*Applied Science and Engineering Progress, Vol. 13, No. 1, pp. 86–93, 2020*

Research Article

# Transferability of Microsatellite Markers from Cucumber (*Cucumis sativus*) to Seven Cultivated Cucurbit Crops

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Received: 13 July 2019; Revised: 25 September 2019; Accepted: 3 October 2019; Published online: 30 January 2020


**Abstract**
Plant breeding relies heavily on genetic resources with high genetic diversity presence in nature. Lack of genomic resources can slow down molecular characterization of any plant species. Transferability of SSR markers is when SSRs developed in one species can cross amplify in other species. Cucumber is an economically important fruit crop in the family Cucurbitaceae with many already developed Simple Sequence Repeat (SSR). We evaluated 515 cucumber-derived SSR markers in seven less studied cucurbit crops consisting of fifty one accessions. The transferability rate was 6.94% in pumpkin, 17.09% in wax gourd, 19.81% in bottle gourd, 13.27% in luffa, 45.05% in melon, 18.55% in watermelon and 8.76% in bitter gourd. Genetic diversity analysis classified tested plant species into five clades corresponding to four tribes. The result indicated that cucumber derived genetic tools are applicable to decipher genetic information in other cucurbit species.

**Keywords**: Cucumber, Microsatellites, Transferability, Cucurbit

## 1 Introduction

The Cucurbitaceae family includes many crops that are well known to consumer worldwide. There are five members that are of great economic importance and are widely cultivated globally which are cucumber (*Cucumis sativus* Linn.), melon (*Cucumis melo* Linn.), squash or pumpkin (*Cucurbita* spp.) and watermelon (*Citrullus* spp.). Other members in this family that are of less economic importance but are still substantial to small farmers and consumers especially in Southeast Asia [1] include bottle gourd (*Lagenaria siceraria* (Mol.) Standl.), wax gourd (*Benincasa hispida* (Thumb.), bitter gourd (*Momordica charantia* Linn.), sponge gourd (*Luffa aegyptiaca* Mill. syn. *L. cylendrica* (Linn.) Roem), ridge gourd (*L. acutangula* Linn.) and snake gourd (*Trichosanthes cucumerina* Linn.) [1], [2]. According to the Food and Agriculture Organization of the United Nations the cucumber ($2n = 2x = 14$) was ranked the fourth most important vegetable worldwide in year 2016 with China was the top leader who produced approximately 85% of the total world production

Please cite this article as: S. Natenuch, C. Nguyen, C. Jantasuriyarat, and S. Samipak, "Transferability of microsatellite markers from Cucumber (*Cucumis sativus*) to seven cultivated cucurbit crops," *Applied Science and Engineering Progress*, vol. 13, no. 1, pp. 86–93, Jan.–Mar. 2020.

followed by Iran, the Russian Federation, Turkey, and the United States of America [3].

Many methods have been employed to assess the genetic relationship among the Cucurbitaceae, ranging from morphological traits to molecular markers. Over the years, molecular markers had gained more popularity and were chosen for most studies because they can reveal high polymorphism and are not interfered by environment factors [4], [5]. Various types of DNA markers have been used to determine genetic relationships in different species of Cucurbitaceae such as RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeats) and SSR (simple sequence repeats) [6]–[10]. SSRs are tandemly repeated sequences of one to six base pairs flanking by unique sequences that are used in primer design [11]. Despite the complex procedures of the development and screening process, SSR remains one marker type that is widely used in a large scale crop research due to its codominant nature, reproducibility and ability to reveal high degree of polymorphism [11], [12]. To maximize the utility of already developed markers, many studies have applied known markers to other species and showed that SSR markers developed from one plant species can be used in other closely related species [13]–[18]. Such examples include one study in monocots showing that SSR markers were transferrable among rye, wheat and triticale [19], while in dicots, cross-genera transferability was observed among cassava, rubber tree and physic nut [20].

