The Horticulture Journal 89 (4): 460–465. 2020.
doi: 10.2503/hortj.UTD-184

The Japanese Society for Horticultural Science
http://www.jshs.jp/

Multiple Non-pungent *Capsicum chinense* Accessions with a Loss of Function *CaKR1* Allele Originating from South America

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In *Capsicum*, loss of function mutation of *acyltransferase* (*Pun1*), *putative aminotransferase* (*pAMT*), *putative ketoacyl-ACP reductase* (*CaKR1*), and *R2R3-MYB transcription factor* (*CaMYB31*) have been reported to be the genetic causes of non-pungency. In the present study, 245 *C. chinense* accessions were initially screened for non-pungency attributes. Six candidates with identification numbers, No. 3327, No. 3356, No. 3529, No. 4026, No. 4028, and No. 4034 were selected by tasting test, and the non-pungency attribute was confirmed by high-performance liquid chromatographic analysis. Expression and sequence analysis inferred that the non-pungency of No. 3529 was due to the non-expression of *Pun1*. Analysis of *pAMT* confirmed that No. 3356 (*pamt*^{5}) and No. 4034 (*pamt*^{9}) had loss of function mutations. Because the non-pungency of No. 3327, No. 4026, and No. 4028 did not seem to be caused by mutation of either *Pun1* or *pAMT*, the *CaKR1* mutation was further examined using a polymerase chain reaction-based, co-dominant marker. Genotyping clarified that No. 3327, No. 4026, and No. 4028 had the same mutated *CaKR1* allele as non-pungent No. 3341. Moreover, a crossing test with a pungent Habanero and No. 3341 clearly revealed that the non-pungency in No. 3327, No. 4026, and No. 4028 was a result of a loss of function mutation of *CaKR1*. Our previous and present studies have shown that non-pungent cultivars of *C. chinense* possessing *pamt* are widely distributed in Central America, South America and the West Indies (Caribbean), while non-pungent cultivars possessing *Cakr1* originate from Bolivia and Peru. Some artificial selection may have occurred that was based on a preference for non-pungent peppers in the local region of origin.

Key Words: *acyltransferase* (*Pun1*), capsaicinoid, pepper, *putative aminotransferase* (*pAMT*), *putative ketoacyl-ACP reductase* (*CaKR1*).

Introduction

Capsicum, a member of the *Solanaceae* family, is categorized into pungent and non-pungent cultivars: the former is used as a spice and the latter as a vegetable. Moreover, peppers are widely used in pharmaceuticals, as natural coloring agents and in the cosmetics industry; they are also used with ornamental plants, and as the active ingredient in most self-defense repellents. According to the statistical data of the Food and Agriculture Organization of the United Nations (FAO; http://www.fao.org), fresh and dried peppers that were produced around the world in the year 2017 totalled 40.7 million tons, implying land under cultivation amounting to 3.8 million hectares.

Based on archeological evidence, *Capsicum* had already been domesticated by around 6000 B. P. in the Americas, which makes it one of the earliest domesticated plant genera (Perry et al., 2007). *Capsicum* is known to consist of several wild species and five domesticated species, *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens* (Bosland and Votava, 2000). The economically important species belong to the *C. annuum* complex (*C. annuum*, *C. chinense*, and *C. frutescens*), and two other species

Received; January 21, 2020. Accepted; April 7, 2020.
First Published Online in J-STAGE on May 26, 2020.
This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers 25850018, 16K07605 for S. Koeda.
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(*C. baccatum* and *C. pubescens*) are known to be cultivated predominantly in Latin America (Pickersgill, 1997). *C. annuum*, which originates from southern Mexico, is the most widely cultivated worldwide. This is probably because *C. annuum* is the best adapted to the cool climate in Europe, where it was first introduced at the end of the fifteenth century from the Americas, following the voyage of Christopher Columbus. *C. chinense* is of Amazonian origin and is indigenous to South America and the Caribbean (Eshbaugh, 1993). *C. chinense* cultivars such as Habanero and Scotch Bonnet are highly pungent and are known to possess highly aromatic flavors that *C. annuum* cultivars lack (Moreno et al., 2012; Koeda et al., 2014).

