAAGAAGCGCGGTACTAGTTATGCGACAAACTCAAGTGATGGATCAAG  120
                  |||||||||||||||||||||||||||| |||||||||||||||||||||| ||||||
Sbjct  201825751  GCACACAAAACTTAAGAAGCGCGGTACTAATTATGCGACAAACTCAAGTGATGAATCAAG  201825810

Query  121        CAAGAAATGTAAGAATAATACTAAGAAGAGGTATACTGAAAGTAATACCAATAAAAATAC  180
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201825811  CAAGAAATGTAAGAATAATACTAAGAAGAGGTATACTGAAAGTAATACCAATAAAAATAC  201825870

Query  181        AAGTCATAATAATATGCAGGAAAATATAGTACTGGAAAGTCCAGAATGGTCACCAAAGGA  240
                  |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  201825871  AAGTCATAATAATATGCAGGAAAATATAGTACTGGAAAGTTTAGAATGGTCACCAAAGGA  201825930

Query  241        ATCATCAAGTGAAGAACTCTCCTCTTACAGTACCACTAATTATCAACAGCAACATAAAGT  300
                  |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201825931  ATCATCAAGTGGAGAACTCTCCTCTTACAGTACCACTAATTATCAACAGCAACATAAAGT  201825990

Query  301        GTTTCAAGAGGAAATAACTAGTGGAAGCTTTTGGACAGAACCATTTGTAGTAGAAAGTTT  360
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201825991  GTTTCAAGAGGAAATAACTAGTGGAAGCTTTTGGACAGAACCATTTGTAGTAGAAAGTTT  201826050

Query  361        CAATACTACTAGAACTGATTTTCTAGCTCCTTCAATTGATTACTGTGGACTTGTGTGTCC  420
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201826051  CAATACTACTAGAACTGATTTTCTAGCTCCTTCAATTGATTACTGTGGACTTGTGTGTCC  201826110

Query  421        ACCTTCACCATATATAGGTCATGAATTTCTTTCCTCCTTTGACTTTGATCATTTTAATTA  480
                  |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  201826111  ACCTTCACCATATATAGGTCATGAATTTCTTTCCTCCTTTGACTTTGATCATTATAATTA  201826170

Query  481        TTAA  484
                  ||||
Sbjct  201826171  TTAA  201826174
```

Figure 23. BLAST results of coding sequence (exon 3) and reference genome L - CM334 (version 1.55).

The high match rate, location (chromosome 7) and equal spacing between the three exons in all three reference genomes indicates that these are the *Pun3* exons. In the three reference genomes, however, their positions on chromosome 7 differ (Figure 24).

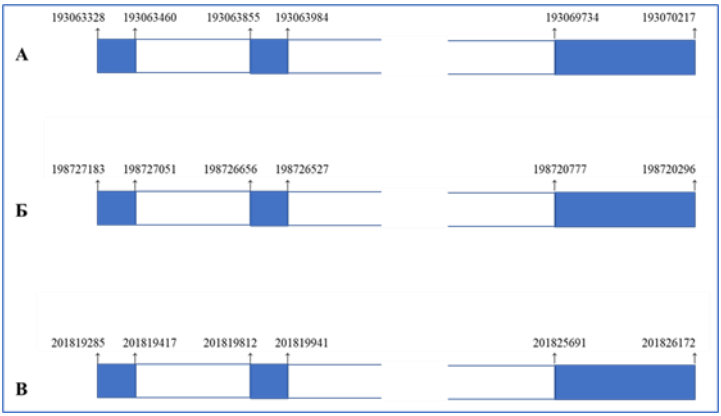


Figure 24. Location of the *Pun3* locus on chromosome 7, in the three reference genomes of *Capsicum annuum* L. - A. Location of exons in reference genome Zunla (version 2.0); B. Location of exons in reference genome UCD10X (version 1.0); C. Location of exons in reference genome CM334 (version 1.55).

Once the location of *Pun3* was determined, the nucleotide sequence of the 873 bp fragment amplified wtih the primers used was found. This fragment included exon1, exon 2, the intron between them, and a portion of the intron between exon 2 and exon 3.

