ASSESSMENT OF GENE PROFILES ASSOCIATED WITH PUNGENCY LEVELS IN CHILI (*CAPSICUM* SPP.)

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Abstract

Chili in the genus *Capsicum* is globally cultivated as an economic vegetable crop, and used in traditionally pungent cuisines, especially in Thailand. Degrees of pungency were positively correlated with capsaicin biosynthesis, which was strongly influenced by genotype and associated with several enzyme-encoding genes. Five candidate genes (*PUN1*, *HCT*, *pAMT*, *CCR* and *KAS*) responsible for capsaicin synthesis were assessed among fourteen *Capsicum* cultivars. Results showed that all putative capsaicin biosynthetic genes were present in five *Capsicum* cultivars of Bhut Jolokia, Orange Habanero (High pungency) and Tubtim Mordindang, Phet Mordingang and Akanee Pirote (Moderate pungency). Two genes (*HCT* and *pAMT*) were absent in all eight low pungent cultivars (Jindanil 80, Bang Chang, Yodson Khem 80, Num Khao Donyang, Num Keowtong 80, Hua-ruea, Huai Si Thon Kham Kaen and Chai Tai). Analysis of the capsaicin biosynthetic genes showed that *Capsicum* cultivar KKU-P28016 (breeding line from Khon Kaen University) contained *PUN1*, *pAMT*, *CCR* and *KAS* genes, suggesting identity as the moderately pungent *Capsicum* group. Mature fruit phenotypic traits among the studied *Capsicum* cultivars showed that a globose-oval shape of mature fruit was detected among highly and moderately pungent *Capsicum* cultivars, while an elongated shape of mature fruit was observed in low pungent cultivars. High correlation between the phenotypic trait of mature fruit shape and gene profiles is a powerful tool to discriminate pungency levels in *Capsicum* cultivars.

Keywords: *Capsicum* cultivars, Capsaicin biosynthesis, Pungency

INTRODUCTION

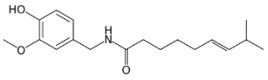
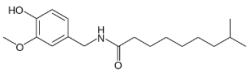
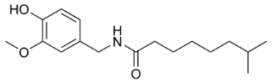
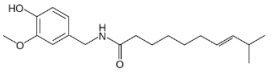
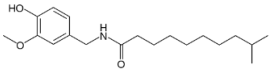
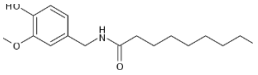
Chili (*Capsicum* spp.), belonging to the *Solanaceae* family, is a significant economic vegetable crop worldwide (Burgos–Valencia et al., 2020). Many *Capsicum* cultivars are cultivated throughout Thailand (Puripanyavanich et al., 2019). The fruits of the *Capsicum* plants contain a great variety of metabolites with nutritional health benefits and bioactive properties (Guzmán & Bosland, 2017) that are commercially used as food ingredients in traditional cuisines and in medicinal applications (Popelka et al., 2017). Among the metabolites, capsaicin has the most significant pharmacological properties and play an important role as pain–relievers (Basith et al., 2016), anti–microbials (Agarwal et al., 2017; Marini et al., 2015), anti–inflammatories (Jolayemi & Ojewole, 2013), antioxidants (Bogusz et al., 2018), anti–cancer (Clark & Lee, 2016) and anti–obesity (Zheng et al., 2017). *Capsicum* fruits containing high capsaicin content are beneficial for health treatments and as functional foods (Naves et al., 2019).

The most common secondary metabolites occurring that make chili pungent are capsaicinoids which comprised variety of analogues, *e.g.*, capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%) and homodihydrocapsaicin (1%). The Scoville scale is a measurement of the pungency (spiciness or "heat") of chili peppers, as recorded in Scoville Heat Units (SHU), based on the concentration of capsaicinoids by HPLC, among which capsaicin is the predominant component. Capsaicin and dihydrocapsaicin (Both 16.0 million SHU) are the most pungent capsaicinoids. Nordihydrocapsaicin (9.1 million SHU), homocapsaicin and homodihydrocapsaicin (Both 8.6 million SHU) are about half as hot. There are six forms of natural capsaicinoids (Table 1). Although vanillylamide of n–nonanoic acid (Nonivamide, VNA, also PAVA) is produced synthetically for most applications, it does occur naturally in *Capsicum* species (Werner, 2021).