The pungency of chili pepper fruits is caused by a group of analogs known as capsaicinoids (Bennett and Kirby, 1968). These unique compounds are selectively produced by *Capsicum* fruits (Andrews, 1984). Given that the pungency of pepper fruit is one of its most important traits, numerous studies have been conducted to better understand this phenomenon. To date, a loss of function mutation of *acyltransferase* (*Pun1*), *putative aminotransferase* (*pAMT*), *putative ketoacyl-ACP reductase* (*CaKR1*), and *R2R3-MYB transcription factor* (*CaMYB31*) have been reported to be the genetic causes of loss of pungency (Stewart et al., 2005; Lang et al., 2009; Arce-Rodríguez and Ochoa-Alejo, 2017; Han et al., 2019; Koeda et al., 2019). Almost all the non-pungent *C. annuum* cultivars possess *pun1*, and only a few cultivars have *pamt* or mutated *CaMYB31*. In contrast, most of the reported non-pungent cultivars of *C. chinense* possess *pamt*, and only single accessions are reported to have *Cakr1* and *pun1*^{2} (Stewart et al., 2007; Tanaka et al., 2010, 2015, 2018; Koeda et al., 2014, 2019). In the present study, non-pungent peppers were screened from 245 accessions of *C. chinense* to reveal the distribution of non-pungency in peppers with different genetic mechanisms and places of origin.

Materials and Methods

Plant materials, crossing combinations, and growth conditions

For initial screening of non-pungent peppers, 245 accessions of *C. chinense* from Antigua and Barbuda, Barbados, Bolivia, Brazil, Colombia, Fiji, Guyana, Jamaica, Peru, Trinidad and Tobago, and Venezuela were used. Initial screening was conducted at the experimental farm of Kyoto University in 2013 and 2014. A pungent pepper, Habanero, and a non-pungent accession, No. 3341 carrying the recessive allele of *putative ketoacyl-ACP reductase* (*CaKR1*; *Cakr1*/*Cakr1*) were used for crossing. F_1 and F_2 populations that were obtained by crossing Habanero with No. 3327, No. 4026, or No. 4028 to determine the inheritance pattern of fruit pungency. F_1 populations were prepared by crossing No. 3341 with No. 3327, No. 4026, or No. 4028. All plants were grown in an unheated greenhouse at Kindai University from March to October in 2018 and 2019.

Phenotyping of fruit pungency

The capsaicinoid contents of the fruits were confirmed using high-performance liquid chromatographic analysis (HPLC). After the fruit had been freeze-dried, the capsaicinoids were extracted and quantified according to the method described by Koeda et al. (2014). The capsaicinoid content was then calculated as the sum of capsaicin and dihydrocapsaicin.

RT-polymerase chain reaction (PCR) analysis and cDNA sequence analysis of Pun1 and pAMT

The full-length cDNA sequences of *Pun1* and *pAMT* were determined for No. 3327, No. 3356, No. 3529, No. 4026, and No. 4028. Pepper fruits were harvested at 25 days post-anthesis (dpa) and the placenta and interlocular septum were separated for RNA extraction. Total RNA was extracted and reverse-transcribed according to the method described by Koeda et al. (2014). For RT-PCR, *CaActin* (AY572427) was used as a positive internal control. The full-length cDNA sequence of *Pun1* was amplified using Pun1-F and Pun1-R primer sets (Koeda et al., 2014), and the full-length cDNA sequence of *pAMT* was amplified using F1 and R1481 primer sets (Tanaka et al., 2010). PCR and electrophoresis were performed according to Koeda et al. (2015). The full-length sequences of *Pun1* and *pAMT* amplified by RT-PCR were cloned into the pTAC-1 cloning vector (BioDynamics Laboratory, Tokyo, Japan). Nucleotide sequencing was performed in an ABI PRISM 3100 genetic analyzer with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

