Capsaicinoid and Carotenoid Composition and Genetic Diversity of *Kas I* and *Ccs* in New Mexican *Capsicum annuum* L. Landraces

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Abstract. Two key fruit qualities in *Capsicum annuum* are fruit pungency and color. We characterize 13 New Mexican landraces for fruit quality traits at both the chemical level measuring the capsaicinoids, dihydrocapsaicin, and capsaicin as well as five carotenoids, β -carotene, β -cryptoxanthin, zeaxanthin, violaxanthin, and capsanthin, and at the genetic level sequencing two genes in these landraces, *Kas I*, a capsaicinoid pathway gene, and *Ccs*, a carotenoid pathway gene. All of the landraces had unusually high levels of dihydrocapsaicin in comparison with capsaicin levels. The levels of the most abundant red pigment, capsanthin, ranged between 468 and 1007 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit in field-grown fruit, whereas levels of β -carotene were more similar in the landraces (13 to 22 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit). Twelve different *Kas I* alleles were found in the landraces, which predicted six novel KAS proteins in these landraces. Seven alleles of *Ccs* were found, which predicted three novel CCS proteins. These results demonstrate that the landraces under cultivation in small farms and pueblos in northern New Mexico are sources of important genetic diversity for *Capsicum* crops.

Chile (*Capsicum annuum* L.) is the iconic crop plant of New Mexico and part of the human diet in the Americas for ≈ 9000 years (Bosland, 1996). Native Americans and the Spanish cultivated this crop. Specific selections of the plant were adopted and maintained by the villages and pueblos of New Mexico to become landraces named for those locations, e.g., Chimayo, Alcalde, and Isleta. Landraces are likely to be genetically heterogeneous with good adaptation to the specific farming system and selected for resistance to

the biotic and abiotic stresses of that location (Portis et al., 2006). In the case of chile, the specific fruit quality traits selected in these landraces would have been red color, early maturity, and heat or pungency.

The pungency or the sensation of heat of chiles is imparted by capsaicinoids, a group of alkaloids unique to the *Capsicum* genus synthesized and accumulated in gland-like structures on the placental tissues (Stewart et al., 2007). Although more than 22 different capsaicinoids have been detected in chile peppers (Bosland and Votava, 2012), the most common capsaicinoids in the fruit are capsaicin and dihydrocapsaicin. In most species and cultivars, capsaicin is the most abundant followed by dihydrocapsaicin (Bosland, 1996; Kozukue et al., 2005; Wahyuni et al., 2011). The red, orange, or yellow color of the mature chile fruit is the result of the accumulation of cultivar- or variety-specific mixtures

of carotenoids in the pericarp. In red fruit, capsanthin is the most abundant carotenoid and this carotenoid generates the red color in the fruit (Daood et al., 2014; Deli et al., 2001). β -carotene and β -cryptoxanthin are orange-colored carotenoids that are essential provitamin A nutrients. They are present in most chile fruit regardless of final fruit color, and their abundance is cultivar-specific (Guzman et al., 2010; Wahyuni et al., 2011; Wall et al., 2001).

The *Capsicum* genus displays extensive variation in fruit shape and size (Andrews, 1995) as well as the fruit color and pungency (Guzman et al., 2011). We have characterized the molecular basis for phenotypic variability in fruit heat and color among a number of chile cultivars in both *C. annuum* and *C. chinense* Jacq. (Aluru et al., 2003; Blum et al., 2003; Guzman et al., 2010; Rodriguez-Uribe et al., 2012; Stewart et al., 2007). These reports include descriptions of specific DNA sequence variations in genes for biosynthetic enzymes on the capsaicinoid and carotenoid pathways.

