Identification of novel SNPs in *Pun1* locus for pungency in *Capsicum* species

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Research Article

Keywords: Capsicum, capsaicin, vitamin C, pungency, sequencing, SNP, Pun 1, amino acid variation

Posted Date: November 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2253725/v1

Abstract

Background

Capsaicin and its analogues known as capsaicinoids are the principal sources of pungency in *Capsicum* spp, detectable by mammalian taste receptors. In this study, a characterization of chilli germplasm was done based on capsaicin concentration. The goal of this study was to figure out what causes *Capsicum* spp. to lose their pungency.

Methods and Results

The experimental material involved forty-nine genotypes of chilli collected from different states of India representing different agro-ecological regions and were evaluated for several quantitative and biochemical traits. Wide variation in capsaicin content was observed among the genotypes. Bhut Jolokia (*Capsicum chinense*) showed highest capsaicin content (10500.75 µg/g). In order to understand the variation in pungency content, molecular analysis of *Pun1* gene was done for discovering SNP in the selected genotypes.The five genotypes namely Bhut Jolokia, Kashmiri-Long-1, Byadgi Dabbi, Byadgi Kaddi and Nishat-1with high, medium and no pungency content were selected for the molecular analysis of *Pun1* gene. Single primer pair was employed for amplification of *Pun1*gene in *Capsicum chinense* and *Capsicum annuum* (with amplicon size of 650bp). However, in the non-pungent variety (Nishat-1), the 650bp DNA fragment was not amplified due to 2.5kb deletion spanning the putative promoter and first exon of AT3. DNA amplification was followed by sequencing. Sequence alignment between *Pun1* gene sequences of genotypes Bhut Jolokia (*Capsicum chinense*) and Kashmiri-Long – 1 (*Capsicum annuum*) with high capsaicin content and medium capsaicin content, respectively, revealed variations at 13 places and alignment of amino acid sequences deduced from nucleotide sequences revealed 5variations in amino-acid sequences. Furthermore, protein structure prediction in *C. chinense* and *C. annuum* identified certain structural changes.

Conclusion

The observed variation in protein structure might be responsible for high capsaicin production in one genotype as compared to the other and hence the protein conformation determines its interaction with the substrate.

Introduction

Chilli (*Capsicum annuum* L.) is an important vegetable-cum-spice crop grown in almost all tropical and subtropical regions of the world. The genus *Capsicum* includes 30 species, five of which are cultivated: *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq, *C. pubescens* R. & P. and *C. baccatum* L. (Perry et al., 2007).

The primary centre of origin of chilli is reported to be Latin American regions of New Mexico, Guatemala and Bulgaria (Salvador, 2002). Chilli forms an indispensable part of every Indian cuisine. Immature chilli fruits contain phytonutrients, ascorbic acid, caretenoids and rutin which are valued for pharmaceutical applications (Purseglove, 1977). It is liked for its pungency and spicy taste besides the appealing colour, which are attributed to capsaicin and capsanthin, respectively. Capsaicinoids, a group of alkaloids, impart pungency to the *Capsicum* spp. and are detected by taste receptor cells in mammals. The perception of pain on contact with this receptor cell during ingestion indicates that capsaicinoids act as deterrents (Jordt and Julius, 2002). The biosynthesis of these compounds occurs in epidermal cells in the interlocular septa of the placental tissues in pepper fruits (Stewart et al., 2007). Capsaicinoids are produced by the condensation of vanillylamine with a branched fatty acid, derived from either valine or leucine, with vanillylamine being derived from phenylalanine (Suzuki et al., 1981). The nutraceutical uses of capsaicinoid as antioxidant, anticancer, anti-arthritic and analgesic are widely identified and exploited (Prasad et al., 2006). Variations in capsaicinoid contents in peppers have been attributed to environmental influences during plant growth and also to the fruit position on the plant (Harvell and Bosland, 1997). The accumulation of capsaicinoids in pepper fruit placental tissues occurs between 20–30 days after flowering in pungent cultivars (Iwai et al., 1986). Capsaicin (8-methyl-N-Vanillyl-trans-6-nonenamide), the most abundant capsaicinoid, is a crystalline acrid volatile alkaloid present in the placenta of fruit and has diverse prophylactic and therapeutic usage in Allopathic and Ayurvedic systems of medicine. As an antioxidant, capsaicin can directly scavenge various free radicals (Bhattacharya et al., 2010).