Genotyping of the CaKR1 allele

DNA was extracted from the pepper leaves using the Nucleon PhytoPure kit (GE Healthcare, IL, USA). Primers 1, 2, and 3 for the PCR-based, co-dominant marker were used to determine the allelic state at *CaKR1* for parental cultivars and crossing progenies according to Koeda et al. (2019).

Real-time qRT-PCR

Pepper fruits were harvested at 10, 25, and 45 dpa and the placenta and interlocular septum were separated for RNA extraction. DNase treatment of RNA, cDNA synthesis, and real-time quantitative reverse-transcription PCR (real-time qRT-PCR) were conducted according to Koeda et al. (2019). At least three biological replicates were analyzed, with three technical replicates for each. The relative abundance of transcripts was normalized to the *C. annuum CaActin* (AY572427) reference control gene, and relative quantities were calculated using the $2^{-\Delta\Delta Ct}$ method.

Results and Discussion

Six candidates, No. 3327, No. 3356, No. 3529, No. 4026, No. 4028, and No. 4034, were screened as non-pungent from the 245 accessions (*C. chinense*) by tasting test. The non-pungency of No. 4034 was shown to be caused by the loss of a functional mutation of *pAMT* (Tanaka et al., 2018). No. 3327, No. 3356, No. 4026, and No. 4028 were shown to have the typical round fruit of *C. chinense*; fruit height and width were approximately 3.5–4.0 cm, and fruit weight was approximately 15 g (Fig. 1). In contrast, the fruit morphology of No. 3529 was distinct, and it had a slender fruit shape. Capsaicinoid accumulations of five candidate accessions were evaluated using HPLC (Table 1). In Habanero, capsaicinoids were detected from mature fruits of 45 dpa. In contrast, capsaicinoids were not detected in No. 3327, No. 3356, No. 3529, No. 4026, or No. 4028.

We investigated the expression levels of *Pun1* and *pAMT* to elucidate the genetic basis of the non-pungent phenotype of No. 3327, No. 3356, No. 3529, No. 4026, and No. 4028. First, the expression of *Pun1* was analyzed by RT-PCR. *Pun1* fragments of 1.3 kbp were amplified from Habanero, No. 3327, No. 3356, No. 4026, and No. 4028, but not from No. 3529 (Fig. 2). The non-expression of *Pun1* seemed to be the underlying mechanism of non-pungency for No. 3529. In *C. annuum* cultivars, deletion mutations in promotor regions caused non-expression on *Pun1*, resulting in non-pungency (Stewart et al., 2005). No. 3529 was originally collected by Norio Yamamoto and categorized as *C. chinense*/*C. frutescens*, but we observed the typical white flowers of *C. annuum* in No. 3529, and No. 3529 had a distinct, slender fruit morphology as compared with the other five accessions, which had the typical round shape of *C. chinense* (Fig. 1). These results strongly suggest that the No. 3529 *C. annuum* accession possesses the *pun1* allele. When cDNA sequences of *Pun1* were compared for Habanero, No. 3327, No. 3356, No. 4026, and No. 4028, the deduced amino acid sequences of Pun1 completely matched. These results indicate that the non-pungency of No. 3327, No. 3356, No. 4026, and No. 4028 was not caused by *Pun1* mutations.