Genetic diversity in landraces of *Capsicum* in Mexico (Aguilar-Melendez et al., 2009; Gonzalez-Jara et al., 2011; Pacheco-Olvera et al., 2012) and in *Capsicum* in home gardens in Guatemala (Guzman et al., 2005) has been investigated. This research concluded that landraces are important sources of genetic diversity for crop improvement breeding programs. Usually in these studies, genetic diversity was measured using anonymous DNA markers; in one case the diversity in three genes, *Dhn*, *G3pdh*, and *Waxy*, was characterized (Aguilar-Melendez et al., 2009). When genetic diversity of chile landraces from northern New Mexico was compared with cultivars and landraces from Mexico using anonymous molecular markers, the New Mexican landraces tended to group together as a distinct clade (Votava et al., 2005). These results indicated that the New Mexican landraces are unique germplasm isolated from modern cultivars and a likely source of important genetic diversity.

The objective of the study reported here was to assess the variability among New Mexican *C. annuum* landraces for traits associated with fruit quality at both the phenotypic level, fruit heat and fruit color, and at the DNA sequence level. In this report the diversity within and among 13 New Mexican landraces was measured for two key traits associated with fruit quality. We determined the genetic variability in two genes, β -ketoacyl-[acyl carrier protein] synthase, *Kas I*, in the capsaicinoid biosynthetic pathway (Aluru et al., 2003) and capsanthin-capsorubin synthase, *Ccs*, in the carotenoid biosynthetic pathway (Guzman et al., 2010; Rodriguez-Uribe et al., 2012), and determined the abundance and composition of capsaicinoids and carotenoids in the fruit of these landraces.

Materials and Methods

Plant material and growing condition. Seed from 13 northern New Mexico *C. annuum* landraces, Alcalde, Casados, Chimayo, Cochiti, Escondida, Isleta, Jarales, Jemez, San Felipe,

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San Juan, Santo Domingo, Velarde, and Zia Pueblo, were purchased from Native Seed/SEARCH (Tucson, AZ). Seed was sown in greenhouses on the New Mexico State University (NMSU) campus and at nearby Fabian Garcia Research Center (FGRC), Las Cruces NM. Seedlings from the FGRC were transplanted in a field at the NMSU Los Lunas Agricultural Experimental Station. After transplanting in Los Lunas, field-grown plants were watered and fertilized as needed and according to standard production practices for the area. Plants were also grown to maturity on the NMSU campus in a greenhouse in 13.5-L pots with SunGro Metro-Mix 360 (Agawam, MA) soilless growing medium, fertilized with Osmocote (14N-14P-14K) (Scotts Miracle-Gro, Marysville, OH), and watered daily. Mature red fruit from at least three independent plants were harvested from both growing areas and processed for pigment and capsaicinoid extractions.

Carotenoid and capsaicinoid extractions. Fruit from the plants grown in the greenhouse were dried in an oven at 50 °C for 2 d, and fruit from the field-grown plants were dried at 40 °C for 1 week. The seeds were removed and five to six dry peppers per plant were pooled and ground to fine powder in a coffee grinder. For each sample, 1 g dry ground fruit was extracted with chloroform (25 mL). An aliquot (≈1 mL) was retained for capsaicinoid composition analysis by gas chromatography flame ionization detection (GC-FID), whereas the remainder was processed for carotenoids. Capsaicinoid separation by GC-FID was achieved on a 30 m × 0.25-mm I.D. Rxi-5 Sil MS column (Restek, <<http://www.restek.com/>>); injection volume, 5 µL, split 20:1; chromatography conditions: 1 min at 80 °C; temperature ramp of 20 °C/min up to 200 °C, then 10 °C/min up to 320 °C, hold for 5 min at 320 °C; and FID detector at 250 °C. Calibration curves (50 to 10,000 mg/L) were generated for capsaicin and dihydrocapsaicin using reference standards (Sigma, <<http://www.sigmaaldrich.com/united-states.html>>); technical replicates were less than 5.0% cv. Representative chromatograms for the GC-FID detection of capsaicin and dihydrocapsaicin are presented (Fig. 1).