A large number of carotenoids provide high nutritional value and colour to chilli. The 60% of the total carotenoids present in the fruits is constituted by capsanthin and capsorubin. Red coloured pigment is used as a natural colour additive in food, drugs and cosmetics. These pigments are also rich in bioflavonoids, which are powerful antioxidants and inhibit the progression of chronic diseases such as muscular degeneration, cardiovascular diseases and cancer. Oleoresin extracted from dried and ground chillies, is gaining more importance especially from export point of view as it offers uniform quality, longer shelf-life, freedom from micro-organisms and lesser freight charges.

Although capsaicinoid contents and the intensity of pungency are quantitative traits, the presence of capsaicinoids is controlled by the single *Pun1* locus. *Pun1* encodes a putative acyltransferase, which acylates vanillylamine with a fatty acid to produce capsaicinoids (Stewart et al., 2005). In the homozygous recessive state, capsaicinoids are not produced by the *Capsicum* spp. Three loss-of-function alleles of *Pun1* have been reported to date, $Pun1^{1-3}$. The allele $Pun1^{1}$ is widely distributed in sweet *C. annuum* cultivars. This type of *Pun1* has a 2.5-kb deletion spanning from the putative promoter region to most of the first exon region (Stewart et al., 2005). The protein is not transcribed or translated. The second type of *Pun1* allele, $Pun1^{2}$ has a 4-bp deletion in the first exon region that creates an early stop codon. In this allele, there is transcription, but no protein product is produced (Stewart et al., 2007). In the third allele, $Pun1^{3}$ has a large deletion in the 2nd exon region leading to the loss of 70 amino acids in the *Pun1* protein (Stellariet al., 2010). This allele is neither transcribed nor translated. However, variations in the loss-of-function allele have not been fully understood.

Materials And Methods

Plant material

Forty-nine diverse chilli genotypes collected from different states of India representing different agro- ecological regions were used in this study. The genotypes belonged to species *Capsicum annuum* and *Capsicum chinense* (BhutJolokia). A code 'C' was assigned to the genotypes used in this study.

Germplasm Propagation

Planting of genotypes was done in Randomized Complete Block Design (RCBD) with three blocks, during Kharif 2020. The seeds were sown in the plots with dimensions 2× 2m and cultivated until they reached the appropriate stage in the raised beds. Seedlings were transplanted in the field after 47 days at a spacing of 45 cm between rows and 45 cm between plants in a row. Ten plants of each genotype were transplanted in Randomised Complete Block Design and five randomly chosen plants from each genotype were used for recording data. So, when fruits were fully mature, they were harvested.

Capsaicin content (µg/g)

Capsaicin content of 49 different chilli genotypes was determined using UV spectrophotometer that revealed a wide range of differences in capsaicin concentration. The concentration of capsaicin was measured using a standard curve and SHU units were calculated. The spicy strength of the samples is calculated using the widely accepted Scoville organoleptic test, which involves converting the capsaicin content expressed in grams of capsaicin per gram of chilli to Scoville heat units by multiplying the capsaicin content in the pepper by the coefficient corresponding to the heat value for pure capsaicin and adjusted for sample extraction efficiency using the formula: 18× µg/ g.

Estimation of Capsaicin (mg/g) by UV spectrophotometer

Capsaicin content was estimated by Phosphomolybdic acid method.

Reagents used:

0.4% NaOH

3% Phosphomolybdic acid

Dry acetone: 25g of anhydrous sodium sulphate mixed into 500ml acetone one day before use.

Method: 0.5g of dry chilli powder was weighed in a volumetric flask and 10ml of dry acetone was added and kept in shaker for an hour. The content was centrifuged at 10000rpm for 10 min. 1ml supernatant was taken in a test tube and evaporated to dryness in hot water bath till residue was left. Then the residue was dissolved in 5 ml of 0.4 % sodium hydroxide solution and 3ml of 3% phosphomolybdic acid was added and kept for an hour and the solution was filtered. The clear blue coloured solution was directly transferred into cuvette and absorbance was read at 650 nm with the help of spectrophotometer against dry acetone as blank.

$$\text{Capsaicin (\%)} = \frac{\text{X} \times \text{dilution} \times 100}{\text{Weight of sample (g)} \times 1000}$$

Where X = ug of capsaicin in sample from standard curve using OD value.

Genomic DNA extraction and quantification

The genomic DNA was extracted by CTAB method as described by Doyle &Doyle (1990). Agarose gel electrophoresis was done to evaluate the quality and quantity of isolated genomic DNA. To assess the concentration of DNA in each sample, DNA ladder (100bp) was used as reference. The electrophoresis was performed for 2 hours at 75V. The gel documentation system (Syngene) was then used to examine the DNA samples for quality and concentration.