In Cucurbitaceae family, many transferability research projects were done. The transferability of 40 EST-SSR markers developed in watermelon were shown to have 35% transferability rate to *C. melo* and 50% transferability rate to *C. sativus* [21]. On the other hand, higher transferability rates of SSR markers were reported between the genus *Cucurbita pepo* and *C. moschata* (88.1% from *C. pepo* to *C. moschata* and 87.3% from *C. moschata* to *C. pepo*) [22]. While 11 SSR markers developed in bitter gourd were transferable to other cucurbit species at various levels (2 SSRs to wax gourd, water melon, and bottle gourd; 3 SSRs to pumpkin, 4 SSRs to luffa and 5 SSRs to cucumber and melon) [23]. Cucurbita SSR markers developed from *C. pepo* (13 SSRs) and *C. moschata* (23 SSRs) were reported to be highly transferrable to bottle gourd (84%) and at a lower rate to luffa (65%) [24]. Another research analyzed 20 cucumber SSR markers and found to be transferrable to melon (65%), bitter gourd (55%), watermelon (50%) and pumpkin (35%) [25]. Since the completion of cucumber genome sequence, 995 SSRs were developed [26] and 28% could be transferred to bottle gourd [27]. Eighty two of these cucumber-derived SSRs (along with 21 eSSRs) were amplified in Luffa, *L. acutangula* (68%), *L. aegyptiaca* (61%) and *L. hermaphodita* (60%) [28].

Although extensive genomic resources (e.g. SSRs markers, SNP genotyping) are available in major cucurbit, genetic studies of minor cultivated cucurbits are limited. Ability to apply well established genetic tools from one species to analyze genetic makeup of other less studied species would allow for speedy research and most effective use of research fund that can be beneficial to many crop scientists worldwide. Here we reported transferability of cucumber SSRs to eight lesser known crop species in the family Cucurbitaceae including pumpkin, wax gourd, bottle gourd, luffa, snake gourd, melon, watermelon and bitter gourd. From this SSR transferability information, evolutionary relationships among Cucurbitaceae were discussed.

## 2 Materials and Methods

### 2.1 *Plant materials and DNA extraction*

A total of fifty three accessions of cultivated cucurbit crops analyzed in this research included thirteen accessions of pumpkin species–four of *Cucurbita moschata*, *C. maxima*, *C. pepo* and one of *C. ficifolia*–, four accessions of wax gourd species *Benincasa hispida*, four accessions of bottle gourd species *Lagenaria siceraria*, twelve accessions of luffa species–four of *Luffa acutangula* (ridge gourd), four of *L. cylindrica* (sponge gourd), and four of *Trichosanthes cucumerina* (snake gourd)–, six accessions of melon species–two of *Cucumis melo* var. inodorus, two of *C. melo var. reticulatus*, and two of *C. melo var. flexosus*, wetermelon–, eleven accessions of watermelon species–six of *Citrullus lanatus subsp. lanatus*, and five of *C. amarus*–, one accession of bitter gourd *Momordica charantia*; and for comparison, two accessions of cucumber *Cucumis sativus* were used in this study (Figure 1 and Table 1). All plant accessions were kindly provided by CHIA TAI CO., LTD, Bangkok, Thailand.

