ORIGINAL ARTICLE

Contrasting modes for loss of pungency between cultivated and wild species of *Capsicum*GM Stellari^{1,4}, M Mazourek^{2,4} and MM Jahn³¹ Department of Biochemistry, Life Science Building, SUNY Stony Brook, Stony Brook, NY, USA; ²Department of Plant Breeding, Bradfield Hall, Cornell University, Ithaca, NY, USA and ³Department of Agronomy and Genetics, Agriculture Hall, Madison, WI, USA

Studies documenting the inheritance of pungency or 'heat' in pepper (*Capsicum* spp.) have revealed that mutations at a single locus, *Pun1*, are responsible for loss of pungency in cultivars of the two closely related species *Capsicum annuum* and *Capsicum chinense*. In this study, we present the identification of an unreported null allele of *Pun1* from a non-pungent accession of *Capsicum frutescens*, the third species in the *annuum*–*chinense*–*frutescens* complex of domesticated *Capsicums*. The loss of pungency phenotype in *C. frutescens* maps to *Pun1* and co-segregates with a molecular marker developed to detect this allele

of *Pun1*, *pun1*³. Loss of transcription of *pun1*³ is correlated with loss of pungency. Although this mutation is allelic to *pun1* and *pun1*², the mutation causing loss of pungency in the undomesticated *Capsicum chacoense*, *pun2*, is not allelic to the *Pun1* locus as shown by mapping and complementation studies. The different origins of non-pungency in pepper are discussed in the context of the phylogenetic relationship of the known loss of pungency alleles.

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Introduction

Capsaicin, an alkaloid derived from the phenylpropanoid and fatty acid biosynthetic pathways, is responsible for the sensation of burning and pain experienced when consuming pungent ('hot') peppers (Bennett and Kirby, 1968; Leete and Loudon, 1968; Nelson, 1919a, b). Peppers are a widely traded spice; perhaps the most commonly consumed spice in the world, making capsaicin one of the most commonly ingested plant secondary metabolites (Govindarajan, 1985; Hadacek, 2002). At the same time, non-pungent peppers are a valuable vegetable crop. This makes understanding the capsaicinoid biosynthetic pathway an extremely important task as both pungent and non-pungent peppers are valuable targets for vegetable crop improvement through plant breeding. Beyond value for applied purposes, the evolution of capsaicinoid biosynthesis is an intriguing example of the origin of a novel metabolism within the well-developed comparative system of the *Solanaceae*.

Biosynthesis of capsaicin is taxonomically restricted to the genus *Capsicum* and furthermore is confined to the developing pepper fruit, specifically the placental dissepiment (Eshbaugh, 1980; Suzuki *et al.*, 1980; Stewart *et al.*, 2007). Naturally occurring genetic variation in capsaicinoid biosynthesis is critical to the study of this biosynthetic pathway as there are only rare examples of the successful generation of transgenic pepper. The utilization of plant collections to further our understanding

of the genetic control of agronomically important traits is a critical component of a forward genetics approach. The *Capsicum* collection in the Plant Genetic Resources Conservation Unit is especially rich, housing over 4000 accessions and is the source of the mutants used in this study (United States Department of Agriculture).

It has been known for almost a century that a mutation at a single genetic locus, *Pun1* formerly *C*, underlies loss of pungency in the widely cultivated bell pepper, the fruit of the plant *Capsicum annuum*, and that this mutation has for an even longer period of time been exploited in the breeding of non-pungent peppers (Webber, 1911; Deshpande, 1934). Through candidate gene analysis, we have identified that the mutation that results in this loss of pungency is a deletion in the gene *AT3*. *AT3* encodes an acyltransferase protein belonging to the BAHD family of acyltransferases (Stewart *et al.*, 2005). *Pun1* is the only locus known to date to have a qualitative effect on pungency accumulation (Blum *et al.*, 2003). Capsaicinoid biosynthesis is environmentally variable, modulated by differences in growing environment, as well as fruit position on the plant (Zewdie and Bosland, 2000). Nonetheless, studies of the quantitative inheritance of capsaicinoid biosynthesis have shown that two major quantitative trait loci (QTL), *caps7.1* and *caps7.2* modulate quantities of capsaicinoid accumulation in red-ripe pepper fruit (Ben-Chaim *et al.*, 2006).

An integration of molecular genetic studies on plant secondary metabolites and their ecological role is somewhat rare. Seminal work has been performed in *Mimulus* where it has been shown that a QTL controlling plant color is responsible for differences in the pollination syndrome seen between species (Bradshaw *et al.*, 1995;

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Schemske and Bradshaw, 1999). In *Capsicum*, recent studies have furthered our understanding of the ecological role of capsaicin in natural populations. In Bolivia, researchers have identified populations of *Capsicum chacoense* and *Capsicum eximium*, two species thought to be basal in the genus, which are polymorphic for levels of capsaicinoid accumulation (Tewksbury *et al.*, 2006). In these populations, it has been shown that the differences in capsaicinoid accumulation are stable and heritable and that both levels of capsaicinoid accumulation and the percentage of plants that are pungent increases along a clinal gradient (Tewksbury *et al.*, 2006).

The goal of our study is twofold. First, we aim to determine the allelic relationship between a non-pungent accession of *Capsicum frutescens*, PI594141, identified in the ARS-Grin repository, and the other earlier characterized null alleles of *Pun1* identified in the cultivated species *C. annuum* (*pun1*) and *Capsicum chinense* (*pun1*²). Second, we aim to understand whether a different genetic locus may underlie loss of capsaicinoid biosynthesis in a non-cultivated species of *Capsicum*, *C. chacoense*, PI260433. This work builds upon the earlier developed genomic and genetic resources available in *Capsicum* and furthers our understanding of capsaicinoid biosynthesis by identifying novel alleles and loci with a qualitative effect on pungency and by describing their relationship with the knowledge gained about the ecology and evolution of the genus.

Materials and methods

Plant growth and tissue collection

The following accessions, F₁ hybrids (see Tables 1 and 4) and select F₂ individuals (see Table 2) were grown from seed in the Guterman facility at Cornell University, Ithaca, NY, USA and SUNY Stony Brook, Stony Brook, NY, USA: *C. frutescens* PI594141 pungent (*C. frutescens* PI594141-P), *C. frutescens* PI594141 non-pungent (*C. frutescens* PI594141-np), *C. chacoense* PI260433 pungent (*C. chacoense* PI260433-P),

C. chacoense PI260433 non-pungent (*C. chacoense* PI260433-np), *C. chinense* Habanero, *C. chinense* NMCA30036 and *C. frutescens* BG2814-6. Growing conditions were approximately 27/18 °C (day/night) with supplemental lighting. Plants were fertilized weekly with Blossom Booster (JR Peters Inc., Allentown, PA, USA) at Stony Brook and continuously at Ithaca with Excel (200 p.p.m.; The Scotts Company, Marysville, OH, USA). For genetic studies in the field, plants were sown in the Ithaca greenhouse and then transplanted to fields located in Varna, NY, USA during the summers of 2007 and 2008.