For detection of carotenoids, the chloroform extract was concentrated under a stream of nitrogen, resuspended in 2-propanol, and an aliquot saponified before separation by high-performance liquid chromatography (HPLC) as described earlier (Rodriguez-Urbe et al., 2012). Calibration curves for the carotenoids on the HPLC were generated at 450 nm using the reference standards β-carotene (Sigma, <<http://www.sigmaaldrich.com/united-states.html>>) and capsanthin, β-cryptoxanthin, violaxanthin, and zeaxanthin (Carotenature GmbH, <<http://www.carotenature.com/>>). Representative chromatograms for the HPLC detection of carotenoids are presented (Fig. 2). Total carotenoids were estimated as the sum of the total peak area in the HPLC chromatogram with absorbance at 450 nm. The calibration curve for β-carotene was used to convert this absorbance to mass units for

Fig. 1. Gas chromatography–flame ionization detection (GC-FID) of capsaicinoids. (A) Capsaicin and dihydrocapsaicin, chemical standards; (B) capsaicinoid extract from field-grown fruit of Jemez landrace; (C) capsaicinoid extract from field-grown fruit of Chimayo landrace.

total carotenoids because there were peaks for unknown carotenoids without calibration standards.

DNA extraction and PCR analysis. Genomic DNA was isolated from pooled leaves collected from three plants from each landrace grown in the greenhouse using a cetyl trimethyl ammonium bromide protocol (Rogers and Bendich, 1985). Polymerase chain reaction (PCR)-based amplification was carried out with Platinum PCR SuperMix High Fidelity (Invitrogen Life Technology, Grand Island, NY). The primers for *Ccs* and *Kas I* were designed to amplify the entire gene. *Ccs* primer sequences were: forward AGCCAT ATGTATAGTTTCAAACACAACCTC and

reverse GTGCCTCGAGTCAAAGGCTCT CTATTGCTAGATTG. The cycle conditions for *Ccs* amplification were: one cycle 95 °C 5 min; 35 cycles 94 °C 30 s, 58 °C 40 s; and a final extension at 68 °C for 2 min. *Kas I* primer sequences were: forward GATTA CAAATGCCAG CAAGCTCTGTTGC and reverse GTCAAGGTTTGTAGGGTGC. The cycle conditions for *Kas I* amplification were: 95 °C 3 min; 35 cycles 94 °C 30 s, 57.5 °C 50 s; and a final extension at 68 °C for 4.3 min.

Amplicons from both genes were cloned into pGEM-T Easy Vector (Promega, Madison, WI) as described by the manufacturer. Plasmid DNA from at least six independent clones of *Ccs* and *Kas I* from each landrace was isolated

and sequenced at MCLAB (San Francisco, CA). To sequence the entire *Kas I* gene, a series of internal primers were used to generate nested sets of sequences: KAS-F-1678 GCAGATG GATAAAACGAGGATCGGTG; KAS-F-2220 CACACGAACAGGTGATGGA; KAS-F-874 GGAGTGAGGCTCTTCC; KAS-F-1462 CTC CTCTGAAGGCTACATAGA; KAS-R-1290 GTAGCTCTCTTAGGAGCTGAGA CAGGC; KAS-R-2534 CAGCCAAATCTCCAGCAAG AGTAG; KAS-R-1790 GACTGATTTTCTT GTATCC. Sequence contigs, protein translation predictions, and multiple sequence alignments were performed using the DNASTar software suite (<http://www.dnastar.com/>). Phylogenetic analysis was performed using software provided by Phylogeny.fr (Dereeper et al., 2008; <http://www.phylogeny.fr/>). The *C. annuum* reference genes for *Ccs* was GenBank X77289 and for *Kas I* was GenBank HQ229922.

Statistical analysis. The mean concentration for each specific metabolite was calculated using the General Linear Model (GLM) procedure (SAS 9.4; SAS Institute Inc., Cary, NC) based on independent chromatographic analysis of at least triplicate individuals for each landrace. Duncan's multiple range test using the GLM procedure determined whether there were significant differences between the means for these metabolite concentrations among the landraces. Unpaired Student's *t* tests were conducted to determine if the metabolite concentration in fruit in a specific landrace was different when grown in the field compared with the greenhouse.

