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Inheritance of Capsaicin and Dihydrocapsaicin, Determined by HPLC-ESI/MS, in an Intraspecific Cross of *Capsicum annuum* L.

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The quantitative inheritance of capsaicin and dihydrocapsaicin contents in fruits has been studied in an intraspecific cross of *Capsicum annuum* L. across two different environments, namely, fruits developed in spring and summer. A liquid chromatography-electrospray ionization/time-of-flight mass spectrometry [HPLC-ESI/MS(TOF)] method was used to identify and quantify capsaicin and dihydrocapsaicin in extracts of pepper fruits. The analytical method used was able to determine the pungency of genotypes that, using other methods, would have been classified as non-pungent. Capsaicin and dihydrocapsaicin contents varied largely among families, and families did not respond similarly in producing these capsaicinoids when their fruits were grown in spring and summer, with some families showing no increase, whereas in others, the increase was more than 2-fold. Heterosis for the pungency trait, assessed by the capsaicin and dihydrocapsaicin contents in fruits, was found, indicating the existence of epistasis, over-dominance, or dominance complementation. Non-pungent parent alleles contributed to the capsaicin and dihydrocapsaicin contents since transgressive segregation did occur. Furthermore, the type of gene action varied between capsaicin and dihydrocapsaicin, and a seasonal effect during fruit development could affect gene action.

KEYWORDS: Capsaicin; dihydrocapsaicin; family−environment interaction; HPLC; intraspecific cross; mass spectrometry; quantitative inheritance

INTRODUCTION

Pepper fruits (*Capsicum* spp.) are among the most consumed vegetables in the world. A significant property of this genus is pungency, which is caused by the presence of alkaloid compounds of the capsaicinoid group in the fruit. Capsaicinoids are only found in the *Capsicum* genus and are bioactive molecules currently relevant in medical and food sciences (*1*−*3*) as well as in the defense weapon industry (*4*). Capsaicinoids occur in the placental tissue of pepper fruits (*5*), and their biosynthesis depends on a complex and still not fully characterized enzymatic pathway. The two major capsaicinoids, responsible for up to 90% of pungency, are capsaicin and dihydrocapsaicin (**Figure 1**) (*6*), with at least nine more minor capsaicinoids occurring in pepper fruits (*7*, *8*). The type and amount of each capsaicinoid affect both the degree and the characteristics of pungency (*9*, *10*). Capsaicinoid levels depend on the genotype (*11*) and also change during fruit development (*12*−*14*). Moreover, environmental and nutritional conditions occurring during the cultivation of peppers can affect the capsaicinoid content. For instance, significant differences in pungency were found in double-haploid chili plants grown in five different plots of the same field (*15*), and the total capsaicinoid content in 'Padrón' pepper fruits developed in summer was found to be larger than in those fruits developed in autumn (*16*). Also, the production of five capsaicinoids in four pepper genotypes was found to depend both on the field location and on the year (*17*).

Genetic mechanisms underlying the inheritance of pungency have long been studied, although they are still poorly understood. Early studies, employing organoleptic tests, found that the presence/absence of pungency was controlled by a single dominant gene, known as *C* (*18*−*20*). This gene, renamed *Pun1*, has been mapped to chromosome 2 of the genus *Capsicum* (*21*) and encodes the acyl transferase enzyme *AT3* (*22*). The recessive gene *pun1*, with a 2.5 kb deletion spanning the putative promoter and first exon, results in the absence of pungency in *Capsicum annuum*.

Other studies have shown that pungency is inherited quantitatively. Since in these studies the determination of capsaicinoid contents is a mandatory requirement, several analytical techniques and different methods, using paper chromatography, thin layer chromatography, and high-performance liquid chromatography (HPLC) as separation techniques and UV−vis spec-