Inverse PCR

Inverse PCR (I-PCR) was performed to identify the novel mutation at *Pun1* in *C. frutescens* PI594141-np based on the protocol by Pham *et al.* (1999) with some modifications. Genomic DNA from *C. frutescens* PI594141 accessions was extracted using a Qiagen Plant DNA extraction kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. Approximately, 250 ng of genomic DNA was digested with *Xho*I (NEB, Ipswich, MA, USA). After heat inactivation of the enzyme, DNA was then purified using a GFX PCR DNA and Gel Band Purification Kit (GE, Healthcare Biosciences Inc., Piscataway, NJ, USA). The purified DNA was self-ligated with T4 ligase incubated at 16 °C overnight and ethanol precipitated. Nested I-PCR amplification primers, anchored in the known region of *pun1*³ in *C. frutescens* PI594141-np were designed using the online module from Primo Inverse 3.4 available through Chang BioScience (Chang BioScience Inc., Castro Valley, CA, USA, <http://www.changbioscience.com/primo/primoinv.html>). A first round of PCR was performed as per the following protocol: 5 µl of 10X PCR Buffer, 4 µl of 2.5 mM dNTPs, 2 µl of 10 µM I-PCR 1F (5-TACCCAACCCCCAACTAT AGG-3), 2 µl of 10 µM I-PCR 1R (5-ACTTGTA GTTTTTCGGAATGAAAAG-3) and in a separate reaction, 2 µl of 10 µM I-PCR 1F with 2 µl of 10 µM I-PCR 2R (5-GTAGTTTTTCGGAATGAAAAGTACTG-3), 0.25 µl

Table 1 Single locus goodness-of-fit test for loss of pungency in *Capsicum frutescens*

Generation	Total	# Pungent		# Non-pungent		Expected ratio	χ^2	df	P-value
		n ^a	Average (low–high range)	n	Average (low–high range)				

<i>C. frutescens</i> BG2814-6 × <i>C. frutescens</i> PI594141-np field grown									
P _a S ₁	4	4	3121 (1877–5492) ^b	0					
P _b S ₁	4	0		4	(ND) ^c				
F ₁	1	1	3294	0					
F ₂	60	48	5220 (672–11475)	12	(ND)	3:1	0.8	1	0.37
<i>C. frutescens</i> BG2814-6 × <i>C. frutescens</i> PI594141-np greenhouse grown									
P _a S ₁	1	1	12794	0					
P _b S ₁	5	0		5	(ND)				
F ₁	1	1	3294	0					
F ₂	41	33	5934 (97–19 557)	8	(ND)	3:1	0.7	1	0.40
<i>C. frutescens</i> BG2814-6 × <i>C. frutescens</i> PI594141-np pooled									
P _a S ₁	5	5	5506 (1877–12 794)	0					
P _b S ₁	9	0		9	(ND)				
F ₁	1	1	3294	0					
F ₂	101	81	5511 (97–19 557)	20	(ND)	3:1	1.46	1	0.22

^an represents the number of plants in each genotypic category.

^bNumbers in parentheses represent the minimum–maximum capsaicinoid levels in parts per million (p.p.m.) for the genotypic category.

^cValues below the detection limit of the assay (1 p.p.m.) were designated non-detectable (ND).

Table 2 Single-locus goodness-of-fit test for loss of pungency in *Capsicum chacoense*

Generation	Total	# Pungent		# Non-pungent		Expected ratio	χ^2	df	P-value
		n ^a	Average (range low–high)	n	Average (range low–high)				
<i>C. chacoense</i> PI260433-np × <i>C. chinense</i> Habanero									
P _a S ₁	4	0		4	5.28 (3–23) ^b				
P _b S ₁	0	5	23 904 (21 737–28 276)	0					
F ₁	7	7	6212 (161–13 292)	0					
F ₂	51	42	7848 (49–42 605)	9	ND (ND–15) ^c	3:1	1.47	1	0.23

^an represents the number of plants in each genotypic category.

^bNumbers in parentheses represent the minimum–maximum capsaicinoid levels in parts per million (p.p.m.) for the genotypic category.

^cValues below the detection limit of the assay (1 p.p.m.) were designated non-detectable (ND).

of ExTaq (Takara Bio Inc. Otsu, Japan), 2 µl of ligated DNA and H₂O to a final volume of 50 µl. A touch-down PCR protocol was used for amplification with cycles as follows: 3 min at 94 °C, 94 °C for 30 s, starting at 60 °C for 1 min, and reduced 1 °C for 9 cycles to reach an annealing temperature of 51 °C, 72 °C for 4 min 30 s, followed by 27 cycles of 94 °C for 30 s, 52 °C for 1 min, 72 °C for 4 min 30 s, with a final extension at 72 °C for 15 min. In all, 2 µl of the primary PCR was amplified with primers I-PCR 2F (5-TAGTATCAACATCACACCTAGAAGATG-3), I-PCR 2R (5-GTAGTTTTTCGGAAATGAAAAGTACTG-3) using the same PCR conditions as stated above. The two reverse primers produced PCR products of roughly the same size. Approximately, 8 kb PCR products were sent for direct sequencing at the SUNY Stony Brook DNA Sequencing Facility (<http://www.osa.sunysb.edu/dna/>). A series of primers were designed sequentially in order to complete the primer walk of the PCR product.

RNA extraction

The tissue was collected and frozen immediately in liquid nitrogen, ground to a powder, and stored at –80 °C. RNA was extracted from all tissue types using a Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. On column digestion of genomic DNA was performed using the Qiagen RNase-Free DNase set following the manufacturer's protocol. Approximately, 100 mg of frozen ground tissue was used for each extraction. RNA was denatured and visualized on an agarose gel to assess quality. Quantity was assessed using a NanoDrop (Nanodrop, Wilmington, DE, USA).