The degree of characteristic spiciness in *Capsicum* mainlydepends on different amounts of capsaicin content, while the biosynthetic pathway is strongly influenced by genotype and associated with at least 14 genes (Arce–Rodriguez & Ochoa–Alejo, 2019). Of these, at least five candidate genes have been predicted to translate key enzymes in the capsaicin biosynthetic pathway such as *HCT*, *CCR*, *pAMT*, *KAS* and *PUN1*, which are

encoded enzymes of hydroxycinnamoyltransferase, cinnamoyl–CoA reductase, putative aminotransferase, ketoacyl–ACP synthase and capsaicinoid synthase, respectively (Burgos–Valencia et al., 2020). Of these five genes, the *pungency 1 (PUN1*) gene is a key enzyme at the final step of capsaicin synthesis by combining both compounds of vanillyamine, originated from the phenylpropanoid pathway and 8–methyl–6–nonenoyl–CoA, derived from the branched–chain fatty acid pathway (Arce–Rodriguez & Ochoa–Alejo, 2019; Naves et al., 2019; Ogawa et al., 2015).

Table 1 Capsaicinoid metabolites occurring in chili (Werner, 2021).

Capsaicinoid name	Abbreviation	Typical relative amount	Scoville heat units	Chemical structure
Capsaicin	C	69%	16,000,000	
Dihydrocapsaicin	DHC	22%	16,000,000	
Nordihydrocapsaicin	NDHC	7%	9,100,000	
Homocapsaicin	HC	1%	8,600,000	
Homodihydrocapsaicin	HDHC	1%	8,600,000	
Nonivamide	PAVA		9,200,000	

Capsicum cultivars, carrying homozygous dominant (*PUN1*/*PUN1*) and heterozygous (*PUN1*/*pun1*) genotypes, successfully produce more capsaicin, expressing a high pungency (hot) phenotype, while the homozygous recessive (*pun1*/*pun1*) genotype cannot synthesize capsaicin, exhibiting a non–pungent (sweet) phenotype (Stewart et al., 2005).

However, the mechanism of the capsaicin biosynthetic pathway has not yet been clearly defined, and increased *Capsicum* pungency through a breeding program still requires knowledge of the genetic resources of major genes associated with capsaicin biosynthesis.

Five candidate genes; *PUN1*, *HCT*, *pAMT*, *CCR* and *KAS*, are responsible for pungency levels and we are going to assess those genes among fourteen *Capsaicum* cultivars. Results will improve knowledge and understanding of genetic resources associated with capsaicin biosynthesis and, also promotes the improvement of a new *Capsicum* cultivars with high capsaicin content for either traditional breeding or a gene transfer approach.

MATERIALS AND METHODS

1. Plant materials

Fourteen different *Capsicum* cultivars, provided from Khon Kaen University, consisted of Bhut Jolokia, Orange Habanero, Tubtim Mordindang, Phet Mordindang, Akanee Pirote, Jindanil 80, Bang Chang, Yodson Khem 80, Num Khao Donyang, Num Keawtong 80, Hua Reu, Huai Si Thon Kham Kaen, Chia Tai (Commercial name) and KKU–P28016. Among these studied cultivars, three levels of pungency were classified using the criteria of Scoville Heat Units (SHU) as high>500,000 SHU, Moderate 100,000–500,000 SHU and low< 100,000 SHU pungency (Othman et al., 2011). All *Capsicum* cultivars were cultivated separately in each pot filled with 4 kg of soil comprises of as the followings: 55% clay, 20% silt and 25% of sand, under favorable greenhouse condition during April at the Faculty of Science, Naresuan University. The conditions were: day length 12–13 h, temperature 30–35°C and humidity 65–70%. Phenotypic traits of mature fruit were observed during cultivation.