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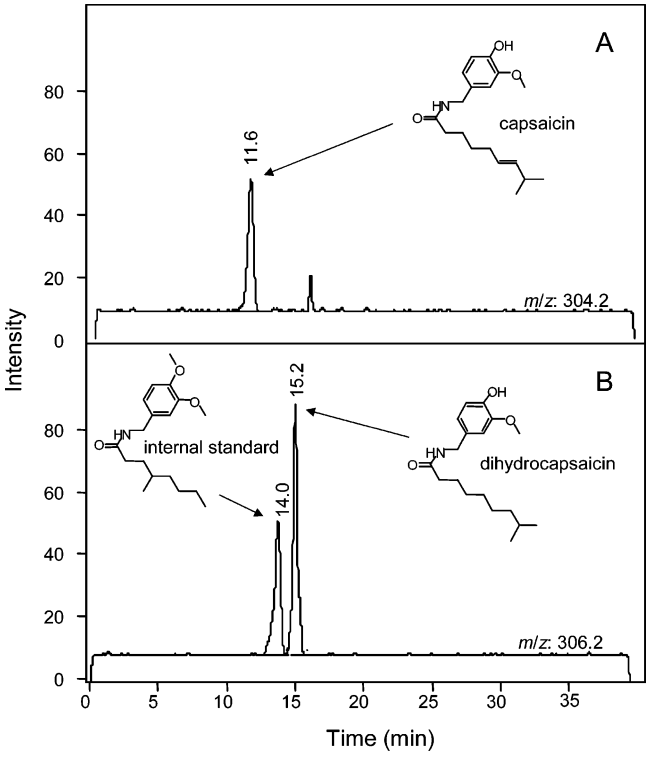

Figure 1. HPLC-ESI/MS chromatogram at *m/z* 304.2 (**A**) and 306.2 (**B**) corresponding to the $[M - H]^{-1}$ pseudo-molecular ions of capsaicin and dihydrocapsaicin, respectively, of a pungent F_1 fruit extract from an intraspecific cross of *C. annuum* L. The chemical structures of capsaicin, dihydrocapsaicin, and internal standard are also depicted.

troscopy as detection techniques, have been employed (*23*−*28*). An HPLC-UV method developed for the determination of five individual capsaicinoids, including capsaicin and dihydrocapsaicin (*29*), was used to confirm that pungency is inherited quantitatively in pepper and also that the biosynthesis of the five capsaicinoids studied was under different genetic control (*26*).

The large variability in capsaicinoid content found naturally in pepper genotypes is a critical point in breeding and production. For instance, capsaicin and dihydrocapsaicin contents ranged from 2 to 6639 mg/kg in eight different pepper genotypes (*30*). Therefore, there is a requirement for analytical techniques able to determine very low amounts of capsaicinoids. Also, these techniques should be capable of determining amounts of the different capsaicinoid molecules, which have very similar chemical structures. These requirements are met by HPLC-MS (mass spectrometry) techniques, which have a high selectivity and sensitivity and have been used for the determination of capsaicinoids in forensic, medical, and food sciences (*30*−*33*). HPLC-MS methods can determine up to six capsaicinoids with limits of detection (LODs) in the range of 0.03−1 μM, better than those of HPLC-UV methods commonly used in genetic analysis, which have LOD values of approximately 9 μM (*29*). The high selectivity and sensitivity of HPLC-MS techniques may also reduce the possibility of assigning false negatives (i.e., when a pungent individual is considered as a non-pungent one), allowing for a more accurate phenotyping, an essential step in capsaicinoid inheritance studies.

So far, only three studies regarding the genetic control of quantitative variation for individual capsaicinoids have been published. Previous studies made use of interspecific crosses of *C. chinense* × *C. annuum* (*26*) and *C. annuum* × *C. frutescens* (*27*, *28*). The aim of the present study was to investigate the inheritance for capsaicin and dihydrocapsaicin contents in an intraspecific cross of *C. annuum*, using liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI/MS), a highly selective and sensitive technique (*30*). In addition, the interaction family−environment was studied, and quantitative results obtained using the HPLC-ESI/MS method were compared to those obtained from a qualitative pungency assessment.

MATERIALS AND METHODS

Plant Material. The *C. annuum* L. non-pungent bell inbred variety 'Yolo Wonder' (Y; *pun1pun1*) and the *C. annuum* L. pungent inbred line 'Serrano Criollo de Morelos-334' (SCM-334; *Pun1Pun1*) were used as parental lines P_1 and P_2, respectively. Families F_1 (Y × SCM-334), F_2, and backcrosses (F_1 × Y and F_1 × SCM-334) were obtained, and these populations and the parents were grown in 2003 and 2004. The number of plants in each family, 7, 7, 26, 32, 18, and 7 for P_1, P_2, F_1, F_2, BC_{P1}, and BC_{P2}, respectively, were evaluated for fruits developed and collected during spring. A different group of plants, 11, 26, 16, 39, 9, and 17 for P_1, P_2, F_1, F_2, BC_{P1}, and BC_{P2}, respectively, were evaluated for fruits developed and collected during summer. Seeds were germinated in Petri dishes, and when cotyledons were developed, each plant was placed on a Jiffy-7 pot (Clause-Tezier Ibérica). When plants had three true leaves, each Jiffy pot was planted into a black plastic pot (11 cm in diameter). Plants were grown randomly distributed in a climatized greenhouse with a substrate mixture of peat, sand, clay−loam soil, and Humin Substrat (Klasman-Deilmann) (1:1:1:1, v/v). Two grams of Osmocote 16N-4P-9K slow-release fertilizer (Scotts) were top-dressed on each pot at the beginning of growth. Plants were watered daily to maintain optimum growth. The average minimum and maximum temperatures in the greenhouse were 14−24 °C during spring and 19−27 °C during summer. Fruits were harvested from each plant when they reached maturity. In 2004, fruits were harvested in spring (from April to June) from a group of plants and in summer (from July to October) from the other one.