Reverse transcriptase-PCR

First strand complementary DNA (cDNA) was synthesized using the Protoscript First Strand cDNA Synthesis Kit from New England Biolabs (New England Biolabs, Ipswich, MA, USA) as per the manufacturer's instructions. In all, 500 ng of total RNA was used as starting material for each reaction. First strand cDNA was diluted in nuclease free water at a 1 to 10 dilution and used for RT-PCR. *Ubiquitin* Conjugating Enzyme (E2) F and R primers were designed as positive controls for cDNA synthesis and amplification based on an expressed sequence tag sequence (accession number DQ924970) identified in GenBank (Benson *et al.*, 2009). *pun1*³-specific PCR primers that span the intron–exon boundaries were designed based on an alignment of the *C. frutescens* PI594141-P and *C. frutescens* PI594141-np *pun1*³

sequences in order to exclude both genomic DNA contamination and contamination from *catf2*, a homolog to *AT3-1* known to be expressed in developing pepper fruits but that does not co-segregate with pungency (Garces-Claver *et al.*, 2007). A PCR reaction designed to simultaneously amplify both the coding region of *pun1*³ (680-base pairs (bp) band) and *Ubiquitin* (129-bp band) was performed as per the following protocol: 5 µl of 10X Buffer, 4 µl of 2.5 mM dNTPs, 1 µl of 10 µM *pun1*³ RT-PCR forward (5-TGGCAGTTTCCCTCTCTC-3), 1 µl of 10 µM *pun1*³ RT-PCR reverse (5-GGGAATAGCCATCAGTGTATGCTTTTCG-3), 1 µl of 10 µM *Ubiquitin* RT-PCR forward (5-TGTGTCTCAACATTCTTCGTGA-3), 1 µl of 10 µM *Ubiquitin* RT-PCR reverse (5-ATACAGCAGCTGCGTCTG-3), 0.25 µl of ExTaq (Takara Bio Inc.), 1 µl of 1:10 cDNA dilution, H₂O to a final volume of 50 µl, and cycling with the following conditions: 94 °C for 3 min, 29 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 30 s, with a final extension at 72 °C for 15 min. Reactions were performed using a PTC 225 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA).

Genotyping of *C. frutescens* BG2814-6 × *C. frutescens* PI594141 non-pungent population

DNA from F₂ segregating populations was extracted using a modified CTAB protocol (Doyle and Doyle, 1990). Polymorphic simple sequence repeat (SSR) markers known to flank the *Pun1* locus on pepper chromosome 2 (<http://www.sgn.cornell.edu>) were used for genotyping: CA514272 (forward: 5-ATCTATTTTCC TCCGGCGAC-3, reverse: 5-CGGTAAGCTGCCTTGATC TC-3) and CA514621 (forward: 5-GTCGAACAAAATGG GGTTTG-3, reverse: 5-GCTGGAGAGTGCTGGTGG-3). PCR conditions were 94 °C for 3 min; 29 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; with a final extension at 72 °C for 5 min. Polymorphic bands were separated using denaturing polyacrylamide gel electrophoresis with 6% acrylamide and visualized using silver staining.

A co-dominant marker specific to the *pun1*³ mutation was designed to genotype the segregating F₂ populations for both the greenhouse and field populations (see Figure 2). The *pun1*³-specific reverse primer was anchored in the inactivating mutation found in *C. frutescens* PI594141-np. A common forward primer was used that would enable amplification of both mutant and wild-type alleles. This forward primer was paired with a reverse primer anchored in the second exon of *C. frutescens* PI594141-P that is located in the deleted region of *C. frutescens* PI594141-np. PCR conditions

were as follows: 2.5 µl of 10X PCR buffer, 2 µl of 2.5 mM dNTPs, 1 µl of 10 µM *Pun1* forward (5-GTAGTTTTTCGG AAATGAAAAGTACTG-3), 1 µl of 10 µM *Pun1* 6R reverse (5-CACGCCTTGCCAGCTTTGTAATCTTTC-3), 1 µl of 10 µM *pun1*³ reverse (5-TCATGTCCATTCGGCCAAACA GTG-3), 0.25 µl of ExTaq (Takara Bio Inc.), 2 µl of genomic DNA solution and H₂O to a final volume of 25 µl. PCR cycles were as follows: 94 °C for 3 min, 34 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 30 s, with a final extension at 72 °C for 15 min. Products were visualized on a 1.2% agarose gel.

Genotyping of *C. chacoense* PI260433 non-pungent × *C. chinense* Habanero

Polymorphic markers co-segregating with the non-pungent phenotype were identified using bulk segregant analysis (Michelmore *et al.*, 1991). Approximately, 30 markers (<http://sgn.cornell.edu>) sampling each of the 12 pepper chromosomes and both chromosome arms were screened using pooled F₂ DNA.

A single marker, HPMS1-172 (forward primer: 5-GGG TTTGCATGATCTAAGCATTTT-3, reverse primer: 5-CG CTGGAATGCATTGTCAAAGA-3) (<http://www.sgn.cornell.edu>) co-segregated with the non-pungent bulk. This marker has been shown to map to Pepper Chromosome 7 in a region with a known QTL affecting capsaicinoid accumulation (Ben-Chaim *et al.*, 2006, Blum *et al.*, 2003). This marker was scored on the entire population along with conserved orthologous sequence (COS)II markers that are known to be located on the same arm of chromosome 7 (Wu *et al.*, 2009) according to protocols described therein. In that study, polymorphisms were selected that discriminated *C. frutescens* and *C. annuum* haplotypes, and therefore the markers needed to be adapted to a cross between *C. chinense* and *C. chacoense*. Amplified fragments were excised and gel purified using the GFX PCR DNA and Gel Band Purification Kit (GE, Healthcare Biosciences Inc.), cloned into a pCR4 TOPO TA vector and used to transform chemically competent TOP10 cells according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA). Individual colonies were prepared by Miniprep using the Bioneer Plasmid Extraction Kit (Bioneer, Alameda, CA, USA) and sequenced at the OSA Sequencing Facility at SUNY Stony Brook (<http://www.osa.sunysb.edu/dna/>).

Restriction enzyme site differences were used to distinguish the *C. chacoense* PI260433-np allele from the *C. chinense* Habanero allele for each marker screened. PCR was performed as above and restriction enzyme digests were performed as follows: 20 µl of PCR product, 3 µl of the appropriate buffer, 1 µl of enzyme, 3 µl of 10X bovine serum albumen and H₂O to a final volume of 30 µl, incubated at 37 °C for 3 h and visualized by agarose gel electrophoresis. One COSII marker polymorphism was found that was linked to pungency, At2g24270. The PCR product obtained from COSII At2g24270 showed a restriction enzyme polymorphism difference when digested with *Afl*III and therefore was used to genotype the *C. chacoense* PI260433-np × *C. chinense* Habanero population.