The expression level of *pAMT* was also analyzed by RT-PCR. Expression of *pAMT* was detected for Habanero and the other five candidates. When cDNA sequences of *pAMT* were compared with pungent Habanero and PI159236, mutations were detected in No. 3356 and No. 3529 (Fig. 3). cDNA sequence analysis revealed that the *pAMT* cDNA of No. 3356 contained a 403-bp insertion, resulting in a frame-shift mutation that was identical to the *pamt*^{5} of Aji Dulse Strain 2 (*C. chinense*) (Tanaka et al., 2010). This insertion led to truncated proteins of 59 amino acids lacking the pyridoxal 5-phosphate (PLP) binding domain (Fig. 3), which is essential for aminotransferase activity and mutations in this domain resulted in loss of pungency (Lang et al., 2009). No. 3529 had two SNPs, and one of them was a guanine (G) to adenine (A) mutation in the PLP domain. However, because this SNP was also observed in pungent PI159236, it could not be the cause of non-pungency in No. 3529. These results indicated that the non-pungency of No. 3327, No. 4026, and No. 4028 was not caused by *pAMT* mutations.

The loss of mutational functionality in *CaKR1* was

Fig. 1. Non-pungent accessions screened from germplasms. Fruit morphology of No. 3327, No. 3356, No. 3529, No. 4026, and No. 4028. Bars indicate 2 cm.

Table 1. Capsaicinoid content in the fruit of Habanero, No. 3327, No. 3356, No. 3529, No. 4026, and No. 4028.

Cultivar and accessions	Capsaicinoid content (μg·g^{-1} DW)
Habanero	15353±2485
No. 3327	N.D.
No. 3356	N.D.
No. 3529	N.D.
No. 4026	N.D.
No. 4028	N.D.

Average and standard deviation were calculated for the results of three plants.
N.D. indicates not detected.

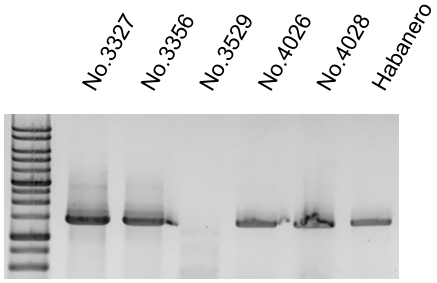

Fig. 2. RT-PCR analysis for full-length *Pun1* in No. 3327, No. 3356, No. 3529, No. 4026, No. 4028, and Habanero.

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PI159236      MANITNEFMGHDMLAPFTAGWQSDMEPLVIEKSEGSYVYDINGKKYLDTLSGLWCATLGGSETRLVEAANKQLNTLPFYHSFWNRTTKPSLDLAKELLNMFTA
Habanero      MANITNEFMGHDMLAPFTAGWQSDMEPLVIEKSEGSYVYDINGKKYLDTLSGLWCATLGGSETRLVEAANKQLNTLPFYHSFWNRTTKPSLDLAKELLNMFTA
No.3327       MANITNEFMGHDMLAPFTAGWQSDMEPLVIEKSEGSYVYDINGKKYLDTLSGLWCATLGGSETRLVEAANKQLNTLPFYHSFWNRTTKPSLDLAKELLNMFTA
No.3356       MANITNEFMGHDMLAPFTAGWQSDMEPLVIEKSEGSYVYDINGKKYLDTLSGLWCATLG----------------------------------------------
No.3529       MANITNEFMGHDMLAPFTAGWQSDMEPLVIEKSGSYVYDINGKKYLDTLSGLWCATLGGSETRLVEAANKQLNTLPFYHSFWNRTTKPSLDLAKELLNMFTA
No.4026       MANITNEFMGHDMLAPFTAGWQSDMEPLVIEKSEGSYVYDINGKKYLDTLSGLWCATLGGSETRLVEAANKQLNTLPFYHSFWNRTTKPSLDLAKELLNMFTA
No.4028       MANITNEFMGHDMLAPFTAGWQSDMEPLVIEKSEGSYVYDINGKKYLDTLSGLWCATLGGSETRLVEAANKQLNTLPFYHSFWNRTTKPSLDLAKELLNMFTA