To test whether CaMYB31 controls pungency in *C. annuum* L. - YCM334, Han and co-authors (Han et al., 2019) designed a CAPS marker based on a SNP in the stop codon they found in this sweet pepper. They subjected the PCR product to the restriction with *TaqI* enzyme and obtained different numbers of fragments, of different sizes, in peppers with different trait expression. The restriction enzyme recognizes and cuts at the T^CGA site. In sweet pepper, they observed three fragments of size 364 bp, 314 bp and 195 bp. In hot pepper they obtained two fragments of 559 bp and 314 bp. The location of the restriction sites in the sequence of the hot pepper reference genomes (Fig. 25) indicates that cutting into these sites would yield three fragments of 364 bp, 314 bp, and 195 bp. However, fragments with these sizes were described by Han and co-authors (Han et al., 2019) in sweet pepper.

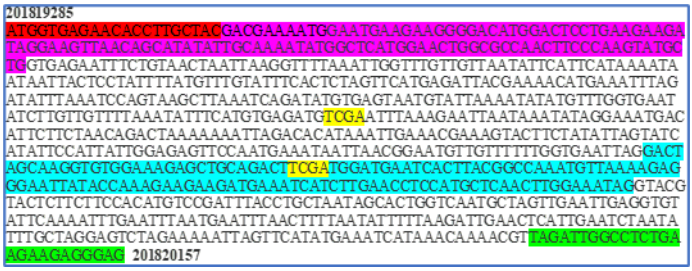


Figure 25. Sequence of a fragment, part of *Pun3*, restricted by primers *Pun-3*-CAPS_F and *Pun-3*-CAPS_R; location at reference genome CM334 (version 1. 55) - *Pun-3*-CAPS_F primer nucleotide sequence - colored red; *Pun-3*-CAPS_R primer nucleotide sequence - colored green; Exon 1 - colored pink; Exon 2 - colored blue; Introns - colored white; *TaqI* restriction site - colored yellow.

4.4. Comparison of sequenced fragments with reference genomes

We compared the sequenced fragments from the non-pungency fruit (*Pun1*/*Pun1* and *Pun1*/*pun1-1* genotype), pungency (*Pun1*/*Pun1* and *Pun1*/*pun1-1* genotype), and non-pungency (*pun1-1*/*pun1-1* allele) plants with the *Capsicum annuum* L. reference genomes CM334 (version 1.55), UCD10X (version 1.0), and Zunla (version 2.0) in the Sol Genomics Network. For all three reference genomes, the concordance is 99%. The location of the sequence on chromosome 7 fully matches the location of the *Pun3* locus. In Figures 43 and 44, the differences between the compared sequences are highlighted in yellow. In exon 1, deletion of T (thymine) and insertion of G (guanine) is observed. Since these point mutations are observed in peppers with different pungency profiles and different *Pun1* genotypes, the deletion and insertion are clearly not the nonsense mutation in exon one that Han and co-authors refer to (Han et al., 2019). No other mutations were observed in exons one and two. A total of five further nucleotide substitutions were detected in our experiments, but they are located in a non-coding region. This suggests that they do not result in a defective (recessive) *Pun3* allele and have no qualitative effect on capsaicin synthesis (Fig. 26 and Fig. 27).