A co-dominant marker specific to the *Pun1* locus was designed to genotype the *C. chacoense* PI260433-np × *C. chinense* Habanero F₂ population. PCR was performed

as stated above using the following primer pair *Pun1* F (5-GGTCTAGCGTTACTCGTGATCATACG-3) and *Pun1* R (5-TCAAACACCACAAAAGACTTGGGA-3) followed by digestion of the PCR product with *Sca*I.

Map location identification

MapMaker/EXP v. 3.0b (MapMaker Exp., The Whitehead Institute, Cambridge, MA) was used to identify map locations and chromosomal distances and results were compared with the pepper FA03 map (<http://www.sgn.cornell.edu>). Map distances for the *C. frutescens* BG2814-6 × *C. frutescens* PI594141-np population were calculated using the following set of commands: 'group', 'pair' and 'sequence' and as only two most likely map orders were identified, both of which were symmetric, the command 'map' was used to identify the best possible map distances.

For the population *C. chacoense* PI260433-np × *C. chinense* Habanero the command 'LOD table' was used to identify even marginal linkage between the chromosome 2, chromosome 7 markers and the capsaicinoid accumulation phenotype. As linkage was found between a COSII marker located on chromosome 7 and the pungency phenotype, the parameters LOD 2, 30 cM were chosen for the 'group' command. Two linkage groups were identified, which were then sequenced using the command 'sequence' followed by the command 'map' to identify the best possible map distances.

Capsaicinoid detection

Capsaicinoids were measured in mature dry fruit using a capsaicinoid detecting enzyme-linked immunosorbent assay kit from Beacon Analytical (Beacon Analytical, Portland, ME, USA). Capsaicinoids were extracted and measured as per the manufacturer's instructions. When feasible each sample was measured multiple times. Capsaicinoid content as low as 1 p.p.m. is consistently and reliably detectable using the method described above, and has been found to be highly correlated with high-performance liquid chromatography measurements of capsaicinoids in earlier studies (Jarret *et al.*, 2003).

Initial alignment

Novel *Pun1* sequences identified in the course of this study, *C. frutescens* PI594141-np and *C. chacoense* PI260433-P were deposited in GenBank (accession numbers FJ871985 and FJ871984, respectively). In all, 21 *Pun1* sequences (accession numbers: AY819026, AY819027, AY819029, FJ687524-FJ687531, FJ755160-FJ755176) that were originally identified as part of another study (Fellman *et al.* unpublished data) were retrieved from GenBank. These sequences included those from *Capsicum* spp. as well as from representative taxa in the Solanaceae and the outgroup sequence from *C. annuum*, *catf2*. The initial alignment was compiled using the program DIALIGN (<http://bibiserv.techfak.uni-bielefeld.de/dialign/>) and refined by hand (Morgenstern, 2004). Despite the absence of full-length sequence, *C. annuum catf2* was chosen as the outgroup for the data set because of its high degree of nucleotide sequence similarity with *AT3. catf2* expression does not co-segregate with pungency and does not map to the same genomic region as *Pun1* (Garces-Claver *et al.*, 2007). This first alignment of 21 sequences and 1876 characters

contained the 5' UTR of *AT3* and the coding regions of *AT3* from the putative start codon to approximately 100 bp upstream of the stop codon, including the intron that is highly conserved and can be easily aligned. The alignment was truncated in the second exon at the site of the mutation where the *C. frutescens* PI594141-np sequence lost all similarity to the remaining sequences.

ModelTest

Before generating the Bayesian phylogenies, the best model of evolution was determined using the program ModelTest v3.7 (Posada and Crandall, 1998). Three separate best-fit models were estimated: the first for the first exon of *AT3*, the second for the intron and the third for the second exon. ModelTest v3.7 estimated three best-fit models for the data. The first exon best fit the model with six rate classes, general time reversible with a gamma distribution of the rate variation among sites and no invariable sites (Tavare, 1986). The intron and second exon sequences best fit the model with a single rate class, F81, equal rates of nucleotide frequencies and the proportion of invariable sites equal to zero (Felsenstein, 1981).

Bayesian analysis

Bayesian analysis of the alignment including the introns was performed in MrBayes v3.1.2 by creating three data partitions in the alignment, one for the first exon, one for the second exon and one for the intron (Ronquist and Huelsenbeck, 2003). Each partition was assigned its best-fit model per ModelTest v3.7 and rates were allowed to vary independently between partitions. *catf2* was set as the outgroup. A total of four chains of the Markov Chain Monte Carlo were run, sampling one tree every 100 generations. The analysis was allowed to proceed until the s.d. of split frequencies fell below 0.01. A fixed proportion of trees comprising 25% of all trees sampled were discarded as the 'burn in' fraction. A majority rule consensus tree of all trees sampled, excluding the burn in fraction, was computed using PAUP v. 4.0b10 (Swofford, 2002).

Results

Tests for linkage between *Pun1* and loss of pungency in *C. frutescens* PI59414 and in *C. chacoense* PI260433

Two non-pungent accession of *Capsicum* were identified in the germplasm collections and in an earlier publications and represent the starting germplasm used for the characterization of loss of pungency in *C. frutescens* and *C. chacoense* (Votava and Bosland, 2002; Tewksbury *et al.*, 2006). PI594141 was originally identified as *C. eximium*, but recently reassigned to *C. frutescens*. A careful inspection of the fruit and flowers agree with this revision (M. Nee and L. Bohs, personal communication). Tasting of ripe fruit in an initial planting of both *C. frutescens* PI594141 and *C. chacoense* PI260433 showed that these accessions contained both pungent and non-pungent individuals as has been noted for *C. chacoense* populations in South America (Tewksbury *et al.*, 2006). A single pungent and non-pungent plant was selected from each accession and propagated by single seed descent for three generations. The *C. frutescens* selection progeny had no detectable capsaicinoids by taste or enzyme-linked

immunosorbent assay for capsaicinoids, but the *C. chacoense* plants consistently accumulated trace levels of capsaicinoids between 1 and 10 p.p.m., which were correlated with a barely detectable organoleptic sensation (see Table 2). Capsaicinoids produce a sensation of pungency at concentrations as low as 10 p.p.m., but are detectable on the tongue at concentrations as low as 1 p.p.m. (Andrews, 1984). Therefore, although we refer to the *C. chacoense* line as 'non-pungent,' it is not a complete knockout mutant but rather a very dramatic knockdown mutant.