Sample Ppreparation and Capsaicinoid Extraction. Mature red fruits were oven-dried at 55 °C for 4−5 days and then ground individually in a Polytron grinder. Ground tissue of each individual fruit was employed for capsaicinoid extraction and qualitative pungency assessment.

Capsaicinoids were extracted from ground fruits according to the method described by Garcés-Claver et al. (*30*). One hundred milligrams of dried tissue samples was extracted with 1 mL of pure acetonitrile, containing a small amount of the internal standard (4,5-dimethoxybenzyl)-4-methyloctamide (DMBMO). Acetonitrile was used for extraction because it gives a high extraction rate, whereas impurities were kept to a minimum. The final DMBMO concentration was 5 μM. The suspension of dried powder in acetonitrile was shaken at room temperature for 60 min in an orbital shaker operating at 250 rpm and then heated in a water bath without shaking at 65 °C for 1 h. The mixture was then shaken again at room temperature for another 60 min in the conditions indicated previously. Then, the suspension was centrifuged for 15 min at 16 000*g*, and the supernatant was collected and brought to a volume of 1 mL with acetonitrile. Finally, the supernatant was filtered successively through a 0.45 and a 0.22 μm PVDF membrane filter (Millipore) before analysis.

Qualitative Assessment of Pungency. A qualitative assessment to distinguish degrees of pungency was carried out using fruits from plants belonging to the F_2 family. One to five fruits per plant were tasted at least by two different persons. When a single fruit was found pungent by the tasters, the genotype was considered pungent, whereas a genotype was considered as non-pungent only when all five fruits were assessed as non-pungent. The degree of pungency was classified into four categories: 1 for non-pungent, 2 for slightly pungent, 3 for pungent, and 4 for extremely pungent.

Quantitative Analysis of Capsaicin and Dihydrocapsaicin. Chemicals and Reagents. All eluents, buffers, and standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore). Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide) (≥97%),

dihydrocapsaicin (8-methyl-*N*-vanillylnonamide) (≥90%), methanol (≥99.9%, LC-MS grade), acetonitrile (LC-MS grade), and lithium hydroxide monohydrate (99.995%) were purchased from Sigma-Aldrich. Standard 1 mM stock solutions of capsaicin and dihydrocapsaicin were prepared by dissolving the appropriate quantity of each compound in 5 mL of acetonitrile. The capsaicin analogue DMBMO was synthesized according to the method of Cooper et al. (*34*) and used as an internal standard. The identity of DMBMO was confirmed by nuclear magnetic resonance (NMR) spectrometry, and its purity was >98%.

HPLC-ESI/MS(TOF) Analysis of Capsaicin and Dihydrocapsaicin. Capsaicin and dihydrocapsaicin were determined in capsaicinoid extracts using the HPLC-ESI/MS(TOF) analytical method developed by Garcés-Claver et al. (*30*). Analyses were carried out with a BioTOF II (Bruker Daltonics) coaxial multipass time-of-flight mass spectrometer [MS(TOF)] equipped with an Apollo electrospray ionization source (ESI) and coupled to a Waters Alliance 2795 HPLC system (Waters). The BioTOF II was operated with endplate and spray tip potentials at 3.0 and 3.5 kV, respectively, in the negative ion mode, with drying gas (N_2) and nebulizer pressures of 30 and 60 psi, capillary voltage of 90 V, and gas drying temperature of 200 °C. Spectra were acquired in the 100−500 mass/charge ratio (*m*/*z*) range. The mass axis was calibrated using lithium−formate adducts. Samples were chromatographed using a 5 μm particle size, 4.6 mm × 250 mm Waters Symmetry C_{18} column coupled with a 5 μm particle size, 3.9 mm × 20 mm Waters Symmetry C_{18} guard column and a gradient of methanol and Milli-Q water, using a flow rate gradient between 0.9 and 1.8 mL/min (*30*). After each injection, the column was re-equilibrated for 10 min with 30% water/70% methanol, at a flow rate of 0.9 mL/min. The total analysis run time was 40 min. The autosampler and column were maintained at 4 and 30 °C, respectively, and the injection volume was 20 μL. The system was controlled with the software packages BioTOF v. 2.2 (Bruker Daltonics) and Hyphenation Star v. 2.3 (Bruker DaltoniK). Data were processed with Data Analysis v. 3.2 software (Bruker DaltoniK).