Molecular analysis of *Pun1*gene from contrasting genotypes with high, medium and no pungency content

The five genotypes namely *Bhut Jolokia*, Kashmiri-Long-1, *Byadgi Dabbi, Byadgi Kaddi* and Nishat-1 with high, medium and no pungency content were selected for the molecular analysis of *Pun 1* gene.

Primer designing

*Pun1*gene primers were designed from the sequence of *Capsicum annuum* cultivar 'Thai Hot'acyltransferase (*Pun1*) gene. Gene sequence was retrieved from the NCBI gene data bank. Primers were designed using the IDT software available online

(https://sg.idtdna.com/pages/tools/primerquest?returnurl=%2FPrimerquest%2FHome%2FIndex).

A single primer pair was used in this investigation that targeted a region of 650base pair.

PCR standardization and amplification

PCR was performed using primer pair given in Table 1. A PCR reaction of total volume 15 µl consisted of 9.3µl Taq mixture(Kapa biosystems), 1.2 µl forward primer(100pmol/µl), 1.2 µl reverse primer(100pmol/µl), 1.5µl DNA template, 1.05 µl molecular grade water and 0.75 µl of DMSO. For DNA amplification, the PCR reaction was performed in a thermal cycler machine (applied biosystems by Thermo Fisher Scientific) with following amplification program. An initial denaturation phase of 5 minute, followed by 35 cycles of denaturation (at 94°C/min), annealing (at 58°C for 1 minute and 30 seconds) and elongation (at 72°C for 30 seconds) with a final extension of 72°C for 10 minutes. Following PCR, the PCR products were analysed on 1.5 % agarose gel and visualised under UV light of gel documentation system (Syngene, UK). The PCR amplicons were produced using the primer pair and genomic DNA taken from three genotypes (Nishat-1, Kashmiri-Long-1 and *Bhut Jolokia*) with varying levels of pungency (no, medium and strong respectively). Only the two accessions produced single amplicons with the primer pair when evaluated by gel electrophoresis and then the amplicons from the PCR products were eluted and purified from the gel and further confirmed by Sequencing with *Pun1*_F and *Pun1*_R (Redcliff Life tech Pvt .Ltd.)

Results

Capsaicin content of the genotypes ranged from 450.00 to 10500.85 µg/g with an overall mean of 2163.58 µg/g. Bhut Jolokia (10500.85 µg/g; 189015.30 SHU) had the highest capsaicin concentration followed by SKAU-HP-21(5530.50 µg/g; 99549.00 SHU) and SKAU-HP-11 (4350.38 µg/g; 78306.90 SHU).

PCR analysis was carried out using *Pun1* primer pair at 15 µl of reaction and the samples were evaluated on a 1.5 percent agarose gel stained with Ethidium Bromide (5 µl) and a 50 bp DNA ladder was employed as a size reference and the gel was run at 100 volt. The gel was evaluated under UV light using a gel documentation system and the results are listed below.

A 650 base pair band was produced for high and medium pungency cultivar and this band was not amplified in no pungency capsicum (*Capsicum annuum* L. *grossum* Nishat-1).

Sequencing

The PCR products from Bhut Jolokia and Kasmiri-Long-1 were sequenced. After aligning the forward and reverse sequences, the common regions were selected as a single sequence. The sequences that were obtained are given below.

1. Forward Sequence of *Capsicum chinense*

GCGGTGGAGGAATAGGAGGAGGAGATTTCCAATGGCATTTCGTGGTAGACGAGGTTTGATCATAGTACGTATGTTTAAGAAGTGAACCAACTTTGATGGTAGCATTGATC

2. Reverse Sequence of *Capsicum chinense*

GAAACATTTTTTTCTACAGATAAGTTAGATGCACTTCGAGCTAAGGTAATACTACCATCGTCCATTATTGTTTGTCTTACGGTATTTTTGAAAAGAATAATATTTAATATTCT

3. Forward Sequence of *Capsicum annuum*

GTTGTGGGAAAATAGGAGGAGAGATTTCCAATGGCATTTCGTGGTAGACGAGGTTTGATCATAGTACGTATGTTAAAGAAGTGAACCAACTTTGATGGTAGCATTGATGA

4. Reverse Sequence of *Capsicum annuum*

AAATAATTTTTTTCTACAGATAAGTTAGATGCACTTCGAGCTAAGGTAATACTACCATCGTCCATTATTGTTTGTCTTACGGTATTTTTGAAAAGAATAATATTTAATAGTCT

Variations (SNPs) in *Pun1* gene and amino acid sequence of *Capsicum chinense* and *Capsicum annuum*