To establish the number of loci responsible for loss of pungency and to identify map location(s) for this trait, *C. frutescens* PI594141-np was crossed to a highly pungent *C. frutescens*, BG2814-6, which has been used extensively in mapping experiments (Ben-Chaim *et al.*, 2006, www.sgn.cornell.edu). Despite performing numerous manual cross-pollinations between *C. frutescens* BG2814-6 and *C. frutescens* PI594141-np, only a single F₁ plant germinated from this cross, likely related to the extremely low fruit set and seed content of PI594141-np. Earlier studies have documented barriers to performing interspecific crosses in *Capsicum*; difficulty in performing crosses seems to depend upon on the direction of the cross and the genotypes chosen as parents (Pickersgill, 1971; Kumar *et al.*, 1988; Walsh and Hoot, 2001). Fruits from this F₁ plant were uniformly pungent and comparable to the levels of pungency seen in the pungent parent, revealing that loss of pungency in *C. frutescens* PI594141 is recessive (Table 1). Segregation of pungency in two F₂ populations grown under field and greenhouse conditions showed that the ratio of pungent to non-pungent plants is consistent with a 3:1 ratio suggesting Mendelian inheritance of a single recessive locus causing loss of pungency in *C. frutescens* PI594141 (Table 1).

As an initial test of the hypothesis that *C. frutescens* PI594141 is non-pungent because of an allele of *pun1*, a map location for loss of pungency was established for the cross between *C. frutescens* BG2814-6 × *C. frutescens* PI594141-np. SSR markers known to flank the *Pun1* locus in the cross between *C. frutescens* BG2814-6 and *C. annuum* 'R Naky' were used to show that loss of pungency in *C. frutescens* PI594141-np maps to the same interval as *Pun1* (Figure 1). Further, a marker based on a polymorphism within the *Pun1* locus perfectly cosegregated with loss of pungency in this cross as described below. This suggested that the earlier unreported loss of pungency event in *C. frutescens* PI594141-np is due to a mutation at the *Pun1* locus and was therefore tentatively designated *pun1*³.

A test of genetic linkage between loss of pungency in *C. chacoense* PI260433-np and the *Pun1* locus was attempted in a cross to *C. frutescens* BG2814-6. Several F₁ plants based on this cross germinated but produced only parthenocarpic fruit. *C. chacoense* PI260433-np was then crossed to the highly pungent *C. chinense* 'Habanero'. Several F₁ individual plants were obtained all of which were pungent, indicating that loss of pungency in this accession is also recessive (Table 2). Although numerous seeds were obtained from these F₁ plants, few germinated (<20%) and of those that germinated, some did not set fruit in the F₂ generation. Despite the difficulty in crossing a wild non-pungent *Capsicum* species to a cultivated variety within the *annuum*, *chinense* and *frutescens* species complex, segregation of

loss of pungency in the F₂ population showed that loss of pungency in *C. chacoense* PI260433-np is consistent with a 3:1 ratio, suggesting that a single recessive mutation controls loss of pungency in this accession (Table 2).

In this population, segregation of loss of pungency with the *Pun1* locus was tested using a CAPS marker designed based on a restriction site polymorphism identified in the intron of *Pun1* (Figure 1b) and the same SSR markers known to flank the *Pun1* locus (Figure 1a).

Figure 1 Map position of loss of pungency in *C. frutescens* PI594141-np. (a) The loss of pungency phenotype in an F₂ population derived from a cross between *C. frutescens* BG2814-6 and *C. frutescens* PI594141-np mapped between simple sequence repeat (SSR) markers CA514272 and CA514621 on pepper chromosome 2. This map order was colinear with the corresponding region in a cross between *C. frutescens* BG2814-6 and *C. annuum* 'R Naky' (www.sgn.cornell.edu, FA03 map). (b) CAPS marker used to genotype the *Pun1* locus in the *C. chacoense* PI260433-np × *C. chinense* 'Habanero' population and in the *C. chacoense* PI260433-np × *C. chinense* NMCA30036 F₂ populations is shown.

The CAPS marker designed to distinguish between the parental alleles of *Pun1* was not linked with the pungency phenotype, nor were the flanking SSR markers, establishing that loss of pungency in *C. chacoense* PI260433-np maps to a region other than the *Pun1* locus and was tentatively designated *pun2*.

Allelism of loss of pungency mutations in *C. frutescens* PI594141 and in *C. chacoense* PI260433

The results of the *Pun1* linkage analysis suggest that loss of pungency in *C. frutescens* PI594141 was due to a mutation at the *Pun1* locus whereas in *C. chacoense* PI260433 the mutation was attributed to a locus other than *Pun1*. This hypothesis was tested by using a complementation test including all known loss of pungency mutations in pepper, all of which are monogenic and recessive (Stewart et al., 2005, 2007). Although interspecific barriers are known in *Capsicum* (Pickersgill, 1971; Onus and Pickersgill, 2004), crosses were performed in all possible combinations and hybrids were recovered between *C. annuum* and *C. chinense*, *C. chinense* and *C. frutescens*, and *C. chacoense* and the *annuum*–*chinense*–*frutescens* species complex. Two complementation groups were defined, *pun1* and *pun2* (Table 3). As no significant or measurable levels of capsaicinoid accumulation were detected in fruit from F₁ plants resulting from the crosses between *C. frutescens* PI594141-np and the other reported non-pungent accessions, the results of the experiment shown in Table 3 show that the mutation in the accession *C. frutescens* PI594141-np fails to complement the *pun1* and *pun1*² mutations (Table 3). This establishes that loss of pungency in *C. frutescens* PI594141-np is allelic to the other mutations at the *Pun1* locus and this novel allele of *Pun1* is named *pun1*³. Loss of pungency in *C. chacoense* PI260433-np complements the *pun1*, *pun1*² and *pun1*³ mutations. This confirms our hypothesis that loss of pungency in *C. chacoense* PI260433-np is due to a mutation at a novel loss of pungency locus, which we name *pun2*. Earlier studies indicated a link between *pun1* mutations and the formation of a capsaicinoid accumulating regions known in the literature as glands, vesicles or blisters (Stewart et al., 2007). We were unable to examine this relationship for the *pun1*³ mutation because of interference from the dominant soft flesh mutation that segregated in this cross (Rao and Paran, 2003).