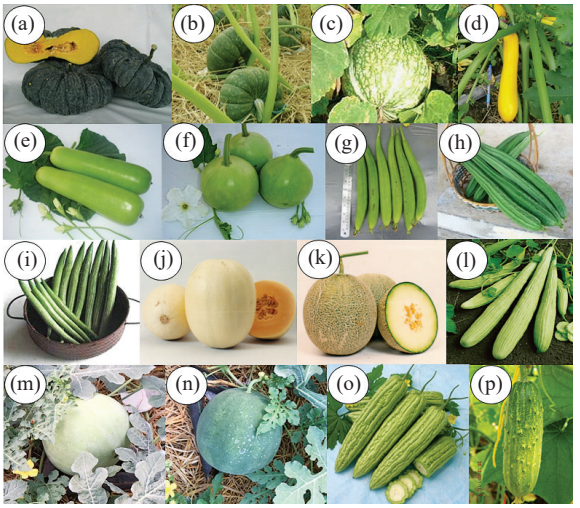


**Figure 1**: Representative characteristics of cucurbit species; *Cucurbita moschata* (a), *C. maxima* (b), *C. ficifolia* (c), *C. pepo* (d), *Benincasa hispida* (e), *Lagenaria siceraria* (f), *Luffa acutangula* (g), *L. cylindrica*l (h), *Trichosanthes cucumerina* (i), *Cucumis melo* var. inodorus (j), *C. melo var. reticulatus* (k), *C. melo var. flexosus* (l), *Citrullus lanatus subsp. lanatus* (m), *C. amarus* (n), *Momordica charantia* (o) and *Cucumis sativus* (p), respectively.

Cucurbit seeds were sown in plastic pots filled with peat moss. Ten days after sowing, young true leaves from five plants of each accession were collected and bulked, total genomic DNA was isolated using CTAB procedure as described by [29] with slight modifications (2x CTAB buffer did not contain 0.2%8 2-mercaptonethanol and wash buffer 76% ethanol with 10 mm ammonium acetate was replaced with 70% ethanol). DNA concentration was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

### 2.2 *Microsatellite marker genotyping*

Five hundred and fifteen cucumber SSR markers distributed across the *C. sativus* genome were selected for this transferability study. These markers included 468 markers selected from 995 cucumber SSR markers reported by Ren *et al*. [26], 3 SSR markers reported by Fazio *et al*. [8], 15 SSR markers reported by Huang *et al*. [30], 6 SSR markers reported by Watcharawongpaiboon and Chunwongse [25], 14 SSR markers reported by Cavagnaro *et al*. [31] and the remaining 5 SSR markers were from unknown published sources. Detailed information of these markers is listed in supplementary table 2. Polymerase Chain Reaction was performed in a total volume of 10 µL reaction containing 1×PCR buffer, 2.5 mm $MgCl_2$, 0.2 mm of dNTPs, 0.25 µm of forward and reverse primers, 50 ng template DNA and 0.25 unit Taq DNA polymerase (Invitrogen, USA). PCR amplification was conducted with GS1 G-Strom (Gene Technologies Thermal Cyclers, UK) or T100TM thermal cycle (Bio-Rad, USA). The amplification condition was 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, with a final extension of 10 min at 72°C. The amplification products were separated on 5% denaturing polyacrylamide

**Table 1**: Sources, origins and number of accessions of each cucurbit species used in this study

| Species | Number of Accessions | Origin (No. of accession) | Chromosome Number | Source |
|---|---|---|---|---|
| *Cucurbita moschata* | 4 | Thailand (4) | n=20 | Chia Tai Co.,Ltd. |
| *C. maxima* | 4 | Japan (4) | n=20 | Chia Tai Co.,Ltd. |
| *C. ficifolia* | 1 | China (1) | n=20 | Chia Tai Co.,Ltd. |
| *C. pepo* | 4 | USA (4) | n=20 | USDA |
| *Benincasa hispida* | 4 | Thailand (4) | n=12 | Chia Tai Co.,Ltd. |
| *Lagenaria siceraria* | 4 | Thailand (4) | n=11 | Chia Tai Co.,Ltd. |
| *Luffa acutangula* | 4 | India (4) from Chia Tai Co., Ltd. | n=13 | Chia Tai Co.,Ltd. |
| *Trichosanthes cucumerina* | 4 | Sri Lanka (3), India (1) | n=11 | Chia Tai Co.,Ltd. |
| *L. cylindrica* | 4 | India (3), Vietnam (1) | n=13 | Chia Tai Co.,Ltd. |
| *Cucumis melo var. inodorus* | 2 | Taiwan (1), Thailand (1) | n=12 | Chia Tai Co.,Ltd. |
| *C. melo var. reticulatus* | 2 | Japan (2) | n=12 | Chia Tai Co.,Ltd. |
| *C. melo var. flexosus* | 2 | India (2) | n=12 | Chia Tai Co.,Ltd. |
| *Citrullus lanatus subsp. lanatus* | 6 | USA (1), India (1), Nigeria (3), Mali (1) | n=11 | USDA |
| *C. amarus Schrad.* | 5 | South Africa (4), Zimbabwe (1 ) | n=11 | USDA |
| *Momordica charantia* | 1 | China (1) | n=11 | Chia Tai Co.,Ltd. |
| *Cucumis sativus* | 2 | Bangladesh (2) | n=7 | Chia Tai Co.,Ltd. |
| Total | 53 | | | |