Capsaicin and dihydrocapsaicin concentrations of the pepper fruit extracts were expressed as milligram per kilogram of dry weight (DW) fruit. The sum of the capsaicin and dihydrocapsaicin contents was calculated and presented as the total capsaicinoid content.

Statistical Analysis. A combined ANOVA for the capsaicin, dihydrocapsaicin, and total capsaicinoid content data for generations across the two environments (spring and summer) was carried out according to the general linear model (GLM), using the SAS software package v. 9.1.3 (SAS Institute). Families and environments were considered as fixed effects. Means were compared using Duncan's LSD test. Correlation analyses were performed with the PROC Corr procedure of the SAS software package using the Pearson correlation test.

To study the quantitative inheritance of pungency, the JNTSCALE software (*35*) was used. A joint scaling test and the individual scaling tests A, B, and C were carried out to provide estimates for the mean, additive effects, and dominance effects. As the family−environment interaction resulted significantly, data of each season were considered separately for generation mean analysis. Tests evaluated the goodness-of-fit of the three-parameter model [midparental value (*m*), additive (*d*), and dominance (*h*) effects] to the observed data, assuming that the sum of squared deviations weighed with the appropriate coefficients follows a chi-squared distribution with three degrees of freedom (*37*), and where a failure of the model is considered as an indication of epistasis. Since the three-parameter genetic model was not adequate to explain the data, the six-parameter genetic model for epistasis was used, incorporating the midparental value (*m*), additive effect (*d*), and dominance (*h*) effect and the three digenic interactions [additive × additive (*i*), additive × dominance (*j*), and dominance × dominance (*l*)].

To estimate the suitability of the qualitative analysis to distinguish degrees of pungency, ANOVA of capsaicin, dihydrocapsaicin, and total capsaicinoid content data for the qualitative categories was carried out. Means were compared using Duncan's LSD test. Statistical analyses were carried out using the SAS software package.

Table 1. CAP, DHC, and Total Capsaicinoid Contents (Results Are Mean ± SE) in Pepper Fruits Grown in Spring and Summer[a]

		contents (mg/kg of DW[b])		
	number of plants	CAP	DHC	total capsaicinoids[c]
		Spring		
P_1	7	0[d] a	0 a	0 a
P_2	7	9.7 ± 3.9 b	9.7 ± 4.1 b	19.4 ± 8.0 b
BC_{P1}	18	12.6 ± 8.5 b	8.5 ± 7.3 b	21.1 ± 15.2 b
BC_{P2}	7	22.8 ± 9.3 c	21.7 ± 7.1 c	44.5 ± 16.3 c
F_1	26	40.0 ± 7.1 d	30.6 ± 6.3 c,d	70.6 ± 12.6 c,d
F_2	32	40.0 ± 7.9 d	37.5 ± 7.7 d	77.5 ± 15.3 d
midparent		**4.9**	**4.9**	**9.7**
		Summer		
P_1	11	0 a	0 a	0 a
P_2	26	9.6 ± 2.2 b	11.1 ± 2.4 b	20.7 ± 4.6 b
BC_{P1}	9	20.5 ± 8.6 c	26.7 ± 10.4 c	47.3 ± 18.9 b
BC_{P2}	17	54.1 ± 9.9 d	38.5 ± 8.5 c	92.6 ± 17.9 c
F_1	16	109.3 ± 9.3 e	87.5 ± 8.9 d	196.8 ± 18.0 d
F_2	39	48.6 ± 7.9 d	31.2 ± 6.0 c	79.8 ± 13.5 c
midparent		**4.8**	**5.6**	**10.4**

[a] Fruits were from the P_1 ('Yolo Wonder') and P_2 ('Serrano Criollo de Morelos-334') parental lines, their F_1 and F_2 families, and the backcrosses (BC_{P1} and BC_{P2}). Means followed by different letters in the same column are significantly different ($p < 0.05$). [b] DW: dry weight. [c] Total capsaicinoids considered as the sum of the capsaicin and dihydrocapsaicin contents. [d] Content below the limit of detection of the method.