Multiple alignments were performed and examined on the acquired sequences using the Clustal W software. The aligned data revealed nucleotide differences in several regions. A total of 13 nucleotide variations were discovered in high and medium pungent genotypes of *Capsicum chinense* and *Capsicum annuum*. (Figure 3 (**a**) and table 3)

The aligned data at protein level also revealed the variation in amino acids. A total of 5 amino acid variations were found in high and medium pungent genotypes of chilli which have been presented in the tabular form. (Figure 3 (**b**) and table 4)

*Pun 1*gene based protein structure in C. *Chinense* and *C. annuum*

The predicted secondary structure indicated that *Pun1* gene in *C. chinense* showed 39.55 % α- helix, 41.36 % random coil, 16.36 % extended strand, and 2.73% β- turn (Fig. 4, a) where incase of *C. annuum*, *Pun1* gene had 37.27 % α- helix, 44.09 % random coil, 15.68 % extended strand, and 2.95 % β- turn (Fig. 4, b).

Pun1 gene sequences from high and medium capsaicin producing genotypes were used to predict the 3-D protein structure. The structure was predicted using the i-tasser software based on the amino acid sequence (Figure 9). The structures were compared and the structural alterations were observed. A variation in protein was found at three places. The putative protein of the medium capsaicin producing genotype (C. *annuum*) showed more coiling at two positions and less coiling at one position when compared with high capsaicin producing genotype (*C. chinense)*. The potential structural alteration might affect its affinity with the subsequent substrate in pathway, resulting in differences in capsaicin levels among genotypes. However, further functional studies are anticipated that may confirm these observations.

Discussion

The goal of this study was allele mining in *Pun1* locus for pungency and to discover the single nucleotide differences (*SNPs*) in the upstream gene of capsaicin production among *Capsicum spp*. with the highest and lowest capsaicin content.

Capsaicin content in the fruits of 49 diverse genotypes was estimated to observe the variation among them. Significant variation was observed in the capsaicin content among the genotypes ranging from 450.00-10500.85 µg/g with an average of 2163.58 µg/g. Highest capsaicin content was recorded in Bhut Jolokia (10500.85 µg/g; 189015.30 SHU). Chilli pepper cultivars' heat levels are known to be affected by the environment (Harvell and Bosland, 1997).Similar results were also found by (Bosland and Baral, 2007) and Popelka et al. (2017). We also observed this phenomenon in our study. Bhut Jolokia, a native of Nagaland and Manipur states of NE India that are largely non-temperate, is known to express pungency of 10,00,000 SHU (Adluri*et al*.,2017). However, in temperate climate of Kashmir, which experiences mild summers, the pungency of Bhut Jolokia drastically dropped to 189015.30 SHU. This ten times decrease in capsaicin content can be attributed only to environmental influence.

The *Pun1*gene (AT3) was targeted in the present study due to its important role in the biosynthesis of capsaicinoids. As detailed in the result section, molecular analysis was carried out on selected five genotypes namely Bhut Jolokia, Kashmiri-Long-1, Byadgi Dabbi, Byadgi Kaddi and Nishat-1 (non-pungent variety used as check for *Pun1*) belonging to *Capsicum chinense* and *Capsicum annuum* species with high, medium and low pungency content. A portion of *Pun 1* gene was targeted for amplification and sequencing in *Capsicum chinense* and *Capsicum annuum* (with amplicon size of 650 bp) as mentioned in the results. However in the non-pungent variety (Nishat-1) the 650bp DNA fragment was not amplified due to 2.5kb deletion spanning the putative promoter and first exon of AT3. These results are in accordance with the previous study (Stewart et al., 2005). Similar results were also obtained by Kirii et al. (2017). The *Pun 1* allele indicated by this significant deletion is the only known mutation that affects the presence or absence of capsaicinoids qualitatively (Webber. 1911; Blum et al., 2002).

Sequence alignment revealed 13 SNPs between *Pun1* gene sequences of genotype with high capsaicin content (Bhut Jolokia, scientifically *C. chinense*) and moderate capsaicin content (Kashmiri-Long-1, scientifically *C. annuum*) that have led to change in amino acid sequence as depicted in Fig. 1. A similar study was carried by Zamora et al. (2020) on *Pun1* gene in pungent cultivars of *Capsicum annuum* of Northern Mexico. They reported 19 single nucleotide polymorphisms in the first exon and 7 in the intron which lead to amino acid changes. Another study was conducted by Yumnam et al. (2012) in which the sequence analysis of the 2, 349 bp AT3 region detected the presence of a total of 79 SNPs.