gel (19 acrylamide: 1 bis-acrylamide) in a TBE buffer using Sequi-Gen GT nucleic acid sequencing system (Bio-Rad, USA) at 80 W for 1–2 h at 50°C and then DNA bands were visualized by staining with silver nitrate solution following the protocol reported by Benbouza *et al*. [32].

### 2.3 *Scoring and data analysis*

The amplified fragments from each accessions were scored as "presence" or "absence" and number of alleles per locus was recorded as described by Kuleung *et al*. and Bhawna *et al*. [19], [27]. The "presence" meant that the amplified fragments appeared strong or clearly distinguishable; while the "absence" meant that the amplified fragments were not detectable or appeared indistinguishable. The number of amplified markers was used to calculate percentage of transferability from cucumber markers to other cucurbit species, as indicates in the Equation (1) below,

$$Transferability\ (\%) = \frac{No.\ amplified\ marker}{Total\ No.\ marker} \times 100 \quad (1)$$

### 2.4 *Statistical and genetic relationship analyses*

The genetic similarity among cucurbit accessions was calculated using Jaccard's coefficient by Numerical Taxonomy System software, version 2.2 (NTSYS-pc 2.2, Exeter Software, Setauket, New York, USA) [33]. A dendrogram was constructed based on principle component analysis (PCA) using Sequential Agglomerative Hierachical Nested (SAHN) and Unweighted Pair-Group Method with Arithmetic mean (UPGMA). Bootstrap values (10,000 replicates) were calculated using WinBoot [34].

## 3 Results

This study was carried out to determine the transferability of cucumber SSRs to other less economically important cucurbits. Total of five hundred fifteen SSR markers were used to screen fifty three accessions in eight cultivated crops of eight genera: pumpkin (*Cucurbita*), wax gourd (*Benincasa*), bottle gourd (*Lagenaria*), luffa (*Luffa* and *Trichosanthes*), melon (*Cucumis*), watermelon (*Citrillus*), bitter gourd (*Momordica*). The transferability of the markers to other cucurbit species was indicated by the presence of distinguishable amplified products. The amplified fragments of the target species were usually weaker than those of the donor cucumber species. Out of the 515 SSR markers tested, 275 markers could positively amplify in other cucurbit species. Of these amplifiable markers, 14 markers (UW006915, UW025857, UW034265, UW040535, UW061643, UW065755, UW068651, UW068779, UW072133, UW072532, UW074494, SSR33278, CUCUM1 and CUCUM4) could amplify all cucurbit species. Most of SSR markers produced similar size PCR products when tested on plants in the same genus/species while the product sizes varied when tested on plants in different genus/species.

The 275 cucumber SSR markers that could amplify all other cucurbit species showed various transferability rates (Supplement Table 2). High percentages of transferability were found in melon species (45.05–45.44%); while wax gourd (17.09%), watermelon (18.06–19.03%), bottle gourd (19.81%) and luffa (12.82–13.98%) showed moderate transferability rates. On the other hand, the bitter gourd (8.76%) and pumpkin (6.41–7.18%) made the lowest transferable plant group (Table 2).