RESULTS

Genotype and Environmental Effects on Pungency. Capsaicin and dihydrocapsaicin were quantified by HPLC-ESI/MS. A chromatogram of a pungent F_1 fruit extract, including the peaks for capsaicin and dihydrocapsaicin at the corresponding *m*/*z* ratios and retention times, is shown in **Figure 1**. In this work, the average coefficients of variation for the capsaicin and dihydrocapsaicin contents of each individual fruit were 8.7 and 9.8, respectively. The analysis of variance for capsaicin, dihydrocapsaicin, and total capsaicinoid contents across the two environments showed very highly significant differences ($p < 0.001$) among families and highly significant differences ($p < 0.01$) between environments (Supporting Information). The family−environment interaction was also very highly significant.

When considering all analyzed fruits over the families, the mean values for capsaicin, dihydrocapsaicin, and total capsaicinoid contents were in the ranges of 9−110, 9−88, and 19−197 mg/kg DW, respectively (Supporting Information). Parental line fruits had total capsaicinoid contents ranging from very low (not detected with the method LOD of 0.6 mg/kg DW) in P_1 to 21 mg/kg DW in P_2. Fruits from the F_1 progeny had values for total capsaicinoids higher than those of the midparent values, and also higher than the P_2 parental line, therefore indicating heterosis. For the F_2 family, values for total capsaicinoid contents shifted toward the P_2 parental line values, reaching higher values than the midparent and surpassing the P_2 parental line contents. Both backcrosses had also higher total capsaicinoid contents than the P_2 parental line.

In three families (F_1, BC_{P1}, and BC_{P2}), fruits harvested in summer had higher total capsaicinoid contents than those collected in spring (**Table 1**). Large increases in capsaicinoid contents were found in summer in the F_1 (2.8-fold), the BC_{P1} (2.2-fold), and the BC_{P2} families (2.1-fold), whereas in the case of the P_2 parental line and the F_2 families, the total capsaicinoid content summer increase was practically nothing.

The frequency distributions for total fruit capsaicinoid contents developed in spring and summer from the segregating families and the P_2 and F_1 families are shown in **Figure 2**.

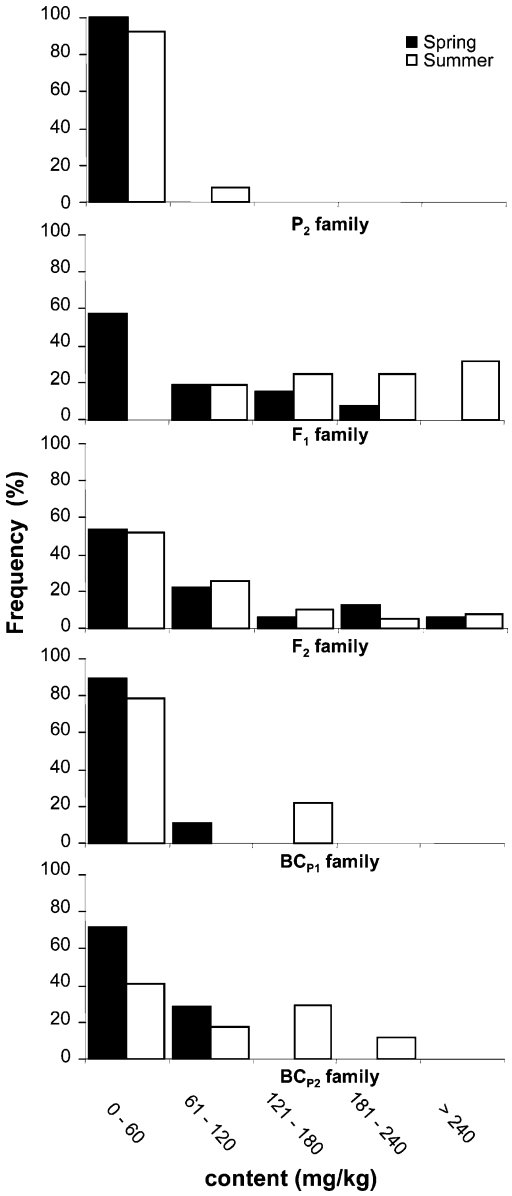

Figure 2. Frequency distribution of P_2, F_1, F_2, BC_{P1}, and BC_{P2} families for total capsaicinoid content in an intraspecific cross of *C. annuum* L. across two environments (spring and summer).