PI159236      NKMAKVFFTNSGSEANDTQVKLVWYYNNALGRPQKKKIIARAKAYHGSTYISAGLSGLPPMHQKFDLPPPFVLHTECPHYWAYHLPGETEEEFSTRLANNLES
Habanero      NKMAKVFFTNSGSEANDTQVKTVWYYNNALGRPQKKKIIARAKAYHGSTYISAGLSGLPPMHQKFDLPPPFVLHTECPHYWAYHLPGETEEEFSTRLANNLES
No.3327       NKMAKVFFTNSGSEANDTQVKLVWYYNNALGRPQKKKIIARAKAYHGSTYISAGLSGLPPMHQKFDLPPPFVLHTECPHYWAYHLPGETEEEFSTRLANNLES
No.3356       ----------------------------------------------------------------------------------------------------
No.3529       NKMAKVFFTNSGSEANDTQVKLVWYYNNALGRPQKKKIIARAKAYHGSTYISAGLSGLPPMHQKFDLPPPFVLHTECPHYWAYHLPGETEEEFSTRLANNLES
No.4026       NKMAKVFFTNSGSEANDTQVKLVWYYNNALGRPQKKKIIARAKAYHGSTYISAGLSGLPPMHQKFDLPPPFVLHTECPHYWAYHLPGETEEEFSTRLANNLES
No.4028       NKMAKVFFTNSGSEANDTQVKLVWYYNNALGRPQKKKIIARAKAYHGSTYISAGLSGLPPMHQKFDLPPPFVLHTECPHYWAYHLPGETEEEFSTRLANNLES

PI159236      LILKEGPETVAAFIAEPVLGAAGVILPPATYFDKVQTILRKYDILFIADEVVCGFGRLGTMFGSDKYNIKPDLVSVAKALSSGYMPIAAVLVSQKISSVILSE
Habanero      LILKEGPETVAAFIAEPVLGAAGVILPPATYFDKVQAILRKHDILFIADEVVCGFGRLGTMFGSDKYNIKPDLVSVGKALSSGYMPIAAVLVSQKISSVILSE
No.3327       LILKEGPETVAAFIAEPVLGAAGVILPPATYFDKVQAILRKHDILFIADEVVCGFGRLGTMFGSDKYNIKPDLVSVGKALSSGYMPIAAVLVSQKISSVILSE
No.3356       ----------------------------------------------------------------------------------------------------
No.3529       LILNEGPETVAAFIAEPVLGAAGVILPPATYFDKVQAILRKHDILFIADEVVCGFGRLGTMFGSDKYNIKPDLVSVAKALSSGYMPIAAVLVSQKISSVILSE
No.4026       LILKEGPETVAAFIAEPVLGAAGVILPPATYFDKVQAILRKHDILFIADEVVCGFGRLGTMFGSDKYNIKPDLVSVGKALSSGYMPIAAVLVSQKISSVILSE
No.4028       LILKEGPETVAAFIAEPVLGAAGVILPPATYFDKVQAILRKHDILFIADEVVCGFGRLGTMFGSDKYNIKPDLVSVGKALSSGYMPIAAVLVSQKISSVILSE

PI159236      SNKIGAFCHGFTYSGHPVACAVALEALKIYKERNITEVVNKISQKFQEGLKAFADSPIIGEIRGTGLALSTEFVDNKSPNDPFPYEWAVGTYFGAQCAKYGML
Habanero      SNKIGAFCHGFTYSGHPVACAVALEALKIYKERNITEVVNKISQKFQEGLKAFADSPIIGEIRGTGLALSTEFVDNKSPNDPFPYEWAVGTYFGAQCAKYGML
No.3327       SNKIGAFCHGFTYSGHPVACAVALEALKIYKERNITEVVNKISQKFQEGLKAFADSPIIGEIRGTGLALSTEFVDNKSPNDPFPYEWAVGTYFGAQCAKYGML
No.3356       ----------------------------------------------------------------------------------------------------
No.3529       SNKIGAFCHGFTYSGHPVACAVALEALKIYKERNITEVVNKISQKFQEGLKAFADSPIIGEIRGTGLALSTEFVDNKSPNDPFPYEWAVGTYFGAQCAKYGML
No.4026       SNKIGAFCHGFTYSGHPVACAVALEALKIYKERNITEVVNKISQKFQEGLKAFADSPIIGEIRGTGLALSTEFVDNKSPNDPFPYEWAVGTYFGAQCAKYGML
No.4028       SNKIGAFCHGFTYSGHPVACAVALEALKIYKERNITEVVNKISQKFQEGLKAFADSPIIGEIRGTGLALSTEFVDNKSPNDPFPYEWAVGTYFGAQCAKYGML