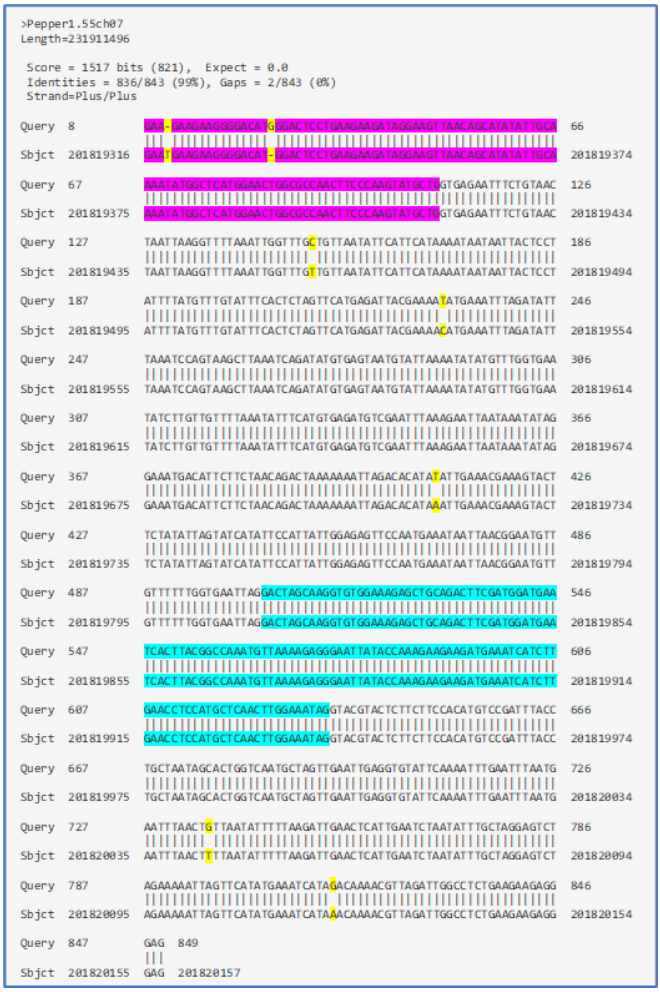


Figure 26. BLAST results of a sequence (851 bp) from *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero (with non-pungent fruits and *Pun1*/*Pun1* genotype) at reference genome *Capsicum annuum* L. - CM334 (version 1.55). The sequence includes: part of the first exon - 103 bp of 133 bp (colored pink), the whole of the second exon of 130 bp (coloured blue), the intron between them and part of the second intron (not coloured).

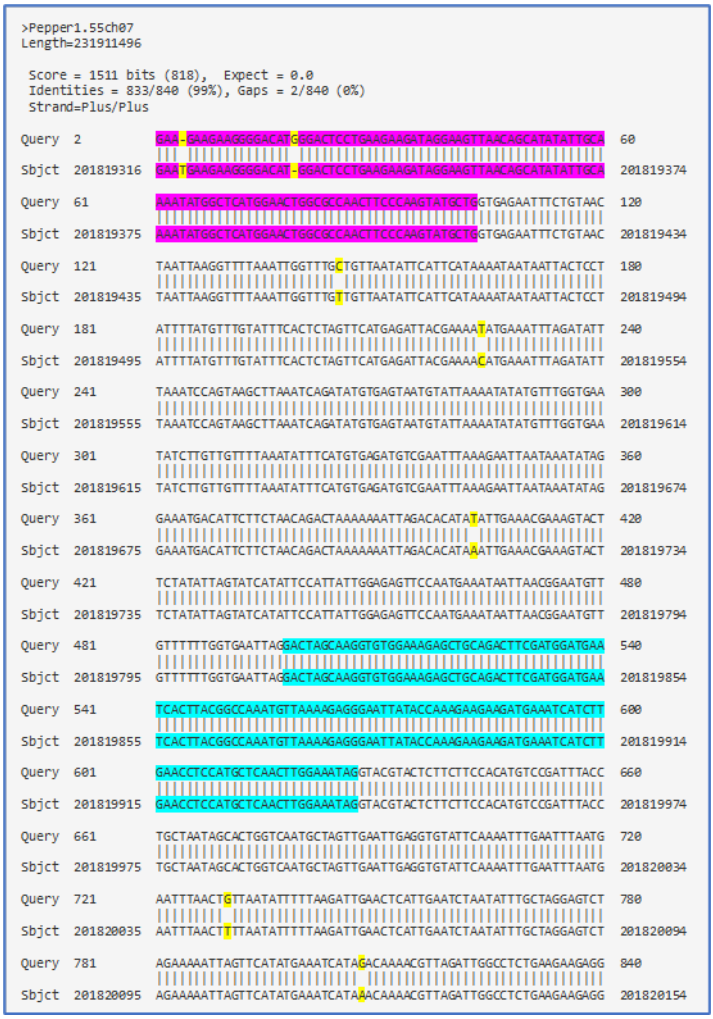


Figure 27. BLAST results of sequence (840 bp) from *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero (with sweet fruits and *pun1-1*/*pun1-1* genotype) at reference genome *Capsicum annuum* L. - CM334 (version 1.55). The sequence includes: part of the first exon, 103 bp of 133 bp (colored pink), the entire second exon, 130 bp in size (colored blue), the intron in between, and part of the second intron (not colored).