Capsaicin contents were in a wide range, from values below the LOD to 166 and 174 mg/kg DW in spring and summer, respectively. Dihydrocapsaicin content ranges varied as broadly as those of capsaicin in both seasons (data not shown). A continuous distribution of the values for capsaicin and dihydrocapsaicin contents was observed when the individuals of the F_2 segregating family were examined.

Quantitative Inheritance of Pungency. The joint three-parameter scaling test indicated that an additive × dominance model based on estimates of χ^2 could be adequate to explain the variation of capsaicin, dihydrocapsaicin, and total capsaicinoid contents in spring and the variation of dihydrocapsaicin in summer because these probabilities were $p > 0.05$ (Supporting Information). However, the additive × dominance model was finally not accepted because some of the individual ABC scaling tests were significantly different from zero, and therefore, a model with epistatic effects was carried out. A five-parameter model could adequately explain the results, showing different epistatic effects for spring and summer seasons (Supporting Information). In spring, significant positive additive (*d*) and negative additive × additive (*i*) effects were shown to occur for capsaicin, dihydrocapsaicin, and total capsaicinoid contents. Although the dominance effect (*h*) was not significant, its value was negative and tended to be larger than both the midparental (*m*) value and the dominance × dominance (*l*) effect. In summer, positive additive (*d*), additive × dominance (*j*), and dominance × dominance (*l*) effects were significant for capsaicin content. Significant positive additive (*d*) and dominance × dominance (*l*) effects were observed for total capsaicinoid and dihydrocapsaicin contents. The dominance effect (*h*) had a non-significant positive value that tended to be higher than the midparental (*m*) value and lower than the dominance × dominance (*l*) effect.

Table 2. CAP, DHC, and Total Capsaicinoid Contents (Means ± SE, with *n* Varying from 12 to 28) of Pepper Fruits from the F_2 Family of a *C. annuum* Intraspecific Cross for Each Pungency Category Assigned by Tasting Fruits[a]

	content (mg/kg of DW[b])		
categories	CAP	DHC	total capsaicinoid[c]
tasted as non-pungent	0.98 ± 0.33 a	0.87 ± 0.30 a	1.80 ± 0.61 a
tasted as slightly pungent	35.2 ± 15.8 b	23.6 ± 10.5 b	58.8 ± 25.8 b
tasted as pungent	48.7 ± 8.2 b	35.5 ± 6.7 b	84.2 ± 14.4 b
tasted as extremely pungent	78.6 ± 9.9 c	63.1 ± 10.5 c	141.6 ± 18.7 c

[a] Means followed by different letters in the same column are significantly different ($p < 0.05$). [b] DW: dry weight. [c] Total capsaicinoid considered as the sum of the capsaicin and dihydrocapsaicin contents.

Correlation Coefficients between Capsaicin and Dihydrocapsaicin Contents. Correlation coefficients between capsaicin and dihydrocapsaicin contents were highly significant ($p < 0.01$) and showed a strong positive correlation between both capsaicinoids, both in spring ($r = 0.859$) and in summer ($r = 0.982$) (Supporting Information). The average ratio between capsaicin and dihydrocapsaicin was close to 1:1 in spring, whereas in summer, the ratio was approximately 1:0.8. A season combined analysis also showed a high positive correlation between both capsaicinoids ($r = 0.892$).

Suitability of a Qualitative Assessment to Distinguish the Degree of Pungency. Means of the capsaicin, dihydrocapsaicin, and total capsaicinoid contents for each qualitative (tasted) category of pungency are presented in **Table 2**. Fruits belonging to the tasted as non-pungent category showed the lowest capsaicin, dihydrocapsaicin, and total capsaicinoid contents, whereas the highest contents were found in the tasted as extremely pungent category. The mean capsaicinoid contents determined by HPLC-MS were 2, 59, 84, and 142 mg/kg DW for the tasted as non-pungent, tasted as slightly pungent, tasted as pungent, and tasted as extremely pungent categories. Values for the tasted as slightly pungent and tasted as pungent categories were not significantly different. Furthermore, a significant number of genotypes in these categories (33 and 39% in the tasted as slightly pungent and tasted as pungent categories, respectively) was incorrectly classified by this qualitative test.