Both *Pun1* and *Pun2* are required for pungency; therefore, in an F₂ population with their mutant alleles segregating, we would expect a 9:7 ratio that is characteristic of complementary gene action. An F₂

Table 3 Complementation test results between *Capsicum* pungency mutants

Genotype	Female parent			
	<i>C. annuum</i> ECW	<i>C. chinense</i> NMCA30036	<i>C. frutescens</i> PI594141-P	<i>C. chacoense</i> PI260433-np
Male parent				
<i>C. annuum</i> ECW	ND			
<i>C. chinense</i> NMCA30036	ND*	ND	ND	
<i>C. frutescens</i> PI594141-np			ND	
<i>C. chacoense</i> PI260433-np	3032*	5122 (4195–6248)	6114 (4465–7812)	1.83 (ND–2.6)

Each cell shows the average value and in parentheses the minimum–maximum capsaicinoid levels in parts per million (p.p.m.) of fruit from duplicate measurements of at least three plants unless three plants were not available. *Cells left blank indicate crosses that could not be performed. ND indicates capsaicinoids not detectable.

Table 4 Epistatic interactions between the *Pun1* and *Pun2* loci

Genotype	n	Pungency (p.p.m.)
<i>Pun1</i> /—	10	1215, (151–5638)
<i>Pun1</i> /—	4	5, (2–11)
<i>pun1</i> ² / <i>pun1</i> ²	5	3, (ND–12)

Each row shows the genotype at the *Pun1* locus, the number of plants in each genotypic category (n), the average capsaicinoid accumulation in parts per million (p.p.m.) and in parentheses the minimum–maximum capsaicinoid levels in p.p.m. ND indicates capsaicinoids not detectable.

population resulting from the cross between *C. chacoense* PI260433-np (*pun2*) and *C. chinense* NMCA30036 (*pun1*²), which is allelic to the mutation in *C. frutescens* PI594141-np, was grown in the field, phenotyped for capsaicinoid accumulation and genotyped at the *Pun1* locus (Table 4). The *pun1*² mutation is found in a *C. chinense* background, and was used as the *Pun1* mutant for this cross because in our hands, *C. chinense* has better interspecific fertility with *C. chacoense* PI260433 than *C. frutescens*, which harbors the *pun1*³ allele. The population that was obtained was too small to rigorously test a genetic ratio, but two pungency classes were observed with roughly equivalent numbers of individuals. Within the non-pungent class, individuals were observed of both *Pun1* and *pun1*² genotypes (Table 4), suggesting that *pun2* indeed has a recessive epistatic interaction with *Pun1*.

Genetic mapping of *pun2*

To identify the map location of *pun2*, bulk segregant analysis was then used to screen over 30 loci distributed throughout the pepper genome, sampling each chromosome arm (Figure 4). By bulk segregant analysis, one marker, Hpms1-172 (www.sgn.cornell.edu), was identified, but could only be genotyped as a dominant marker as more than one locus was amplified in this cross. Data from other mapping populations showed that the marker Hpms1-172 maps to the upper arm of pepper chromosome 7 on the FA03 map (www.sgn.cornell.edu). Additional markers were sought for this region to confirm this map location for *pun2*. As few SSR markers were available for this genomic region that were conserved or polymorphic between the two parents, COSII markers known to map near Hpms1-172 were screened (Mueller et al., 2005; Wu et al., 2009). Despite the use of an interspecific cross for this population, polymorphism was limiting. However, linkage between COSII marker C2_At2g24270 and loss of pungency was observed (19.8 cM, LOD 2.3). A major QTL controlling levels of capsaicinoid accumulation has been mapped to this region in a segregating population between *C. frutescens* BG2814-6 × *C. annuum* 'R Naký' (Ben-Chaim et al., 2006) suggesting that *pun2* may therefore represent an allele of that locus.

Sequence of the *Pun1* locus in *C. frutescens* PI594141 non-pungent and in *C. chacoense* PI260433 non-pungent. Having established that loss of pungency in *C. frutescens* PI594141 mapped to the same genomic region as the *Pun1* locus, *AT3*, the candidate gene underlying the *Pun1*

locus, was sequenced to identify any possible sequence polymorphisms that could be associated with loss of pungency in this accession (Stewart et al., 2007). I-PCR revealed that in *C. frutescens* PI594141-np *AT3* shows a large sequence deletion when compared with the wild-type sequence obtained in the sister line *C. frutescens* PI594141-P and other known *Pun1* sequences. *AT3* from *C. frutescens* PI594141-np has a deletion of terminal 70 amino acids that are replaced by a repetitive region showing numerous SSRs and long repetition of the bases adenine and thymine (Figure 2). The repetitive sequence identified modifies the putative amino acid sequence at this locus and introduces a stop codon 64 amino acids from the position of the wild-type stop codon. This would lead to a truncated protein of 376 amino acids and lacking the DFGWGKP, which is characteristic for the BAHD family of acyltransferases and is present in the wild-type sequence from sister line *C. frutescens* PI594141-P (St-Pierre and De Luca, 2000). Despite obtaining over 3 kb of sequence downstream from the beginning of the interruption from *C. frutescens* PI594141-np *AT3*, we could not recover the final 210 bp for this sequence, and therefore could not detect the origin of the genomic interruption causing the mutation of this novel allele of *Pun1*.

Through comparative sequence alignment with the wild-type sequence in *C. frutescens* PI594141-P as well as other wild-type *Pun1* alleles, we were able to design a reverse primer anchored in the mutation in *C. frutescens* PI594141-np that in combination with primers anchored in conserved region of *Pun1*, allows for co-dominant genotyping (Figure 2). It further shows that loss of pungency in *C. frutescens* PI594141 can be attributed to a deletion in the coding region of the gene, consistent with the earlier identified mutations at the *Pun1* locus. In contrast, the coding region of *Pun1* in *C. chacoense* PI260433-np was established to be wild type and no obvious sequence polymorphisms were identified other than a 36-bp insertion in the intron, which is conserved between the pungent and non-pungent *C. chacoense* PI260433 lines, further suggesting that the *Pun1* locus is not responsible for loss of pungency in *C. chacoense* PI260433-np.

It is known that expression of the *Pun1* locus as well as other capsaicinoid structural genes mirrors the location and developmental time course of capsaicinoid accumulation in pepper fruit (Curry et al., 1999; Aluru et al., 2003; Stewart et al., 2005, 2007). The beginning of *Pun1* expression coincides with the beginning of capsaicinoid accumulation, peaking at around 20 days post anthesis. *Pun1* expression begins to decline around 40 days post anthesis when capsaicinoid accumulation has arrived at its maximum. RT-PCR using primers specific to the *Pun1* locus shows that *pun1*³ in *C. frutescens* PI594141-np is not expressed (Figure 3). In contrast, expression of *Pun1* in *C. frutescens* PI594141-P is prominent around 20 days post anthesis, declines significantly by 40 days post anthesis, and is overall consistent with the pattern of *Pun1* expression that has earlier been reported (Stewart et al., 2005). This is in contrast with what has been reported earlier for the *pun1*² mutation in *C. chinense* NMCA30036. In this non-pungent cultivar *pun1*² is weakly expressed as detected by northern blot hybridization (Stewart et al., 2007). However, translation of the locus appeared to be absent and no protein accumulation was detectable by western blot hybridization.