The amino acid sequences obtained from the nucleotide sequences were aligned. They also showed the variation in both the species. The alignment revealed 5 variations in amino acids as depicted in Fig. 2. A study was carried out by Kirii et al. (2017) to validate the impact of single nucleotide insertion on the amino acid sequence and a subsequent alteration in amino acid sequence was observed owing to a frameshift mutation.

The amino acid sequences of high and medium capsaicin producing genotypes were used to predict the protein structure. Variations in certain regions were identified in the putative proteins. When compared to the high capsaicin producing genotype, the putative protein of the medium capsaicin producing genotype showed more coiling at two positions. In the high capsaicin producing genotype, more coiling was also found at one region, but no such coiling was observed in the medium capsaicin producing genotype. Variations in amino acid sequences have an influence on the predicted protein. Changes in binding sites might be caused by the high and low coiled regions. Because protein structure and conformation impact its interaction with the substrate, change in the putative protein structure might easily be the basis for higher capsaicin synthesis in one genotype compared to the other. However, further functional studies will confirm these observations.

Conclusions

The genotypes like Bhut Jolokia, SKAU-HP-21, SKAU-HP-11, SKAU-HP-20 that expressed high capsaicin content could be exploited in a hybridization program to produce superior recombinants in the next generation and molecular analysis of *Pun1* gene helped in understanding the possible molecular basis of variation in pungency content among various genotypes of chilli.

Declarations

Author contribution statement:

AG did the experimental work, molecular analysis and wrote the manuscript; GM, KH, NN and IA helped in biochemical and statistical analysis; UG, AH and RM helped in experimental work and analysis; SB helped in manuscript writing; AM and SMZ developed the concept and guided all the field and laboratory work and finalized the manuscript.

Acknowledgment: AM and SMZ acknowledge Honorable Vice Chancellor SKUAST-K (Prof. Nazir A. Ganai) for the support and encouragement in carrying out research on applied aspects.

Conflict of interest

Authors declare that there are no known conflicts of interest associated with this publication.

References

Adluri, PK, Borah AR, Nath PD (2017) Study of Genetic Diversity of Bhut Jolokia Germplasm in North East India by SSR Markers and Morphology. International Journal of Pure and Applied Bioscience 5(4): 1657-1665

Bhattacharya A, Chattopadhyay A, Mazumdar D, Chakravarty A, Pal S(2010) Antioxidant constituents and enzymes activities in chilli peppers. International Journal of Vegetable Science 16(3): 201-211

Bosland PW, Baral JB (2007) Bhut Jolokia-the World's hottest known chile pepper is a putative naturally occurring interspecific hybrid. Horticultural Science 30: 137-139

Blum E, Liu K, Mazourek M, Yoo EY, Jahn M, Paranl(2002) Molecular mapping of the C locus for presence of pungency in Capsicum. Genome 45: 02-705

Doyle J J, Doyle J L (1990) Isolation of plant DNA from fresh tissue. Focus 12:13-15

Harvell K P, Bosland PW (1997) The environment produces a significant effect on pungency of chiles (Capsicum annuum L.). Hort Science 32:1292

Iwai K, T Suzuki, H Fujiwake(1986) The formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogues in Capsicum annuum var. annuum cv. Karayatsubusa at different growth stages after flowering. Agricultural and Biological Chemistry 43: 2493–2498.

Jordth S E, Julius D (2002) Molecular basis for species specific sensitivity to 'Hot' chilli peppers. Cell 11: 421-430

Kirii E, Goto T, Yoshida Y, Yasuba K, Tanaka Y(2017) Non-pungency in a Japanese Chilli Pepper Landrace (Capsicum annuum) is caused by a Novel Loss-of-function *PUN1* Allele. The Horticulture Journal 86:61-69

Perry L, Dickau R, Zarrillo S, Holst I, Pearsall D M, Piperno D R, Berman M J, Cooke R G, Rademaker K, Ranere A J, Rademaker K, Ranere A J, Raymond J S, Sandweiss D H, Scaramelli F, Tarble K, Zeidler J A (2007)Starchfossils and the domestication and dispersal of chilli pepper (Capsicum spp. L). Americas Science 315: 986-988

Popelka P, Jevinova P, Smejkal K, Roba P (2017) Determination of capsaicin content and pungency level of different fresh and dried chilli peppers. Folia Veternaria, 2:11-16

Prasad NBC, Gururaj HB, Kumar V, Giridhar P, Parimalan R, Sharma A, Ravi shankar GA. (2006) Influence of 8-methyl-Nonenoic acid on capsaicin biosynthesis invivo and invitro cell cultures of Capsicum Spp. Journal of Agricultural Federation Chemistry 54:1854-1859