2. DNA extraction

Young leaves of the chili samples pooled from each cultivar in triplicates. Approximately 1 g from each triplicate was ground to a fine powder in liquid nitrogen and applied for isolating genomic DNA (gDNA) using the InnuPrep Plant DNA Kit (Analytik Jena, Germany) according to the manufacturer's instructions. The gDNA was quantified and qualified by a UV–spectrophotometer at OD_{260} and OD_{280} nm (Microplate Reader, Synergy H1 Biotek, USA). The gDNA integrity was examined by 1.0% (w/v) agarose gel electrophoresis assay and stained with 0.5 µg/ml ethidium bromide (EtBr) (Invitrogen™ UltraPure™, USA). The gel image was visualized by the gel documentation system (Thermo Fisher Scientific, USA) and the gDNA was stored at –20°C until required for further use.

3. PCR amplification

The PCR amplifications were performed using OnePCR Master Mix (GeneDireX, USA). According to the manufacturer's instructions, each PCR reaction (25µL) is assembled the following components in a 0.2 mL PCR tube on ice; 12.5µL of the OnePCR Master Mix, individual primer pair (10 µM) of the five candidate genes associated with capsaicin biosynthesis (Table 2), DNA template (25 ng/µl), and adjusted its final volume to 25µL nuclease–free water (Life Sciences, USA). The PCR reaction was carried out under the following conditions: pre–denaturation 1 cycle of 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55–60°C for 40 seconds, extension at 72°C for 1 minute and final extension for 1 cycle of 72°C for 5 minutes in a thermal cycler (Bio–Rad T100™, USA). The PCR products were separated on 1.5% (w/v) agarose gel, with 1X TAE buffer, with 0.5 µg/ml ethidium bromide (EtBr) (Invitrogen™ UltraPure™, USA). The gel image was visualized under ultraviolet light by the gel documentation system (Thermo Fisher Scientific, USA). The presence of the expected PCR products, amplified by individual genes, was recorded to assess the genetic profile associated with the capsaicin biosynthetic pathway.

Table 2 Primer list for gene amplification.

Gene	Accession No.*	Primer	Base sequence (5'–3')	Product size (primer location)
PUNI	AY819029.1	F	GAGCTGAGTTCTTGAGTGTTCG	990 bp
		R	TGTCCTGCATGTTAGTTGCTTC	(2209 to 3198)
HCT	EU616565.1	F	TGAGAGAATCGACGATGGTGC'	595 bp
		R	TACTCGATGTGGGGGAACTT	(14 to 608)
pAMT	AY819029.1	F	GCAACATTAGGGGGAAGTGAG	517 bp
		R	CTGCTCCTAGGACTGGTTC	(240 to 758)
CCR	EU616565.1	F	CGGAGGTTTCATTGCTTCTTGG	379 bp
		R	GTCCACAACTTTCTCGGTGG	(42 to 420)
KAS	EU616565.1	F	GCTGTAAAATGTGGAAGAGTCAGGG	519 bp
		R	AGCCAACAATGCTGATCCCA	(1226 to 1744)

*Individual gene accession number used to validate its PCR amplification was publicized in NCBI database (https://www.ncbi.nlm.nih.gov/).

RESULTS

1. Analysis of gene profiles

The *PUN1*, *HCT*, *pAMT*, *CCR* and *KAS* genes were considered as candidate genes, mainly encoded with key enzymes associated with the capsaicin biosynthetic pathway that determined the degree of pungency traits in *Capsicum.* Therefore, the profiles of these five candidate genes were assessed among fourteen different *Capsicum* cultivars. Results showed that all putative capsaicin biosynthetic genes (*PUN1*, *HCT*, *pAMT*, *CCR* and *KAS*) were present in *Capsicum* cultivars of Bhut Jolokia, Orange Habanero (high pungency) and Tubtim Mordindang, Phet Mordingang, and Akanee Pirote (moderate pungency) (Figure 1 and Table 3).