Figure 2 *pun1*³ genotyping. (a) The *pun1*³ mutation is a large insertion or deletion that results in a truncated second exon. Genotyping primers were designed based on this mutation that discriminated wild-type *Pun1* and *pun1*³. Primers 1 and 2 are anchored in exons, but primer 3 is located in the genomic sequence past the mutation site in *pun1*³. (b) The primer set shown in A allows for co-dominant genotyping of populations segregating for *pun1*³. Primer pair 1 and 3 produces a band of 1033 bp in the presence of the *pun1*³ allele, while primer pair 1 and 2 produces a band of 586 bp in the presence of a wild-type *Pun1* allele. The parental genotypes of *C. frutescens* BG2814-6 (P₁), *C. frutescens* PI594141-np (P₂) and their F₁ hybrid used as the parent of the F₂ can be distinguished. F₂ segregants are shown: *Pun1*/*Pun1* and pungent (P), *Pun1*/*pun1*³ and pungent and *pun1*³/*pun1*³ and non-pungent (np). No bands are found in the negative control (NC). (c) Capsaicinoid accumulation was measured by enzyme-linked immunosorbent assay (ELISA) in red-ripe fruits of the plants shown in B. No capsaicinoids were observed in *C. frutescens* PI594141-np or *pun1*³/*pun1*³ F₂ individuals. *Pun1*/*Pun1* and *Pun1*/*pun1*³ individuals had a wide range of capsaicinoid levels from less than 200 p.p.m. to more than 1000 p.p.m.

Figure 3 *Pun1* expression in fruit of wild type and mutant peppers. Pepper fruit were collected 20 and 40 days post anthesis (dpa) and RT-PCR was performed using primers that provided PCR amplification of both *Pun1* and *pun1*³ alleles from genomic DNA. Robust expression of *Pun1* was observed from 20 days post anthesis fruit that were pungent, but no expression was observed in *pun1*³, non-pungent fruit. Expression diminished in 40 days post anthesis pungent fruit. An internal *ubiquitin* conjugating enzyme (E2) control was equivalent in all samples. Neither band was produced in a negative control without complementary DNA (cDNA) (NC).

Genetic architecture of known *Pun1* alleles

Although cultivars of *C. annuum*, *C. chinense* and *C. frutescens* all share mutations at the same locus that

are responsible for loss of pungency, the nature and putative origin of each mutation seems to be quite different (Figure 4a). In *C. annuum* ECW (and other

Figure 4 Relationship of *Pun1* alleles in *Capsicum*. (a) *Pun1* alleles from *C. annuum*, *C. chinense* and *C. frutescens* differ by single-nucleotide polymorphisms (SNPs) scattered throughout the gene. The *Pun1* allele recovered in *C. chacoense* PI260433-np contains an intron insertion and several SNPs in the intron that were used for genotyping. As observed in *C. chacoense* and *C. chinense* F₂ progeny, pungency requires both *Pun1* and *Pun2* alleles. A large five' deletion produced *pun1*¹, a frameshift mutation in the second exon is found in *pun1*² and an indel truncates the second exon of *pun1*³. The *pun1*¹ and *pun1*³ mutations are not transcribed, while transcription but not translation has been observed for *pun1*². (b) Phylogeny of *Pun1* alleles of selected *Solanaceae* (GenBank accession numbers: FJ871984, FJ871985, AY819026, AY819027, AY819029, FJ687524-FJ687531, FJ755160-FJ755176). Bayesian phylogenetic analysis of 21 *Pun1* alleles from a diverse sampling of species and cultivars within *Capsicum* as well as from domesticated and representative genera in the *Solanaceae* shows that the *pun1* series of null alleles are scattered and not clustered together into a single clade suggesting multiple origins of the loss of pungency alleles followed by lineage sorting. *Pun1* from *C. chacoense* PI260433-np is most closely related to the sister line *C. chacoense* PI260433-P suggesting possible gene flow between the two populations for this allele.

non-pungent cultivars), a 2.5kb deletion removes a majority of the first exon, and the putative promoter region thereby eliminating transcription and translation

of the locus. In *C. chinense* NMCA30036, a 4-bp deletion causes a frameshift mutation thereby reducing transcription and eliminating translation of the locus. Similarly,

in *C. frutescens* PI594141-np, the deletion of part of the second exon, including the stop codon introduces a frameshift mutation that correlates with loss of transcription of the locus. This mutation also perfectly cosegregates with loss of pungency in segregating populations contrasting for the trait. Although it is possible that the mutation in *C. annuum* was caused by secondary deletion after the mutation in *C. chinense*, the mutation in *C. frutescens* is clearly distinct from either of the other two as it occurs in the second exon, thus cannot have arisen from either the *pun1* or *pun1*² mutations, except by an unlikely process of reversion.

To test whether the *Pun1* null alleles all share a common origin despite showing different sequence interruptions, Bayesian phylogenetic analysis of the mutations was conducted using a data set of full-length *Pun1* sequences assembled in the course of a different study (Fellman, Stellari and Jahn, unpublished data). The phylogeny suggests that each null *Pun1* allele has a distinct phylogenetic origin and that each mutation arose independently. At the same time, the *Pun1* locus in *C. chacoense* PI260433-np, which is wild type in sequence is shown to be most closely related to the *Pun1* sequence from its pungent sister line *C. chacoense* PI260433-np, thereby suggesting gene flow between the pungent and non-pungent *C. chacoense* populations for this allele. This is consistent with the observation of intermixed populations of these plants in Bolivia (Tewksbury *et al.*, 2006). The placement of *C. frutescens* PI594141-np in the phylogeny suggests instead that this null-allele may not be most closely related to the allele identified in the pungent sister line. Furthermore, its phylogenetic placement implies that the allele arose sometime before the origin of the *annuum*, *chinense* and *frutescens* species complex and may have been maintained in *C. frutescens* PI594141 by lineage sorting. Although sequence sampling within the genus is scant in this study and does not allow for a detailed analysis of the evolution of *Capsicum*, the phylogeny suggests that the *pun1* and *pun1*² alleles are of ancient origin and predate the diversification of some of the species we currently recognize in the genus *Capsicum*.