Results and Discussion

Carotenoid composition in New Mexican landraces. The fruit in all 13 New Mexican chile landraces are pungent and mature to a red color. Tables 1 and 2 report the mature fruit carotenoid content in field-grown and greenhouse-grown plants, respectively; the entries in the tables are ordered alphabetically by landrace name. Across all landraces and in both environments, capsanthin was the most abundant carotenoid, representing close to one-third of the total carotenoid content, whereas β -carotene was usually the least abundant carotenoid. Between landraces within an environment, there was a great deal of overlap in the abundance levels of the carotenoids. For example, in the field (Table 1) there were no differences between landraces for the accumulation of β -carotene or violaxanthin. In the greenhouse (Table 2), there were no differences between landraces for the accumulation of either total carotenoids or zeaxanthin. For the red pigment capsanthin, fruit from field-grown Alcalde and San Juan landraces had higher levels than Chimayo or Escondida. In the greenhouse environment, capsanthin levels were higher in Jemez fruit than in Cochiti or Jarales.

To investigate the effect of environment on accumulation of carotenoids, unpaired, one-tailed Student's *t* tests were run on the specific carotenoids accumulated by specific landraces in the field vs. the greenhouse. The levels for β -cryptoxanthin in 12 landraces (in

all but San Felipe) and capsanthin (San Juan, Casados and Zia Pueblo), violaxanthin (Chimayo, Casados, and Santo Domingo), or β -carotene in three landraces (Isleta, Jarales, and Escondida) were higher in field-grown fruit than greenhouse-grown fruit.

The carotenoid levels reported here are similar to those found for cultivars of *C. annuum*. The cultivar, NuMex Garnet, which was bred for

low pungency and high pigment content (Walker et al., 2004), accumulated 116 $\mu\text{g}\cdot\text{g}^{-1}$ fresh weight of capsanthin and 36 $\mu\text{g}\cdot\text{g}^{-1}$ fresh weight of β -carotene (Rodríguez-Urbe et al., 2012). These values would be ≈ 10 -fold higher if expressed on a fruit dry weight basis as is presented in Tables 1 and 2. Other reports of carotenoid levels in red-fruited *Capsicum* sp. determined capsanthin levels at 180 to

Fig. 2. High-pressure liquid chromatography of saponified carotenoid extract. Sample is from greenhouse-grown fruit of Chimayo landrace; peaks eluting at same retention time and with the same photodiode array spectra as reference standards are labeled: 1 = violaxanthin; 2 = capsorubin; 3 = capsanthin; 4 = zeaxanthin; 5 = β -cryptoxanthin; 6 = β -carotene.

Table 1. Carotenoids in field-grown mature fruit of New Mexican *Capsicum annuum* landraces.^z

	β -carotene	β -cryptoxanthin	Zeaxanthin	Violaxanthin	Capsanthin	Total
Alcalde	18.4 a ^y	67.6 abcd	160.9 ab	117.0 a	1,006.8 a	3,481.6 a
Casados	14.4 a	53.3 bcde	168.0 a	105.8 a	918.2 ab	2,860.1 abc
Chimayo	13.8 a	39.1 cde	82.8 bc	62.5 a	467.8 b	1,808.2 bc
Cochiti	17.6 a	56.1 bcde	77.3 bc	78.4 a	771.1 ab	2,502.9 abc
Escondida	21.6 a	37.4 cde	73.8 c	72.4 a	465.2 b	2,183.4 abc
Isleta	22.0 a	97.3 a	115.2 abc	96.0 a	872.5 ab	2,910.1 abc
Jarales	15.5 a	56.2 bcde	127.2 abc	66.7 a	673.0 ab	2,216.9 abc
Jemez	13.0 a	39.8 cde	143.7 abc	109.8 a	869.2 ab	2,736.2 abc
San Felipe	12.5 a	19.2 e	81.8 bc	61.8 a	518.5 ab	1,697.7 c
San Juan	18.5 a	87.8 ab	169.0 a	125.8 a	977.0 a	3,186.0 ab
Santo Domingo	13.9 a	38.7 cde	116.2 abc	63.2 a	671.0 ab	2,166.8 abc
Velarde	16.8 a	72.6 abc	116.4 abc	125.2 a	882.0 ab	2,876.8 abc
Zia Pueblo	14.2 a	29.9 de	150.6 abc	88.8 a	897.0 ab	2,511.9 abc

^zHigh-performance liquid chromatography was used to quantify pigments expressed as $\mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit.