The lack of differences between sequences of plants with different pungency profiles and with different *Pun1* allele compositions indicates that the second factor controlling the lack of pungency is not located in the part of *Pun3* we considered. Contrary to expectations, mutation in the first and second exons of *Pun3* is not associated with the absence of pungency in the F_2 hybrid population studied. The second gene remains unknown for now, its identification is pending. Its identification would help in marker-assisted selection and could shed light on the evolution of Bulgarian sweet pepper.

5. Studying the relationship between genetic identity and capsaicin biosynthesis in the different parental forms, F_1 and F_2.

5.1 ISSR analysis

A set of ISSR primers was used to assess the heterogeneity present in each of the genotypes studied, and parental forms of *C. annuum* L. – var. Plovdivska kapiya and *C. annuum* L. – var. Familiya and F_2 of the crosses *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. and *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero. Individual plants from each of them were examined to confirm the ability of the selected marker system to reveal a sufficient number of polymorphisms. After an initial screening of the primers, it was found that some of them did not result in amplified fragments. This may be due to the lack or low number of the corresponding microsatellite sequence in the genome of the tested peppers.

Running the reactions with the selected primers resulted in one or more polymorphic reaction fragments. Obtaining such results was to be expected due to the low levels of genetic diversity in a facultative self-pollinating plant such as pepper (Fig. 28).

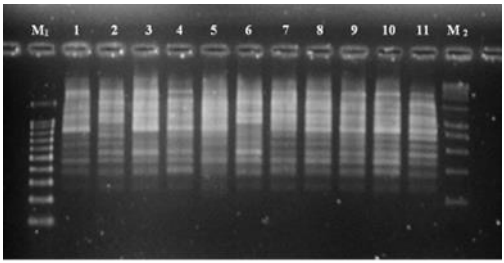


Figure 28. Results of ISSR reactions performed with ISSR primer P14: M_1-100 bp DNA molecular marker (RAINBOW extended DNA Ladder - Bioron, Cat. No. 304225); M_2 1 kb. molecular marker (Hyper Ladder – Bioline Lot. № H_1 618106A)-1 kb; Lanes 1-11 analyzed pepper plants.

Determining whether individual fragments are specific to each genotype involves using pooled samples. On the other hand, the use of pooled samples would hide the level of heterogeneity across genotypes, which would interfere with the reproducibility of the results if there are heterogeneous individuals in the sample. Because the likelihood of such heterogeneity within each genotype is not negligible, as shown by the studies of Cooke and co-autors (Cooke et al., 2003) and Bredemeijer and co-autors (Bredemeijer et al., 2002), and because we had no prior information on the genotypes that were analyzed in this study, we resorted to comparing only individual plants from each of the peppers examined (parental forms and F_2 of the crosses *C. annuum* L. – var. Plovdivska kapiya x *C. frutescens* L. and *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero).

5.2. Analysis of genetic diversity

The use of the ISSR primers resulted in a number of polymorphic bands. The results of the initial screening demonstrated the potential of the selected marker system to identify differences between pepper species and varieties. The presence of a larger number of polymorphic bands is a desirable and necessary condition for a more accurate identification of genetic diversity among them. Based on the 5 polymorphic ISSR markers used, a total of 65 polymorphic bands were obtained. Fragments ranging in size from 350 to 2000 base pairs were clearly distinguished. The average number of bands generated with the polymorphic primers was 6 with a maximum of 9 for

primer PE8. The average number of products obtained per primer was 8 and the detected polymorphism level was 4.4.