PI159236      VSSTGDHVNMAPPFMLSLEELDELIRIYGKALKDTEKRVEELKSQKK
Habanero      VSSTGDHVNMAPPFILSLEELDELIRIYGKALKDTEKRVEELKSQKQ
No.3327       VSSTGDHVNMAPPFILSLEELDELIRIYGKALKDTEKRVEELKSQKQ
No.3356       -------------------------------------------------
No.3529       VSSTGDHVNMAPPFTLSLEELDELIRIYGKALKDTEKRVEELKSQKK
No.4026       VSSTGDHVNMAPPFILSLEELDELIRIYGKALKDTEKRVEELKSQKQ
No.4028       VSSTGDHVNMAPPFILSLEELDELIRIYGKALKDTEKRVEELKSQKQ
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Fig. 3. Alignment of the deduced amino acid sequences of *pAMT* from pungent PI159236 (*C. chinense*), Habanero and non-pungent No. 3327, No. 3356, No. 3529, No. 4026, and No. 4028. the underlined part indicates the PLP-binding domain. The amino acid residues that were minor in the compared cultivars are shaded black.

shown to cause non-pungency in a single *C. chinense* accession, No. 3341, in our previous study (Koeda et al., 2019). No. 3341 had an insertion of a 4.5-kb transposable element (TE) sequence in the first intron, resulting in the production of a truncated transcript missing the region coding for the catalytic domain. A co-dominant marker system for *CaKR1* based on the TE insertion was utilized for No. 3327, No. 4026, and No. 4028 (Fig. 4A). A 599-bp amplicon was detected for Habanero, while amplicons of 1447-bp were detected for No. 3341, No. 3327, No. 4026, and No. 4028, indicating that these newly studied non-pungent peppers have the same mutated *CaKR1* allele as No. 3341. Moreover, when gene-specific primers amplified the 3′ end of *CaKR1* and it was used for real-time qRT-PCR, expression of *CaKR1* was detected only in Habanero and not in No. 3341, No. 3327, No. 4026, or No. 4028 (Fig. 4B). These results strongly suggest that the mutation in *CaKR1* is responsible for the non-pungency in No. 3327, No. 4026, and No. 4028.

We further conducted crossing tests with Habanero, No. 3341, No. 3327, No. 4026, and No. 4028 (Table 2). F_1 progenies obtained by crossing No. 3327, No. 4026, or No. 4028 with Habanero were pungent, and F_1 progenies obtained by crossing with No. 3341 were non-pungent. Moreover, F_2 progenies obtained by crossing with Habanero were pungent when individuals possessed wildtype *CaKR1* in homozygous or heterozygous states, but non-pungent when they possessed a mutated allele (*Cakr1*) in homozygous state, which represents complete co-segregation between the phenotype and genotype. These results clearly show that non-pungency in No. 3327, No. 4026, and No. 4028 is the result of a *CaKR1* loss of function mutation.