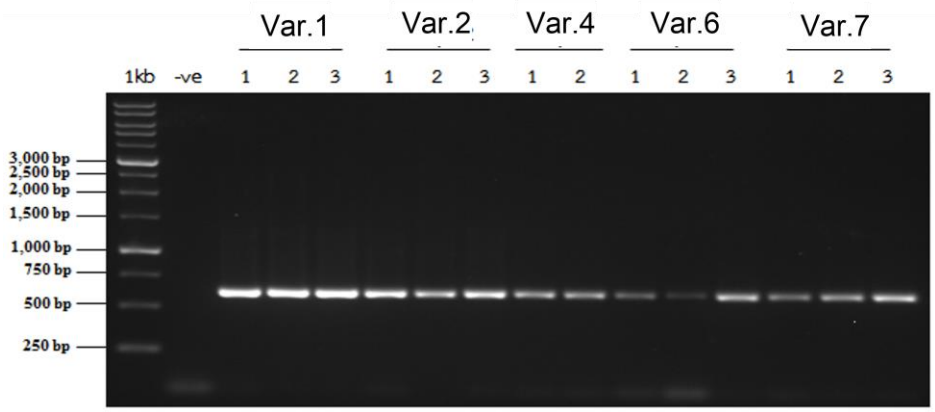


Figure 1 Representative PCR product profiles, amplified with gene specific primer of *KAS* gene, among 14 different chili cultivars. Other genes, i.e., *PUN1*; *HCT*; *pAMT*; and *CCR* had been amplified with gene specific primer in the same way.

Note: One representative from three samples per chili cultivars was showed to identify *KAS* gene through PCR assay. Expected PCR amplicons, corresponding to 519 bp, were fractionated on 1.5% agarose gel, then DNA bands were strained with EtBr, and visualized under UV light. M expresses the 100 bp DNA ladder (OneMarker 100, Gene Dire X, Taiwan). N represents negative control without DNA template in the PCR reaction. Numbers 1–14 represent chili cultivars, related to the list shown in Table 3.

Only two (*HCT* and *pAMT*) genes were absent in all eight low pungent cultivars (Table 3). Of these low pungent cultivars, three (Jindanil 80, Bang Chang and Yodson Khem 80) contained two (*PUN1*, and *KAS*) genes, while five *Capsicum* cultivars (NumKhao

Donyang, Num Keowtong 80, Hua–ruea, Huai Si Thon Kham Kaen and Chai Tai) carried three (*PUN1*, *CCR* and *KAS*) putative capsaicin biosynthetic genes (Figure 2 and Table 3).

According to the gene profile analysis, *Capsicum* cultivar KKU–P28016 carrying *PUN1*, *pAMT*, *CCR* and *KAS* genes was identified as the moderately pungent *Capsicum* group.

Results indicated that all five candidate genes (*PUN1*, *HCT*, *pAMT*, *CCR* and *KAS*) were strongly related to capsaicin biosynthesis. An unknown *Capsicum* cultivar KKU–P28016 carrying four putative capsaicin biosynthetic genes (*PUN1, pAMT, CCR* and *KAS* (Table 3) was predicted as a moderately pungent *Capsicum* cultivar.

2. Phenotypic variations of mature fruits

Mature fruits among the studied *Capsicum* cultivars showed phenotypically diverse traits in size, weight, pericarp thickness, shape, and color. Of these traits, fruit shape and color were related to degree of pungency. Results illustrated that fruit shapes could be classified into short–ovate, oval–triangular and elongated. The short–ovate to oval–triangular mature fruits were found in both highly and moderately pungent *Capsicum* cultivars of Bhut Jolokia, Orange Habanero (highly pungent) and Tubtim Mordindang, Phet Mordingang and AkaneePirote (moderately pungent), while mature fruits found in low pungent *Capsicum* cultivars of Jindanil 80, Bang Chang, Yodson Khem 80, NumKhao Donyang, Num Keowtong 80, Hua–Ruea, Huai Si Thon Kham Kaen and Chia Tai were more elongated and resembled KKU–P28016 from *Capsicum* breeding (Figure 3 and Table 4). Fruit colors varied from light yellow to dark red. Highly and moderately pungent *Capsicum* cultivars exhibited light–yellow, light–orange and reddish mature fruits, while low pungent *Capsicum* cultivars had dark red mature fruits (Figure 3 and Table 4).

These results indicated that phenotypic traits as short–ovate to oval–triangular fruit shape and light–yellow to reddish mature fruits were determined as highly and moderately pungent *Capsicum* cultivars, while elongated fruit shape with dark–red mature fruits was determined as low pungent *Capsicum* cultivars.