^yMean values within a column followed by different letters are significantly different Duncan's multiple range test ($P = 0.05$).

Table 2. Carotenoids in greenhouse-grown mature fruit of New Mexican *Capsicum annuum* landraces.^z

	β -carotene	β -cryptoxanthin	Zeaxanthin	Violaxanthin	Capsanthin	Total
Alcalde	17.3 b ^y	9.6 ab	96.6 a	33.6 b	530.5 ab	2,215.5 a
Casados	13.3 b	7.4 abc	121.1 a	48.5 ab	651.1 ab	1,870.4 a
Chimayo	10.2 b	6.3 b	84.3 a	50.8 ab	635.4 ab	2,012.1 a
Cochiti	14.5 b	7.8 ab	131.2 a	46.2 ab	330.4 b	2,348.2 a
Escondida	7.7 b	9.8 ab	133.9 a	84.3 a	659.1 ab	2,366.1 a
Isleta	15.6 b	12.8 a	112.6 a	35.7 b	627.1 ab	2,186.3 a
Jarales	5.0 b	6.1 b	89.9 a	34.3 b	444.8 b	1,347.5 a
Jemez	13.5 b	6.8 b	154.8 a	59.9 ab	811.6 a	2,295.5 a
San Felipe	8.2 b	10.2 ab	115.3 a	63.7 ab	681.2 ab	2,376.5 a
San Juan	16.1 b	9.8 ab	109.9 a	35.2 b	673.5 ab	2,392.6 a
Santo Domingo	12.6 b	9.1 ab	112.3 a	33.3 b	670.4 ab	2,108.9 a
Velarde	34.9 a	11.6 ab	132.9 a	47.2 ab	687.5 ab	2,285.6 a
Zia Pueblo	12.3 b	6.2 b	136.3 a	35.5 b	672.9 ab	2,295.5 a

^zHigh-performance liquid chromatography was used to quantify pigments expressed as $\mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit.

^yMean values within a column followed by different letters are significantly different Duncan's multiple range test ($P = 0.05$).

1013 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight depending on variety (Ha et al., 2007); several New Mexican landraces had capsanthin levels close to 1000 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight (Table 1).

Capsaicinoid composition in New Mexican landraces. Table 3 reports the mature fruit capsaicin and dihydrocapsaicin content in field-grown and greenhouse-grown plants; the entries in the table are rank-ordered by the total capsaicinoid content in field-grown fruit. The capsaicinoid levels of the landrace fruit were variable and the levels were higher in fruit grown in field conditions as compared with the greenhouse. Under field conditions, the fruit of the landrace San Felipe had the highest level of capsaicinoids; in contrast, this landrace grown in the greenhouse had very low levels of capsaicinoids. Under greenhouse conditions, fruit of the landrace San Juan had the highest levels of capsaicin. Landraces Casados, Alcalde, and Isleta consistently had mild or low capsaicinoid levels in their fruit grown in either the field or the greenhouse. To investigate the effect of environment on accumulation of capsaicinoids, unpaired, one-tailed Student's *t* tests were run on the capsaicin and dihydrocapsaicin amounts accumulated by specific landraces in the field vs. the greenhouse. Four landraces, Zia Pueblo, Jemez, Casados, and Isleta, all accumulated higher levels of both capsaicinoids in the field as compared with the greenhouse. Three landraces, Escondida, Santo Domingo, and Chimayo, accumulated higher levels of dihydrocapsaicin in the field compared with the greenhouse.