Discussion

The *Pun1* locus had been the only locus identified that had a qualitative effect on pungency (Blum *et al.*, 2002). Identification of two instances in sister taxa where loss of pungency is caused by a mutation at the *Pun1* locus suggested that all naturally occurring loss of pungency mutations in the domesticated *Capsicums* would be mutations in the *Pun1* locus (Stewart *et al.*, 2005, 2007). In the effort to complete the characterization of loss of pungency in known non-pungent *Capsicum* accessions, we described the genetic basis for this trait in two non-pungent accessions. One of these was first identified in this study from the ARS-GRIN repository, *Capsicum frutescens* PI594141. The other, *C. chacoense* PI260433, was known from the literature to be the subject of extensive studies analyzing the ecological function of capsaicin (Votava and Bosland, 2002; Levey *et al.*, 2006; Tewksbury *et al.*, 2006). We hypothesized that there would be a difference in the genetic basis for loss of pungency in cultivated versus non-cultivated species in the genus

Capsicum consistent with the phylogenetic groupings of the species.

Our results show that the mutation in *C. frutescens* PI594141-np is allelic to the other two earlier identified loss of pungency loci found in the cultivated species of pepper whereas the mutation in *C. chacoense* PI260433-np is due to a mutation at a different genetic locus (Table 3). Interestingly the species *C. annuum*, *C. chinense* and *C. frutescens* have a close phylogenetic relationship, dubbed the *annuum*, *chinense* and *frutescens* species complex (Pickersgill, 1971; Walsh and Hoot, 2001; Olmstead *et al.*, 2008). The propensity for these three domesticated species to all share mutations at the *Pun1* locus leading to loss of pungency may reflect differences in the nature of selection that distinguishes wild populations from cultivated varieties or a genomic factor within the domesticated clade that predisposes mutations at the *Pun1* locus.

Alternatively, the *pun2* mutation could be part of another allelic series transferred to domesticated peppers. Loss of pungency in *C. chacoense* PI260433-np may be related to the *caps7.1* QTL as evidenced by preliminary map data (Ben-Chaim *et al.*, 2006). *C. chacoense* has been used extensively in the breeding of modern pepper cultivars as a source of genetic resistance to common pathogens. The *Bs2* gene on the lower arm of pepper chromosome 9 that confers resistance to bacterial spot caused by *Xanthomonas euvesicatoria* has been introgressed into *C. annuum* from *C. chacoense* PI 260435 (Tai *et al.*, 1999). The *L4* gene from *C. chacoense* PI260429 has been similarly used for resistance to several tobamoviruses including *Tobacco mosaic virus* and *Pepper mild mottle virus*. *L4* is thought to be a member of the NB-LRR class of plant resistance genes and occur in a cluster of paralogs on the bottom of pepper chromosome 11 (Tomita *et al.*, 2008). The *caps7.1* QTL could, therefore, be an ortholog of *pun2* with different expressivity or the result of a historical introgression of the *pun2* allele that has a qualitative rather than quantitative behavior in a Habanero background. Furthermore, the results of our experiments suggest that the *Pun2* locus is epistatic to *Pun1*. The trace levels of capsaicinoids detectable in the *pun2* mutant would be consistent with it being part of an allele series with different strengths of expression depending on the genetic background. The identification of the genetic basis for loss of pungency in *C. chacoense* PI260433-np and the *caps7.1* QTL should resolve this relationship.

This study shows that there is a difference in the genetic basis for loss of pungency in a 'wild' *Capsicum* species compared with that seen in non-pungent accessions from cultivated species. The resources described here and in earlier studies will allow the trends we observed regarding the association between species and loss of pungency mutations to be tested in other populations of un-cultivated peppers that are known to be polymorphic for pungency, such as *C. eximium* whose range overlaps with that of *C. chacoense* PI260433-np (Tewksbury *et al.*, 2006; Stewart *et al.*, 2005, 2007). It remains intriguing to speculate as to why the *Pun1* locus seems to be favored in generating null capsaicinoid biosynthetic mutants in cultivated species. Similar allelic series that affect anthocyanin biosynthesis have been found to be naturally occurring in grape (This *et al.*, 2007) and have been artificially generated by transposon

mutagenesis to affect carotenoid accumulation in maize (Singh *et al.*, 2003).

The history of artificial selection by humans in *C. annuum*, *C. chinense* and *C. frutescens* should not be discounted as a factor influencing *Pun1* either. Selection for fruit or seed characteristics could have affected the *Pun1* locus by virtue of their genetic linkage on pepper chromosome 2 (Ben Chaim *et al.*, 2001; Ben-Chaim *et al.*, 2006). The capsaicinoid biosynthetic pathway may also be metabolically linked to a trait that is under strong selective pressure for an agronomically desirable trait. There is evidence suggesting that natural selection on secondary metabolites has a heritable effect on plant biomass accumulation, highlighting that a tradeoff between carbon allocation to plant secondary metabolites and biomass exists in natural populations (Han and Lincoln, 1994). One could imagine a similar tradeoff existing under artificial selection for agronomically important traits at the expense of secondary metabolite accumulation. Interestingly, it has been found that the fruits of both pungent and non-pungent *C. chacoense* plants have a higher proportion of lipids, a characteristic of fruits favored by avian frugivores, than cultivars of *C. annuum* (Levey *et al.*, 2006); one moiety of the capsaicinoid molecule is a fatty acid. It is possible that the relationship between the capsaicinoid biosynthetic pathway and other traits of agronomic importance are mediated either through genes with pleiotropic function or by selection on related traits (Purugganan and Fuller, 2009).

Conclusions

The *Pun1* locus has a qualitative effect on capsaicinoid biosynthesis in cultivated varieties belonging to the species *C. annuum*, *C. chinense* and *C. frutescens*, which are thought to form a closely related species complex. In the course of this study, we have shown that loss of pungency in the accession *C. frutescens* PI594141-np is allelic to the mutations *pun1* and *pun1*², and thus designated *pun1*³, whereas the mutation in *C. chacoense* PI260433-np is not allelic to the other known *Pun1* mutations, and thus represents the identification of *pun2*. The loss of function mutation in *pun1*³ is a deletion of the terminal region of the second exon, including the canonical stop codon and the DFGWGKP site, characteristic of the BAHD enzyme family to which *Pun1* belongs. *pun1*³ perfectly co-segregates with loss of pungency and furthermore is associated with a loss of transcription of the *Pun1* locus. A phylogeny of the null alleles of *Pun1* suggests that each mutation has an independent origin predating the diversification of the species complex and that some alleles may have been maintained by lineage sorting.

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