DISCUSSION

Gene–specific markers are a powerful tool to discriminate genetic diversity among *Capsicum* plants. For example, capsaicin biosynthetic genes were positively correlated with divergent degrees of pungency levels among *Capsicum* cultivars (Guillen et al., 2018; Werner, 2021). The capsaicin biosynthetic pathway is regulated by multiple genes (at least 14) (Arce–Rodríguez & Ochoa–Alejo, 2019) of which at least five candidate genes have been predicted to translate key enzymes in the capsaicin biosynthetic pathway such as *HCT*, *CCR*, *pAMT*, *KAS* and *PUN1* (Burgos–Valencia et al., 2020). In agreement with this result, the assessment of genetic profiles revealed that five highly and moderately pungent *Capsicum* cultivars of Bhut Jolokia, Orange Habanero, Tubtim Mordindang, Phet Mordindang and Akanee Pirote had similar profiles in the presence of five candidate genes as *PUN1*, *HCT*, *AMT*, *CCR* and *KAS*.

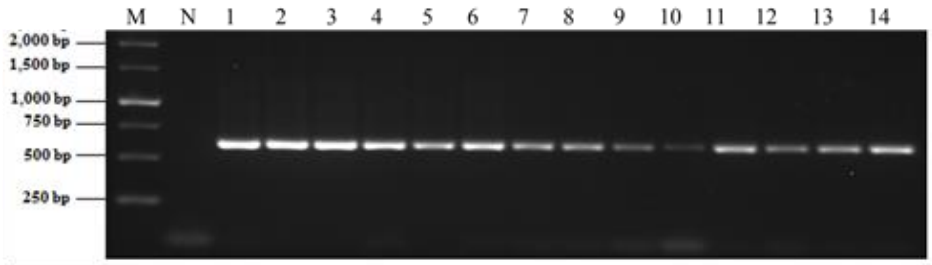


Figure 2 PCR product profiles, amplified with gene specific primer of *KAS* gene, among 14 different chili cultivars

Note: One representative from three samples per chili cultivars was showed to identify *KAS* gene through PCR assay. Expected PCR amplicons, corresponding to 519bp, were fractionated on 1.5% agarose gel, then DNA bands were strained with EtBr, and visualized under UV light. M expresses the 100 bp DNA ladder (OneMarker100, GeneDireX, Taiwan). N represents negative control without DNA template in the PCR reaction. Numbers 1–14 represent chili cultivars, related to the list shown in Table 3 and Table 4.

This finding supported that capsaicin biosynthesis in *Capsicum* fruits involved two major pathways as the phenylalanine pathway and the pyruvate pathway, producing two substrate compounds of vanillylamine and branched chain fatty acid, respectively, as described in Figure 2 (Naves et al., 2019; Zhang et al., 2016). The vanillylamine compound was synthesized under sequential regulation of *HCT, CCR* and *pAMT* genes in the phenylalanine pathway (Tsurumaki & Sasanuma, 2019), while branched chain fatty acid

(such as 8–methyl–6–nonenoyl–CoA) was synthesized under expression of the *KAS* gene through the pyruvate pathway (Reddy et al., 2014; Tanaka et al., 2017).

Table 3 Profiles of candidate genes associated with the capsaicin biosynthetic pathway among fourteen *Capsicum* cultivars.

Capsicum cultivar	Species	Pungency	Presence of Gene				
			PUN1	*HCT*	*pAMT*	*CCR*	*KAS*
Bhut Jolokia	*C. chinense*	High	•	•	•	•	•
Orange Habanero	*C. chinense*	High	•	•	•	•	•
Tubtim Mordindang	*C. chinense*	Moderate	•	•	•	•	•
Phet Mordindang	*C. chinense*	Moderate	•	•	•	•	•
Akanee Pirote	*C. chinense*	Moderate	•	•	•	•	•
Jindanil 80	*C. annuum*	Low	•	O	O	•	•
Bang Chang	*C. annuum*	Low	•	O	O	•	•
Yodson Khem 80	*C. annuum*	Low	•	O	O	•	•
Num Khao Donyang	*C. annuum*	Low	•	O	O	O	•
Num Keowtong 80	*C. annuum*	Low	•	O	O	O	•
Hua Ruea	*C. annuum*	Low	•	O	O	O	•
Huai Si Thon Kham Kaen	*C. annuum*	Low	•	O	O	O	•
Chia Tai (commercial)	*C. annuum*	Low	•	O	O	O	•
KKU–P28016	*Capsicum spp.*	expected moderate	•	O	•	•	•