Purseglove JW (1977) Tropical Crops. Dicotyledon—2. Longman. Green & Co. Publisher, London, pp 524-530

Salvador MH (2002) Genetic resources of chilli (Capsicum annuum L.) in Mexico. Proceedings of the 16th International Pepper Conference, Tampico, Tamaulipas, Mexico,pp 10-12

Stellari GM, Mazourek M, Jahn. M (2010) Contrasting modes for loss of pungency between cultivated and wild species of Capsicum. Heredity 104:460 471

Stewart C, Kang BC, Liu K, Mazourek M, Moore SL, Yoo EY, Kim BD, Paran I, Jahn MM (2005) The *PUN1* gene for pungency in pepper encodes a putative acyltransferase. The Plant Journal 42:675-688

Stewart C, Mazourek M, Stellari GM, Connell MO, Jahn M (2007) Genetic control of Pungency in C. chinense via the *PUN1* locus. Journal of Experimental Botany 58: 979-991

Suzuki T, Fujiwake H, Iwai K (1980) Intracellular localization of capsaicin and its analogues, capsaicinoid, in *Capsicum* fruit 1. Microscopic investigation of the structure of the placenta of *Capsicum annuum* var. *annuum* cv. Karayatsubusa. *Plant and Cell Physiology 21:839-853*

Suzuki T, Kawada T, Iwai K (1981) Biosynthesis of Acyl Moieties of Capsaicin and its Analogues from Valine and Leucine in *Capsicum Fruits. Plant and Cell Physiology22: 23-32*

Webber H J (1911) Preliminary Notes on Pepper Hybrids. Journal of Heredity, 7:188-199

Yumnam JS, Tyagi W, Pandey A, Ngasepam, T M (2012) Evaluation of Genetic Diversity of Chilli Landraces from North Eastern India Based on Morphology, SSR Markers and the Pun 1 Locus. Plant Molecular Biology Reporter 30:1470-1479.

Zamora AG, Olvera C, Calleros AA, Hernandez J LG (2020) Genetic variability of Pun 1 gene (Capsaicin synthase) in pungent cultivars of Capsicum annuum of Northern Mexico. Tropical and Subtropical Agroecosystems 23:1-15

Tables

Table 1 Mean performance of *Capsicum* spp. with respect to Capsaicin concentration.

No.	Chilli Genotypes	Capsaicin (µg/g)	Capsaicin in SHU	S. No.	Chilli Genotypes	Capsaicin (µg/g)	Capsaicin in SHU
	SKAU-HP-1	3150.61	56710.98	27.	SKAU-HP-25	1350.65	24311.70
	SKAU-HP-2	2970.55	53469.90	28	SKUA-HP-26	3360.50	60489.00
	SKAU-HP-3	450.52	8109.36	29.	SKAU-HP-27	1120.65	20171.70
	SKAU-HP-4	1780.28	32045.04	30.	SKAU-HP-28	1510.53	27189.54
	SKAU-HP-5	2290.50	41229.00	31.	SKAU-HP-29	870.50	15669.00
	SKAU-HP-6	1630.67	29352.06	32.	Kashi-Anmol	900.50	16209.00
	SKAU-HP-7	1150.50	20709.00	33.	Arka Lohit	3100.42	55807.56
	SKAU-HP-8	1600.62	28811.16	34.	Bhut Jolokia	10500.85	189015.30
	SKAU-HP-9	2130.57	38350.26	35.	SKAU-HP-30	500.32	9005.76
.	SKAU-HP-10	3780.50	68049.00	36.	SKAU-HP-31	1080.58	19450.44
.	SKAU-HP-11	4350.38	78306.84	37.	SKAU-HP-32	2890.43	52027.74
.	SKAU-HP-12	2680.50	48249.00	38.	SKAU-HP-33	920.55	16569.90
.	SKAU-HP-13	860.56	15490.08	39.	SKAU-HP-34	870.57	15670.26
.	SKAU-HP-14	2430.58	43750.44	40.	SKAU-HP-35	750.28	13505.04
.	SKAU-HP-15	2030.74	36553.32	41.	Byadgi Dabbi	1200.75	21613.50
.	SKAU-HP-16	1870.42	33667.56	42.	SKAU-HP-36	730.52	13149.36
.	Kashmiri-Long-1	3050.62	54911.16	43.	SKAU-HP-37	920.50	16569.00
.	Pusa Sadabahar	3100.52	55809.36	44.	SKAU-HP-38	3490.48	62828.64
.	SKAU-HP-17	3360.72	60492.96	45.	SKAU-HP-39	1810.50	32589.00
.	SKAU-HP-18	1570.40	28267.20	46.	Byadgi Kaddi	1620.28	29165.04
.	SKAU-HP-19	2600.53	46809.54	47.	SKAU-HP-40	800.32	14405.76
.	SKAU-HP-20	3980.63	71651.34	48.	SKAU-HP-41	1610.44	28987.92
.	SKAU-HP-21	5530.50	99549.00	49	SKAU-HP-42	1110.52	19989.36
.	SKAU-HP-22	2170.57	39070.26				
.	SKAU-HP-23	1710.54	30789.72				
.	SKAU-HP-24	760.40	13687.20				
)(P< 0.05)		28.424					