Based on the genotypic profiling of the parental forms and F_2 of the performed crosses using the five ISSR primers, a cluster analysis was performed. The 1/0 system was used to generate the dendrogram, with one indicating the presence of an ISSR fragment and zero indicating the absence of an amplified fragment.

The subcluster divisions show the relative genetic distances between the individual samples analyzed (Fig. 29). Clustering of parental lines and F_2 progeny was observed, and it is interesting to note that *C. annuum* L. – var. Familiya and *C. chinense* Jacq. - type Habanero (the two parental lines in this cross) appear to be relatively distant from the F_2 generation. This seemingly unusual fact can be explained by the high levels of genetic diversity obtained in the offspring from which the most outstanding individuals were selected for this analysis. It is evident from the presented dendrogram, in terms of the studied traits, that the population of F_2 individuals is distinguished by even greater genetic distance between individuals than is that between parental forms. On the other hand, the group of F_2 individuals as a whole appears to be substantially different from each of the two parents.

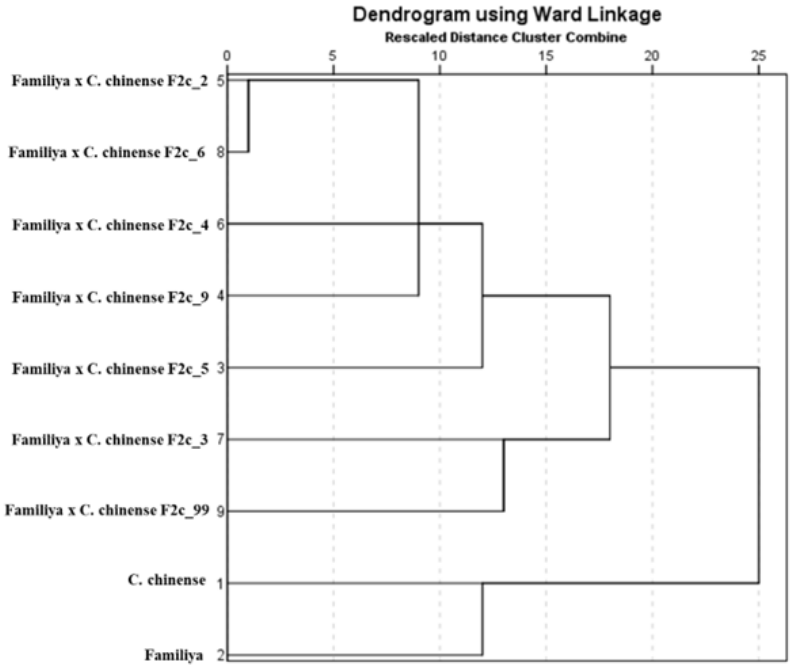


Figure 29. Clustering of parental forms with F_2 individuals from the Familiya x *C. chinense* Jacq. cross based on the identified ISSR polymorphisms.

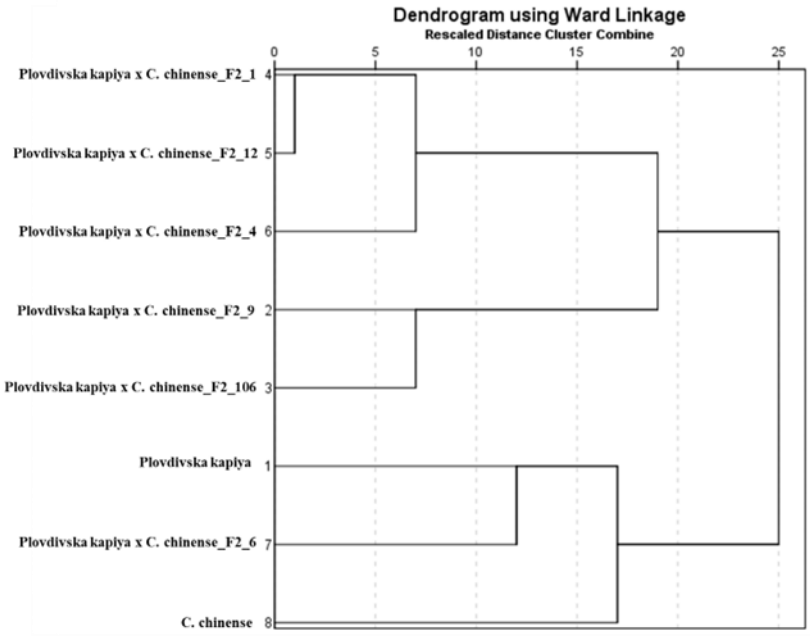


Figure 30. Clustering of parental forms with F_2 individuals of the Plovdivska kapiya x *C. chinense* Jacq. cross based on the identified ISSR polymorphisms.