• found O not found

Finally, the capsaicin in the *Capsicum* fruit was synthesized by catalyzing the condensation of these two substrate compounds regulated by capsaicin synthase (translated from the *PUN1* gene) (Arce–Rodriguez & Ochoa–Alejo, 2019). The gene analysis profile also showed that eight *Capsicum* cultivars contained *PUN1* and *KAS* genes but did not contain *HCT* and *pAMT*, which were characterized as low pungent cultivars. One possible explanation is that only one (8–methyl–6–nonenoyl–CoA) of the two substrate compounds for capsaicin biosynthesis was synthesized by *KAS* (encoded ketoacyl–ACP synthase) (Burgos–Valencia et al., 2020; Reddy et al., 2014; Prasad et al., 2006), but another substrate compound (Vanillylamine) for capsaicin biosynthesis was reduced due to loss of

the functions of *HCT* (Encoded hydroxycinnamoyltransferase) and *pAMT* (Encoded putative aminotransferase). These results concurred with previous findings that functional loss of *HCT* and *pAMT*by gene mutation affected biosynthesis of capsaicin in *Capsicum annuum* (Lang et al., 2009; Reddy et al., 2014; Tsurumaki & Sasanuma, 2019). This suggested that the *HCT*, *pAMT, KAS* and *PUN1* genes could play an important role in the capsaicin biosynthetic pathway, being responsible for determining degrees of pungency levels in *Capsicum*. Interestingly, mature fruit phenotypic traits among the studied *Capsicum* cultivars showed a highly positive correlation to the gene profile pattern associated with the capsaicin biosynthesis pathway. Results showed that a globose–oval shape of mature fruit was detected among highly and moderately pungent *Capsicum* cultivars, while an elongated shape was observed in low pungent cultivars. These results agreed with previous findings that a triangular–ovate shape of mature fruits was detected in high pungency *Capsicum chinense*, while an elongated mature fruit was detected in low pungency *Capsicum annum* (Sapras et al., 2016).

Table 4 Phenotypic traits of mature fruits among *Capsicum* cultivars used in this study.

Capsicum cultivar	Species	Degree of pungency	Fruit characteristics	
			Shape	Color
BhutJolokia	*C. chinense*	High	Rough ovate	Light orange
Orange Habanero	*C. chinense*	High	Triangular	Light yellow
TubtimMordindang	*C. chinense*	Moderate	Rough ovate	Red
PhetMordindang	*C. chinense*	Moderate	Rough ovate	Red
AkaneePirote	*C. chinense*	Moderate	Rough triangular	Light red
Jindanil 80	*C. annuum*	Low	Elongated	Orange–red,
Bang Chang	*C. annuum*	Low	Elongated	Dark red
YodsonKhem 80	*C. annuum*	Low	Elongated	Red
NumKhaoDonyang	*C. annuum*	Low	Elongated	Light red
NumKeowtong 80	*C. annuum*	Low	Elongated	Light red
HuaRuea	*C. annuum*	Low	Elongated	Red
Huai Si Thon Kham Kaen	*C. annuum*	Low	Elongated	Dark red
Chia Tai (commercial name)	*C. annuum*	Low	Elongated	Dark red
KKU–P28016	*Capsicum spp.*	expected moderate	Elongated	Red

Note: Degree of pungency was considered by Scoville Heat Units (SHU); high>500,000 SHU, moderate 100,000–500,000 SHU and low<100,000 SHU (Othman et al., 2011).