Table 2: The designed primers along with their sequences

S. No	Primer	Sequence Text (5'-3')	Annealing Temperature (°C)	Target Chilli species
1	*PUN1*_F	CAACTCCATGTCCTGCATGTTA	58	*Capsicum annuum* and C. *chinense*
	*PUN1*_R	TTGTCCGATCTCAACCAGTGCG		

Table 3 Nucleotide variation in high and medium pungent genotypes of chilli

S. No.	Base pair	Variation of nucleotides in *Capsicum chinense*	Variation of nucleotides in *Capsicum annuum*
1	40	Guanine	Adenine
2	80	Adenine	Cytosine
3	267	Adenine	Thymine
4	572	Adenine	Guanine
5	583	Guanine	Adenine
6	618	Thymine	Adenine
7	631	Adenine	Cytosine
8	648	Thymine	Cytosine
9	679	Guanine	Cytosine
10	777	Adenine	Thymine
11	1099	Guanine	Adenine
12	1137	Guanine	Adenine
13	1176	Adenine	Guanine

Table 4 Amino-acid variation in high and medium pungent genotypes of chilli

S. No.	Base pair	Variation of amino acids in *Capsicum chinense*	Variation of amino acids in *Capsicum annuum*
1	27	K (Lysine)	T(Threonine)
2	191	H (Histidine)	R (Alginine)
3	195	A (Alanine)	T (Threonine)
4	211	K (Lysine)	Q (Glutamine)
5	367	E (Glutamic acid)	K (Lysine)

Figures

Figure 1

Figure legend not available with this version.

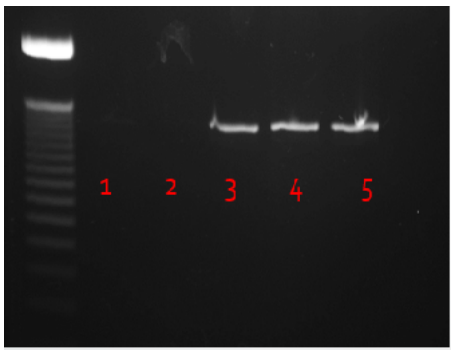


Figure 2

Figure legend not available with this version.