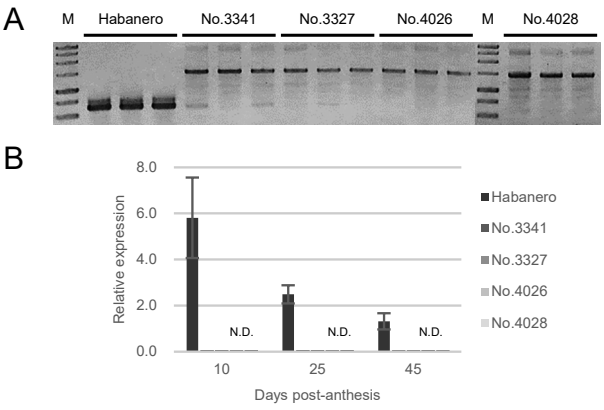

Fig. 4. Genotyping and expression analysis of the *CaKR1 allele*. (A) Agarose gel showing PCR bands from *CaKR1* genotyping of Habanero, No. 3341, No. 3327, No. 4026, and No. 4028. M represents a 1-kb DNA ladder. (B) Real-time qRT-PCR analysis of *CaKR1* in fruits of Habanero, No. 3341, No. 3327, No. 4026, and No. 4028, at 10, 25, and 45 dpa. Three biological replicates were analyzed with three technical replicates for each. N.D. indicates not detected. Error bars indicate standard deviations.

In the present study, 245 *C. chinense* accessions were used for preliminary screening for non-pungent accessions, and this is a relatively large number. We have reported many non-pungent peppers (*C. chinense*) for

Table 2. Phenotypic segregation of the non-pungent phenotype of No. 3327, No. 4026, and No. 4028.

Parental cultivar and cross combination	Genotype of *CaKR1* locus[z]	Population size (n)	Number of plants[y]	
			Pungent	Non-pungent
P_1 Habanero	*WT/WT*	10	10	0
P_2 No. 3341	*mu/mu*	10	0	10
P_3 No. 3327	*mu/mu*	10	0	10
P_4 No. 4026	*mu/mu*	10	0	10
P_5 No. 4028	*mu/mu*	10	0	10
F_1 (P_1×P_3)	*WT/mu*	7	7	0
F_1 (P_1×P_4)	*WT/mu*	7	7	0
F_1 (P_1×P_5)	*WT/mu*	7	7	0
F_1 (P_2×P_3)	*mu/mu*	15	0	15
F_1 (P_2×P_4)	*mu/mu*	15	0	15
F_1 (P_2×P_5)	*mu/mu*	15	0	15
F_2 (P_1×P_3)	*WT/WT*	8	8	0
	WT/mu	9	9	0
	mu/mu	15	0	15
F_2 (P_1×P_4)	*WT/WT*	9	9	0
	WT/mu	10	10	0
	mu/mu	17	0	17
F_2 (P_1×P_5)	*WT/WT*	11	11	0
	WT/mu	7	7	0
	mu/mu	10	0	10

[z] Genotyping was conducted by PCR using primers 1, 2, and 3 reported in Koeda et al. (2019). *WT* and *mu* indicate wildtype and mutated allele of *CaKR1*.

[y] Pungency of each individual was evaluated using HPLC.