DISCUSSION

A genetic analysis of the inheritance of capsaicin and dihydrocapsaicin has been carried out using an intraspecific cross of *C. annuum* across two seasons. Capsaicinoids were determined using a validated HPLC-ESI/MS method, which allowed reliable identification and quantification of capsaicin and dihydrocapsaicin. This analytical method uses for compound determination not only a HPLC retention time but also the exact *m*/*z* ratios of capsaicinoids, leading to a high selectivity that

avoids errors in capsaicinoid peak assignment during the analysis of complex matrices such as pepper fruit extracts. This method also has a high sensitivity (LODs of 0.6 and 3 mg/kg DW for dihydrocapsaicin and capsaicin, respectively), minimizing the possibility of assigning false negatives (individuals with low capsaicinoid contents) in inheritance studies. In this work, 67 out of 184 pungent individuals had total capsaicinoid contents between 0.6 (LOD of our method) and 30 [LOD of the Collins et al. (*29*) method] mg/kg DW. Thus, in this specific cross, 36% of individuals considered as pungent with our methodology would have been considered non-pungent individuals using the HPLC-UV method applied until now in capsaicinoid quantitative inheritance genetic studies in pepper (*29*).

The highest individual and total capsaicinoid contents corresponded to the F_1 family, and they were higher than the midparental values, in agreement with the studies of Zewdie and Bosland (*26*) and Blum et al. (*27*). The capsaicinoid contents of the F_1 family were also higher than those of the pungent parental line, a fact that was not observed in previous studies (*26*, *27*). This observation would infer the presence of heterosis in this cross, which could be attributed to the existence of interactions among alleles of different loci (epistasis), to the superiority of the heterozygote above the homozygote (overdominance), or to dominance complementation (*36*). The total capsaicinoid content of the F_2 family was higher than that of the pungent parental line, indicating transgressive segregation. Therefore, non-pungent parental line ('Yolo Wonder') alleles could contribute to increase the capsaicinoid content. Transgressive segregation for the pungency trait was also found in the F_2 family in a cross between Maor (a non-pungent *C. annuum* parent) and BG2816 (a pungent *C. frutescens* parent), and this was attributed to the Maor alleles (*27*).

The family−environment interaction was also found to be significant, and consequently, the families did not respond similarly in producing capsaicin and dihydrocapsaicin when fruits were developed in spring and summer. The family−environment interactions for total and individual capsaicinoids also have been examined by other authors. The contents of capsaicin, dihydrocapsaicin, and three minor capsaicinoids behaved differently in two double haploid lines, an F_1 hybrid, and an open-pollinated cultivar when grown in two New Mexico locations with either furrow or drip irrigation during 1996 and 1997 (*17*). In contrast, the family−environment interaction was not significant for major and minor capsaicinoid contents in parental lines and families obtained from an interspecific cross when grown either in a greenhouse or in an open field (*26*). Also, capsaicin and dihydrocapsaicin contents did not show significant family−environment interactions in fruits harvested in summer and winter, using families from another interspecific cross and their parental lines (*27*).

The environmental factor affected capsaicin and dihydrocapsaicin production, and in three of the families (BC_{P1}, BC_{P2}, and F_1), fruits grown in summer had markedly higher capsaicinoid contents than those developed in springtime. For BC_{P1} and BC_{P2}, these differences in the capsaicinoid contents also could have been affected by the limited segregating families size used since they could have contained a subset of the genetic variation. An effect of the fruit growing season was observed in previous studies. For instance, fruits have been found to have higher capsaicinoid contents in summer than in autumn (*16*), and the capsaicin content was increased in the warmer season as compared to the cooler one (*27*).

Positive correlations between the contents of capsaicin and dihydrocapsaicin were found both in spring and in summer. Blum et al. (*27*) found similar results for fruits harvested in summer and winter from an interspecific cross (*C. annuum* Maor × *C. frutescens* BG2816). This supports the hypothesis that the identification and selection of plants with high capsaicin contents could be linked to selection based on high dihydrocapsaicin contents, regardless of the harvest season. The fact that the ratio between capsaicin and dihydrocapsaicin was lower in summer than in spring deserves further investigation.