One novel feature of the capsaicinoid content of the landrace fruit was the very high dihydrocapsaicin concentration. Typically capsaicin is twice as abundant as dihydrocapsaicin in *C. annuum* cultivars (Bosland, 1996; Kozukue et al., 2005; Wahyuni et al., 2011). Ratios of the capsaicin to dihydrocapsaicin were calculated and were compared with three *C. annuum* cultivars: Jalapeno PX206, Jalapeno PX211 (Keyhaninejad et al., 2014), and Santa Fe Grande (Fig. 3). Under field conditions, all of the landraces had higher concentrations of dihydrocapsaicin than capsaicin. Under greenhouse conditions, these compounds were present at similar relative abundances, resulting in ratios close to 1.0. One landrace, Jarales, when cultivated in the greenhouse had a 2-fold ratio of capsaicin to dihydrocapsaicin.

The capsaicinoid content for the New Mexican landraces was similar to the level reported for cultivars of the "New Mexican type." The total capsaicinoid content in these landraces ranged between 250 and 1750 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit. A mild cultivar grown in the area, 'NuMex Heritage 6-4', has total capsaicinoids at $\approx 100 \mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit (Bosland, 2012), whereas a slightly hotter type, 'NuMex Heritage Big Jim', has total capsaicinoids at close to 600 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit (Bosland and Coon, 2013). However, the high levels of dihydrocapsaicin in the landraces were unusual for *C. annuum* lines. High ratios of dihydrocapsaicin to capsaicin, as observed in these landraces, have been reported in *C. pubescens* Ruiz

and Pavon (Collins et al., 1995; Zewdie and Bosland, 2001).

Genetic variability among the New Mexico landraces capsaicinoid biosynthetic gene *Kas I*. Genomic DNA, isolated from the 13 landraces, was amplified using the primers for *Kas I*. The amplicon products were 3.4 kb and contained all of the exons and seven introns in this gene. A total of 12 distinct *Kas I* alleles (a, c to m) were detected within the 13 landraces (GenBank accessions: KM037707 to KM037726). Table 4 lists the alleles observed within each landrace sample. Half of the DNA polymorphisms that produced these alleles were located in the intronic regions. DNA polymorphisms were detected in introns 1, 2, 5, and 7. Some alleles, like *Kas I* allele a, were detected in five different landraces, but most of the other alleles were detected in only one landrace. Six independent

clones were sequenced for each population, but for an allele to be scored as present, that unique sequence variant needed to be detected in two or more clones. This filter was applied to rule out technical sources of sequence variability. Because our biological sample size was limited to only three individuals for each landrace, these results likely underestimate the *Kas I* allelic diversity within these populations.

The cDNA sequences for the *Kas I* genes were assembled from the genomic sequences and a multiple sequence alignment of those sequences was obtained, which included the cDNA version of *Kas I* from *C. annuum* cv. Yidu-Red (GeneBank HQ229922). A phylogenetic tree was generated using this multiple sequence alignment (Fig. 4). The exons for alleles d and j were identical as were the exons for alleles d and e and alleles f, i, k, and m.

Table 3. Capsaicinoids in mature fruit of New Mexican *Capsicum annuum* landraces.^z

	Field		Greenhouse	
	Capsaicin	Dihydrocapsaicin	Capsaicin	Dihydrocapsaicin
San Felipe	719.5 a ^y	1,024.9 a	13.4 b	13.3 b
Zia Pueblo	410.4 ab***	921.4 ab**	159.9 b	253.1 b
Escondida	399.6 ab	508.6 abc**	93.3 b	125.6 b
Jemez	332.6 ab**	477.0 abc**	149.3 b	148.4 b
Cochiti	284.9 b	405.0 bc	60.8 b	71.3 b
Velarde	280.6 b	436.0 abc	520.3 ab	684.0 a
Santo Domingo	252.5 b	499.4 abc**	289.4 ab	272.2 b
San Juan	236.4 b	323.6 bc	710.0 a	369.4 ab
Jarales	216.5 b	329.2 bc	169.1 b	84.1 b
Chimayo	142.4 b	293.7 c**	215.2 ab	206.4 b
Casados	126.6 b**	167.6 c**	80.3 b	49.2 b
Alcalde	118.7 b	158.5 c	128.9 b	145.0 b
Isleta	103.1 b**	146.7 c**	10.6 b	12.1 b

^zGas chromatography-flame ionization detection was used to quantify capsaicinoids expressed as $\mu\text{g}\cdot\text{g}^{-1}$ dry weight fruit.