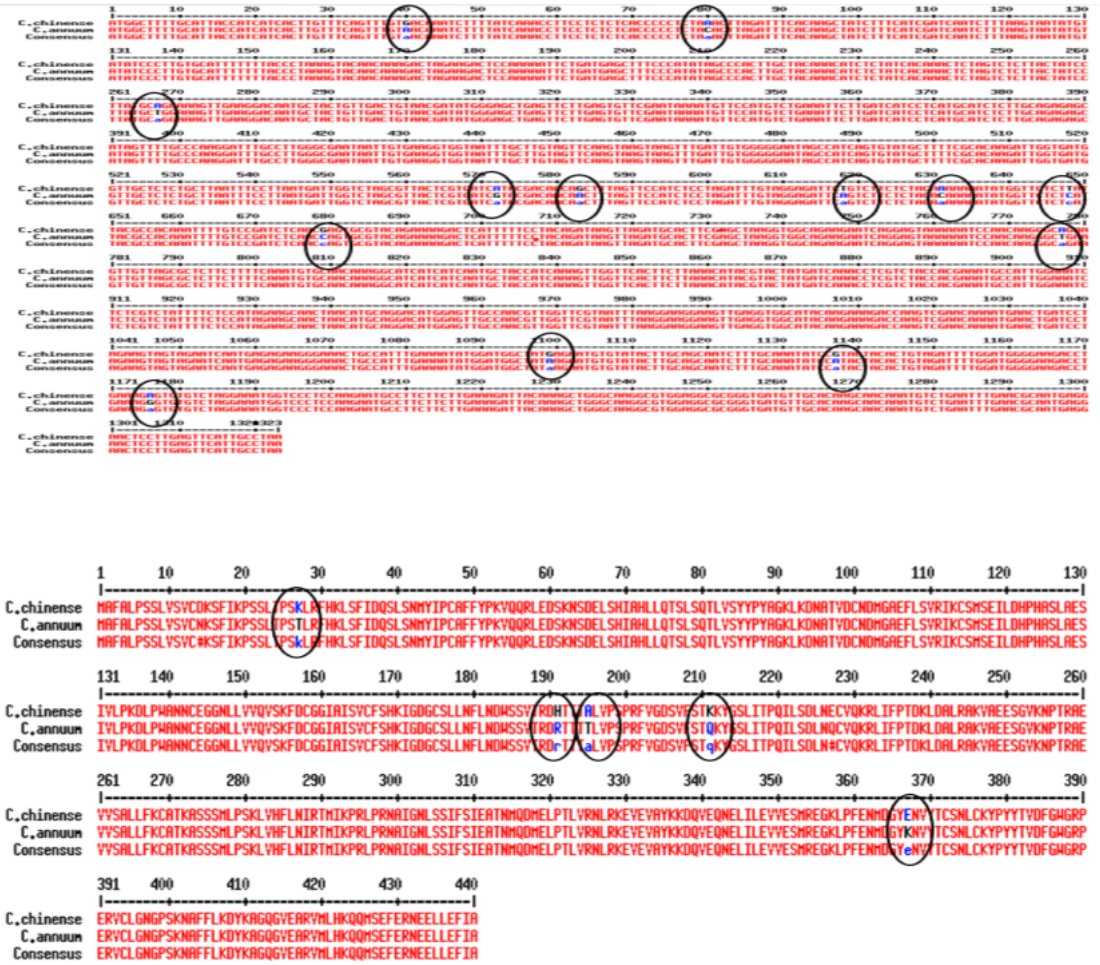
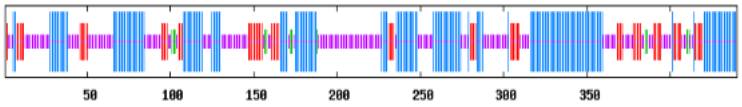


Figure 3

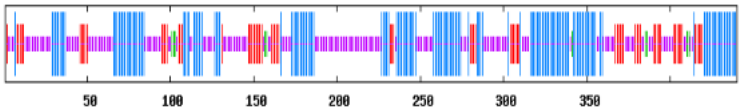
Figure legend not available with this version.

a




```
SOPMA :
   Alpha helix      (Hh) :   174 is  39.55%
   3₁₀ helix        (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    72 is  16.36%
   Beta turn        (Tt) :    12 is   2.73%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   182 is  41.36%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

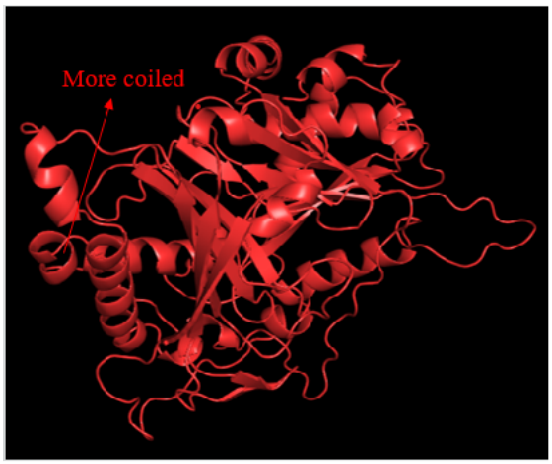
b




```
SOPMA :
   Alpha helix      (Hh) :   164 is  37.27%
   3₁₀ helix        (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    69 is  15.68%
   Beta turn        (Tt) :    13 is   2.95%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   194 is  44.09%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

Figure 4

Figure legend not available with this version.



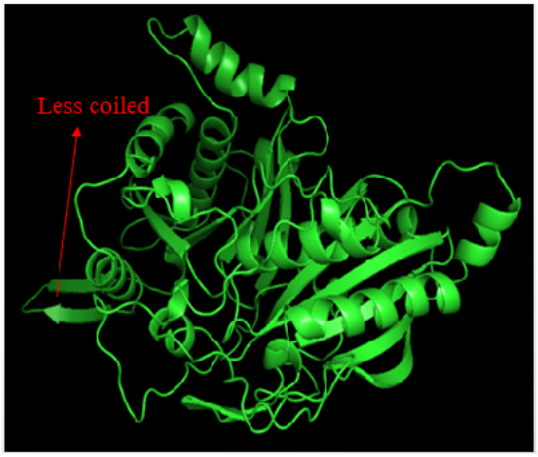


Figure 5

a (*Capsicum chinense)*

b (*Capsicum annuum)*