Capsaicin and dihydrocapsaicin quantitative inheritance in spring and summer cannot be adequately explained by a digenic model without epistasis (additive × dominance model). Instead, a model with interactions could explain the data. A negative additive × additive interaction occurred in spring, whereas both positive dominance × dominance and additive × dominance interactions were found in summer for total capsaicinoid content. Therefore, genetic control for the two capsaicinoids studied appears to depend on the season. The type of gene action did not vary between capsaicin and dihydrocapsaicin in spring, whereas in summer, different interactions were observed for both capsaicinoids. The gene pairs seem to be in a dispersive form (*37*) since both the additive × additive interaction and the dominance effect had the same sign for capsaicin and dihydrocapsaicin inheritance in spring. Gene interaction is considered to be complementary when the dominance and dominance × dominance estimates have the same sign and to be duplicated when the signs differ (*37*). On this basis, whereas duplicate gene action was observed for capsaicin, dihydrocapsaicin, and total capsaicinoid in spring, complementary action for both capsaicinoids and total capsaicinoid was observed in summer.

Zewdie and Bosland (*26*) did not find an environmental effect, and interactions were also different from those observed in this work. Possible explanations for the differences between both studies could be attributed to (i) the different genotypes used [an intraspecific cross between *C. annuum* 'Serrano Criollo de Morelos-334' and *C. annuum* 'Yolo Wonder' in this work and an interspecific cross between *C. annuum* PI298646 and *C. chinense* 'Habanero' in the work of Zewdie and Bosland (*26*)]; (ii) the different environments (spring and summer harvest time in our study and open field and greenhouse growth conditions in their study); and (iii) the different selectivity and sensitivity of the analytical methods for capsaicinoid determination [the Garcés-Claver et al. (*30*) method in our work and the Collins et al. (*29*) method in their work]. Therefore, the pungency trait may be inherited differently in specific environments and genotypes.

The pungency inheritance is likely to be polygenic, as supported by the continuous distribution of the F_2 capsaicinoid contents, the transgressive segregation in the F_2 progeny, and the different type of gene action between capsaicin and dihydrocapsaicin in summer. However, the number of effective factors controlling capsaicin and dihydrocapsaicin inheritance could not be accurately estimated in this study because of the presence of a major gene (*Pun1*) and the dominance and epistatic effects, as concluded by Kondra and Tomas (*38*) and Falconer (*39*).

The HPLC-ESI/MS method was much more powerful than the qualitative assessment method used to distinguish between capsaicinoid contents. First, many genotypes considered as non-pungent had a measurable amount of capsaicinoids. Because of this fact, within the tasted as non-pungent category, two types of individuals, in both cases not producing an organoleptic pungent sensation, were included: those individuals where no capsaicinoid was detected and those individuals with certain low amounts of capsaicin and dihydrocapsaicin that could be

quantified with the analytical method used. Also, a significant number of genotypes in the tasted as slightly pungent and tasted as pungent intermediate categories (>30% in both cases) were incorrectly classified by our qualitative test.

In summary, this is the first time that a pungency quantitative inheritance study in an intraspecific cross of *C. annuum* was carried out using a highly reliable method for the identification and quantification of capsaicin and dihydrocapsaicin. Capsaicin and dihydrocapsaicin contents varied largely among families, and these families did not respond similarly in producing these capsaicinoids when their fruits were grown in spring and summer. The contribution of the environment could be only important in some families (BC_{P1}, BC_{P2}, and F_1). The heterosis for the pungency (capsaicin and dihydrocapsaicin contents) trait was found, which indicated the existence of epistasis, overdominance, or dominance complementation. Also, non-pungent parent alleles appear to contribute to the capsaicin and dihydrocapsaicin contents since transgressive segregation occurred. The selection of genotypes with high capsaicin contents from the studied population imposes the selection of high dihydrocapsaicin contents regardless of the fruit growing season. Finally, the type of gene action varied between capsaicin and dihydrocapsaicin, and a seasonal effect could suggest additional differences in gene action.

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Supporting Information Available: Mean squares for capsaicin, dihydrocapsaicin, and total capsaicinoid contents of six families of an intraspecific cross of *C. annuum* L. across two environments. Joint scaling test and estimates obtained using a three-parameter model and individual scaling test A, B, and C models. Joint scaling test using a six-parameter model. Correlation coefficients between capsaicin, dihydrocapsaicin, and total capsaicinoid contents for pepper fruits grown in spring and summer. This material is available free of charge via the Internet at http://pubs.acs.org.

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