^yMean values within a column followed by different letters are significantly different Duncan's multiple range test ($P = 0.05$).

^zMean values followed by ** are higher in field- vs. greenhouse-grown fruit; Student's *t* test, unpaired, one-tailed ($P \geq 0.05$).

Fig. 3. Ratio of capsaicin to dihydrocapsaicin in *C. annuum* fruit. Capsaicinoid content was determined in fruit collected from field- (dark gray) or greenhouse- (light gray) grown plants. Student's *t* test (unpaired, two-tailed) on the means of the ratio of capsaicin to dihydrocapsaicin were calculated on each landrace grown in the two environments; significantly different means are indicated (****).

The alleles c, g, h, and l were unique. None of the landraces were identical to the *Kas I* sequence published in GenBank for *C. annuum*; the reference sequence was the most distant sequence on the phenogram.

From a functional perspective, the DNA sequence polymorphisms detected in the *Kas I*

Table 4. Distribution of *Kas I* and *Ccs* alleles in the New Mexican landraces.

Landrace	<i>Kas I</i> alleles	<i>Ccs</i> alleles
Alcalde	h	f
Casados	a	f
Chimayo	a	f, 4
Cochiti	d	f
Escondida	j, k	e, f
Isleta	i	f
Jarales	k, l	f, g
Jemez	c	a, b
San Felipe	a, g	f
San Juan	a	f
Santo Domingo	f, m	f
Velarde	a	a, c
Zia Pueblo	e	f

alleles resulted in only a few amino acid changes; altogether there were six distinct predicted KAS I protein products (KAS I A to F). KAS I encodes a protein 489 amino acids long; all of the landraces had a *Kas I* with amino acid changes as a result of DNA sequence variability. The positions of amino acid changes in KAS I and the landraces with these variants are presented in Table 5. None of these changes altered the amino acids at the active site motifs TACAT (aa 237 to 241) and the histidines at positions 351 and 317 (Aluru et al., 2003; Siggaard-Andersen et al., 1991).

Genetic variability among the New Mexico landraces carotenoid biosynthetic gene Ccs. A similar DNA sequence analysis was conducted on the *Ccs* gene in the landraces. This gene is comprised of a single exon, and the genomic amplicon was 1.5 kb. The *Ccs* gene in *C. annuum* cv. Yolo Wonder (GenBank X77289) was used as the reference sequence. Eight different single nucleotide polymorphisms at nucleotide positions 235, 376, 609,

630, 703, 808, 1447, and 1477 were identified resulting in seven alleles within the landraces for this gene (GenBank accessions: KM037687 to KM037706). Table 4 lists the *Ccs* alleles observed within each landrace sample. Allele f was the most common version of *Ccs* because it was detected in 11 of the 13 landraces. Three of these nucleotide changes (630, 703, and 1447) were silent mutations; the nucleotide changes at 235, 376, 609, 808, and 1447, however, resulted in amino acid changes and thus variant CCS proteins, CCS-A, -B, and -4. The positions of amino acid changes in CCS and the landraces with these variants are presented in Table 6. The truncated CCS-4 protein, if expressed with only 203 amino acids in length, would be missing the domains essential for enzyme activity.

The amino acid sequences of CCS-A and -B both had the conserved domains for function identified earlier (Jeknic et al., 2012; Mialoundama et al., 2010). Specifically, the putative cleavage site of the N-terminal plastid transit peptide (FLDLA aa 54 to 58), the domain essential for B- and K-cyclase activity (FLEET motif aa 293 to 297), the conserved amino acids with important catalytic activity (MDW aa 258 to 260, E 332, and VHPS 359 to 362), and the potential dinucleotide binding site (aa 88 to 103 GTGPAGLRRLAEQVSKYG) were found in all the CCS proteins except in CCS-4. The amino acid substitutions that were detected were conservative replacements at three of the four sites with changes in CCS-A and CCS-B: V126I, Y270H, and T483A.