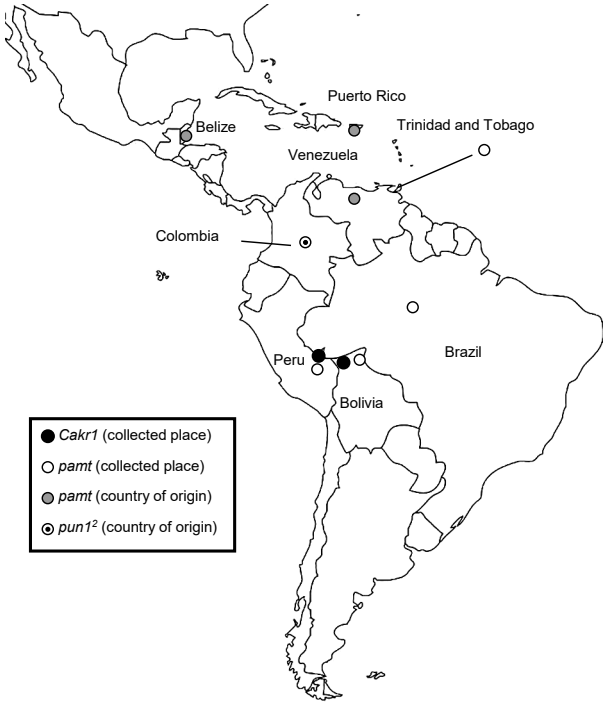

Fig. 5. Places of origin for non-pungent *C. chinense* cultivars carrying the loss of function allele of *CaKR1*, *pAMT*, and *Pun1*. Black circles and white circles indicate the original collection place for the *Cakr1* mutant and *pamt* mutant. Gray circles indicate country of origin for the *pamt* mutant. Double circles indicate country of origin for the *pun1²* mutant.

which the country of origin is evident (Fig. 5). No. 3341 (*Cakr1*) and No. 3327 (*Cakr1*) originated from the suburbs of Cobija in Bolivia. No. 4026 (*Cakr1*), No. 4028 (*Cakr1*), and No. 4034 (*pamt⁹*) originated from the suburbs of Alto Rio Purus, located near the border of Peru and Brazil, which is an upstream region of the Rio Purus River. No. 2 (*pamt⁵*) originated from Manaus in Brazil, No. 80 (*pamt⁶*) from Trinidad island in Trinidad and Tobago, No. 3356 (*pamt⁵*) from Guayaramerin in Bolivia, Belize sweet (*pamt³*) from Belize, LP2 (*pamt³*) from Puerto Rico, and the Aji dulce strain 2 (*pamt⁵*) from Venezuela, NMCA30036 (*pun1²*) from Colombia (Koeda et al., 2014; Bosland and Coon, 2015). These results show that non-pungent cultivars possessing *pamt* are widely distributed in Central America, South America and the West Indies (Caribbean). In contrast, non-pungent cultivars with *Cakr1* originated only from Bolivia and Peru, which is a relatively limited region. Moreover, NMCA30036 is the only non-pungent cultivar possessing *pun1²*, and we could not find additional accessions with *pun1²*, even though we screened a large number of germplasms. Phylogenetic research inferred that the origin of *Capsicum* is Bolivia (McLeod et al., 1982; Moscone et al., 2007), a continuous belt of land from south-eastern Brazil to the Andes (Bianchetti, 1996; Pozzobon et al., 2006), or regions of Peru, Ecuador and Colombia (Carrizo García et al., 2016). In Central America, South America and the West Indies,

the majority of peppers are pungent cultivars. On the other hand, although they represent a minority compared to the pungent cultivars, several non-pungent cultivars are also important in local cuisine (Koeda, 2012; Koeda et al., 2014). Some artificial selection may have occurred with these non-pungent peppers motivated by preference in the local region of origin. Although there are many possibilities, we hypothesize that differences in mutated genes (*Pun1*, *pAMT*, or *CaKR1*) may be affecting other fruit characteristic traits. *C. chinense* cultivars such as Habanero and Scotch Bonnet are highly pungent and have highly aromatic flavors, which *C. annuum* cultivars lacks (Moreno et al., 2012; Koeda et al., 2014). There may be some overlap or indirect interaction between the capsaicinoid biosynthetic pathway and volatile aroma-producing compound biosynthetic pathways. Our team is currently analyzing the relationship between pungency/non-pungency and the aroma of *C. chinense* fruit.

Acknowledgements

Research materials were kindly provided from Norio Yamamoto (National museum of ethnology, Japan) and Sota Yamamoto (Kagoshima University, Japan). We would like to thank Toshio Sakakibara and Masaru Matsuda (Experimental farm of Kyoto University) for supporting the initial screening of non-pungent accessions.

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