Figure 30 shows a dendrogram with the distribution of parental genotypes and F_2 from the *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero cross. Here again there is considerable genetic diversity in the F_2 progeny, and it is interesting to note that *C. annuum* L. - variety Plovdivska kappiya and *C. chinense* Jacq. - type Habanero (the two parental lines in this cross) are again relatively less distant from each other than from the F_2 generation. Also interesting is the fact that one of the F_2 individuals falls into the cluster of parental forms. This is not an unexpected result, since with respect to at least some of the traits, individuals of the F_2 offspring should exhibit intermediate levels to their parents. The very fact that the crossing of genetically so distant parents is found to produce genetic diversity beyond the direct differences between the two parents provides one

possible explanation for the significant differences observed between cultivated forms of pepper in different geographical regions of the world. It can be speculated that the induction of such significant genetic diversity in interspecific crosses was used during the period of empirical crop selection to produce forms with a variety of characteristics ranging in fruit shape, size, and coloration, but also in their levels of pungency. In this respect, this dissertation contributes to the elucidation of the functioning mechanisms of regulation of capsaicin levels by revealing the presence and mode of functioning of alleles of the *AT3* gene available in Bulgarian breeding, as well as the presence in at least some of the samples of functional alleles of a second, epistatic gene.

CONCLUSIONS:

Based on the results obtained from the experiments and analyses conducted in this dissertation, the following conclusions can be drawn:

1. The allelic composition of the *Pun1* gene in pepper parental forms, F_1, and F_2 was determined by the applied molecular method.

2. Bulgarian pepper varieties - Plovdivska Kapiya, Familiya and IZK Delicates, of the species *C. annuum* L., the loss of pungency is due to the presence of the most widespread mutant recessive allele - *pun1-1*. They are of the *pun1-1/pun1-1* genotype. In the analysed *C. chinense* Jacq. - type Habanero and *C. frutescens* L., the dominant *Pun1* allele is in the homozygous state (*Pun1*/*Pun1*).

3. The artificial hybridization technology of pepper was successfully applied. After the organoleptic and laboratory analyses, the hybrid nature of F_1 and the heterozygous state of the *Pun1* gene was confirmed and the hybrids were of *Pun1*/*pun1-1* genotype.

4. In F_2 of the analyzed crosses *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero and *C. annuum* L. – var. IZK Delicates x *C. frutescens* L. the ratio of 3:1 between plants with pungent fruits and those with non-pungent fruits and the ratio of 1:2:1 between plants with the genotype *Pun1*/*Pun1* : *Pun1*/*pun1-1* : *pun1-1/pun1-1* in F_2 of the cross *C. annuum* L. – var. Delicates x *C. frutescens* L., once again demonstrates the involvement of a single gene (*Pun1*) controlling fruit pungency.

5. Despite the widespread occurrence of the *Pun1* major allele, its presence in non-pungent pepper *C. annuum* L. - cultivar Plovdivska kapiya has not prevented the spread of a new resource controlling pungency. In F_2 of the cross *C. annuum* L. – var. Plovdivska kapiya x *C. chinensis* Jacq. - type Habanero, the 9:7 ratio between hot and sweet fruit plants and the diverse genotype (both *pun1-1*/*pun1-1*, *Pun1*/*pun1-1* and *Pun1*/*Pun1*) of sweet fruit plants indicate the presence of a second gene determining the lack of pungency in these plants.