High correlation between the phenotypic trait (Mature fruit shape) and gene profile are a powerful tool to discriminate degrees of pungency levels in *Capsicum* cultivars and can provide useful information for farmers and breeders

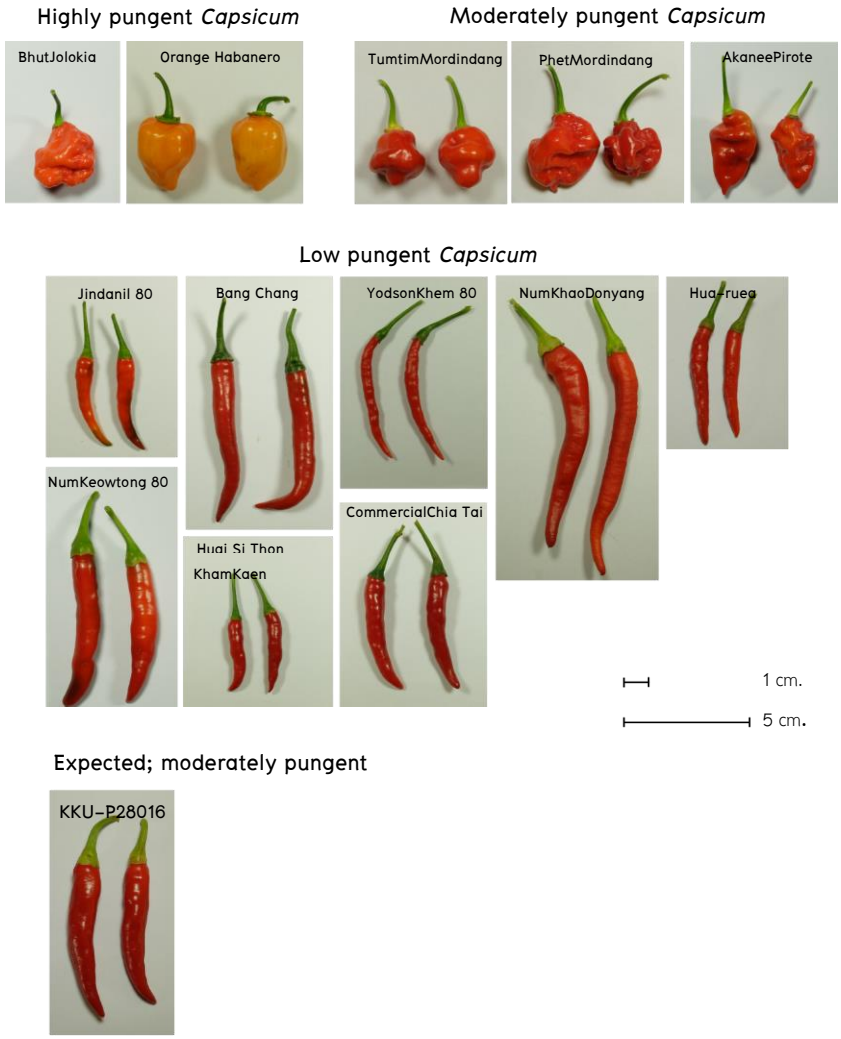


Figure 3 Mature fruit phenotypic diversity in size, Shape and Color among *Capsicum* cultivars.

Note: Bar scale equals either 1 or 5 centimeters. Degrees of pungency levels were determined according to Scoville Heat Units (SHU) with high pungency as more than 500,000 SHU, moderate pungency ranging from 100,000 to 500,000 SHU and low pungency less than 100,000 SHU (Othman et al., 2011).

CONCLUSIONS

Pungency levels among fourteen Thai *Capsicum* cultivars were screened using PCR assay with gene–specific primers associated with the capsaicin biosynthetic pathway. Results showed that highly and moderately pungent levels of *Capsicum* cultivars contained five candidate capsaicin biosynthetic genes (*PUN1*, *HCT*, *pAMT*, *CCR* and *KAS*), while low pungency was recorded in *Capsicum* cultivars that did not contain two (*HCT and pAMT*) genes. Furthermore, mature fruit phenotypic traits among the studied *Capsicum* cultivars showed that a globose–oval shape of mature fruit was detected among highly and moderately pungent *Capsicum* cultivars, while an elongated shape was observed in low pungent cultivars. Findings showed high correlation between the phenotypic trait (mature fruit shape) and gene profiles as a powerful tool to discriminate degrees of pungency levels in *Capsicum* cultivars. This information can assist in selection to further improve new Thai *Capsicum* cultivars with high capsaicin content by considering both expression levels and presences of genes associated capsaicin synthesis.

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