Conclusions

The recurrent selection for red pungent fruit among the New Mexican chile landraces has generated a set of germplasm that appears to be suited to the environment of New Mexico. The fruit quality of these landraces measured in terms of pigment accumulation was better in fruit cultivated in fields in New Mexico than under greenhouse conditions, and the carotenoid content approached the levels reported for in cultivars bred for high pigment. The content of one of the capsaicinoids, dihydrocapsaicin, was also higher in fruit of landraces cultivated in fields in New Mexico in contrast to greenhouses. The genetic variability between landraces was very low, measured in terms of DNA sequence differences in the *Kas I* and *Ccs* genes. All of the amino acid sequences identified as important in CCS function were conserved among the two functional CCS variants detected in the landrace populations. Similarly, among the more polymorphic alleles for *Kas I*, there were very few changes in gene sequence that resulted in amino acid differences in the predicted protein; again, none of these changes involved amino acids identified as important in enzyme activity. The polymorphisms for *Kas I* and *Ccs* were independently observed in the populations; in some landraces, for example Velarde, only one allele for *Kas I* was detected, but this

Fig. 4. Phylogenetic relationship of *Kas I* in *C. annuum* New Mexican landraces. The analysis was performed using the Phylogeny.fr platform (MUSCLE, PHYLIP, and Drawgram). The exonic sequences were assembled from the genomic DNA sequences of the 12 *Kas I* alleles (a, c to m) and the sequences were aligned with MUSCLE; the phylogenetic tree was constructed using the PHYLIP neighbor joining method, and a phenogram was generated using Drawgram. The landrace source of the allele is indicated in the figure.

Table 5. Amino acid differences in KAS I proteins in New Mexican landraces.^a

	Amino acid position							Landraces
	55	105	140	220	327	361	434	
wt KAS I	G	R	H	A	S	M	A	
KAS IA	E	S	H	A	S	K	T	Alcalde
KAS IB	G	S	H	A	S	K	T	Casados, Escondida, Isleta, Jarales, Santo Domingo
KAS IC	V	S	R	A	S	K	T	Jemez
KAS ID	G	S	R	A	S	K	T	Chimayo, Escondida, San Felipe, San Juan, Velarde
KAS IE	G	S	R	A	A	K	T	Cochiti, Zia Pueblo
KAS IF	G	S	R	T	S	K	T	San Felipe

^aSingle letter codes for amino acids are indicated: A = alanine; E = glutamic acid; G = glycine; H = histidine; K = lysine; M = methionine; R = arginine; S = serine; T = threonine; V = valine.

Table 6. Amino acid differences in CCS proteins in New Mexican landraces.²

	Amino acid position					Landraces
	79	126	203	270	483	
wt CCS	G	V	C	Y	T	Alcalde, Casados, Chimayo, Cochiti, Escondida, Isleta, Jarales, San Felipe, San Juan, Santo Domingo, Zia Pueblo
CCS-A	R	V	C	Y	T	
CCS-B	R	I	C	H	A	Jarales
CCS-4	R	V	stop			Chimayo

²Single letter codes for amino acids are indicated: A = alanine; C = cysteine; G = glycine; H = histidine; I = isoleucine; R = arginine; T = threonine; V = valine; Y = tyrosine.

sample had two alleles for *Ccs*. A full analysis of the genetic diversity at the DNA sequence level within these landrace populations will need larger sample sizes and more loci screened.

The New Mexico landrace population does present a novel trait, high dihydrocapsaicin content, which will be interesting to investigate in the future. The biosynthetic pathway for capsaicin is partially described, but the regulation of this pathway needs further study. Comparisons between these landraces and selected cultivars may reveal important aspects about the control of the capsaicin and dihydrocapsaicin content.

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