6. Based on sequencing of a portion of the *Pun3* gene and bioinformatics analyses performed, no mutations causing blockage of capsaicin synthesis were found on this fragment. Mutations in the first and second exons of *Pun3* were not associated with the lack of pungency in the F_2 hybrid population studied *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero.

7. In the present study, ISSR markers were successfully used to assess genetic diversity among the analyzed pepper species and cultivars and the hybrid lines *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. and *C. annuum* L. – var. Familiya x *C. chinense* Jacq. - type Habanero.

8. The results obtained provide a basis for the development of future breeding programs by applying MAS (marker-assisted selection).

CONTRIBUTIONS

Scientific and theoretical contributions:

1. For the first time the allelic status of the *Pun1* gene was determined in Bulgarian pepper varieties - Plovdivska kapiya, Familiya and IZK Delicates, of the species *C. annuum* L.

2. For the first time, the inheritance of the genes and their mutant alleles responsible for the lack of hotness in F_1 and F_2 in *C. annuum* L. – var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero, *C. annuum* L. - variety Familiya x *C. chinense* Jacq. - type Habanero and *C. annuum* L. – var. IZK Delicates x *C. frutescens* L.

3. The hypotheses regarding the inheritance of pungency and lack of pungency in the studied peppers, validated by applying the $\chi 2$ statistic, can be used in future studies by other authors.

4. For the first time, the presence of additional loci with effects on capsaicin synthesis in the F_2 population of the cross *C. annuum* L. - var. Plovdivska kapiya x *C. chinense* Jacq. - type Habanero.

5. Methods applied to other crops were modified and adapted to conduct molecular ISSR analysis in pepper.

Scientific and applied contributions:

1. For the first time, a successful hybridization was carried out between mother plants of the Bulgarian non-pungent pepper varieties Plovdivska kapiya, Familiya and IZK Delicates of the species *C. annuum* L. and pungent parent plants of the species *C. chinense* Jacq. - type Habanero and *C. frutescens* L.

2. The analyzed pepper species and varieties can be used as potential donor in future breeding programs.

3. The methodology applied to determine the allelic status of the *Pun1* gene is a good strategy when testing seed lots in purity and quality programs.

4. The data obtained from the sequencing and processed by the bioinformatics tools provide the basis needed to conduct further analyses of the genes responsible for capsaicin synthesis in pepper (genus *Capsicum*).

LIST OF SCIENTIFIC PUBLICATIONS RELATED TO THE DISSERTATION:

1. Srebcheva, T., & Bojinov, B. (2018). Genetic control of non-pungency in pepper (*Capsicum* sp.)(mini-review). *Ştiinţa Agricolă*, (2), 57-63. https://sa.uasm.md/index.php?journal=sa&page=article&op=view&path%5B%5D=615

2. Srebcheva, T. & Bojinov, B. (2019). Identification of allelic State of the *Pun-1* gene associated with the capsaicin synthesis in selected *Capsicum* (pepper) species. *Научни трудове на Съюза на учените–Пловдив. Серия В: Техника и технологии*, *17*, 257-260. https://cyberleninka.ru/article/n/identification-of-allelic-state-of-the-pun-1-gene-associated-with-the-capsaicin-synthesis-in-selected-capsicum-pepper-species?fbclid=IwAR14xQKa9VRsYEVL2xO5kVW71J2FYHjFyXF6Ta2NqOoN61QBzMUhma565yw

3. Srebcheva, T., & Kostova, M. (2022). Influence of the *Pun1* gene on capsaicin synthesis in hybrid lines of the genus *Capsicum*. *Trakia Journal of Sciences*, *20* (1), 37. DOI:10.15547/tjs.2022.01.005, ISSN 1313-3551 (online).

4. Srebcheva, T., Kostova, M. (2022). Study of the inheritance of pungency in a hybrid pepper lines (genus *Capsicum*). Изследване унаследяването на лютивината в хибридна линия пипер (род *Capsicum*). *Journal of Mountain Agriculture on the Balkans (JMAB)* Journal of Mountain Agriculture on the Balkans, 2022, 25 (1), 407-422. ISSN 1311-0489 (Print), ISSN 2367-836 (Online).