**Table 2**: Transferability of 515 cucumber SSR markers to cucurbit species

| Cucurbit Species | No. Markers | % Transferability |
|---|---|---|
| **Pumpkin** | | |
| - *Cucurbita moschata* | 33 | 6.41 |
| - *C. maxima* | 37 | 7.18 |
| - *C. ficifolia* | 37 | 7.18 |
| - *C. pepo* | 36 | 6.99 |
| **Wax gourd** | | |
| - *Benincasa hispida* | 88 | 17.09 |
| **Bottle gourd** | | |
| - *Lagenaria siceraria* | 102 | 19.81 |
| **Luffa** | | |
| - *Luffa acutangula* | 66 | 12.82 |
| - *L. cylindrica* | 72 | 13.98 |
| - *Trichosanthes cucumerina* | 67 | 13.01 |
| **Melon** | | |
| - *Cucumis melo* var. inodorus | 232 | 45.05 |
| - *C. melo var. reticulatus* | 234 | 45.44 |
| - *C. melo var. flexosus* | 230 | 44.66 |
| **Watermelon** | | |
| - *Citrullus*. amarus | 98 | 19.03 |
| - *C lanatus subsp. lanatus* | 93 | 18.06 |
| **Bitter gourd** | | |
| - *Momordica charantia* | 43 | 8.76 |
| **Cucumber** | | |
| - *Cucumis sativus* | 515 | 100 |

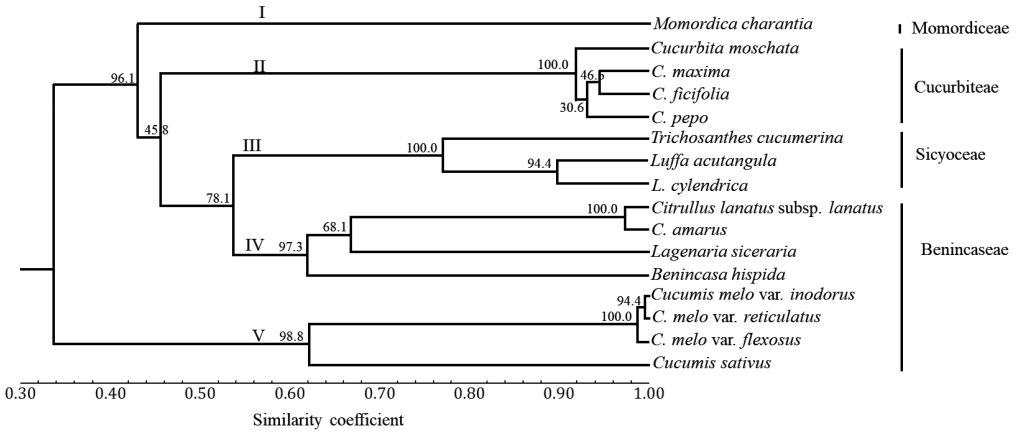


**Figure 2**: Dendrogram showing relationships of Cucurbit species constructed by UPGMA based on cross-amplification results of 515 cucumber SSRs. The values between branches are the bootstrap values generated by 10,000 resamplings. Tribal classification is displayed on the right.

Phylogenetic relationship of 16 cucurbits species were established using information obtained from these 515 cucumber SSR markers. A dendrogram representing genetic similarity based on the amplifiable markers between species was constructed which clustered all accessions into 5 clades at the similarity level of 0.60 (Figure 2). The five clade separation corresponded well with five major tribes in Cucurbitaceae [35], [36]. Clade I contained only *M. charantia* species which is in tribe Momordiceae. The second clade (clade II) included all four species of the genus Cucurbita: *C. moschata*, *C. maxima*, *C. ficifolia* and *C. pepo*, which are all in the tribe Cucurbiteae. The third clade (clade III) constituting of all three species of luffa, *Luffa acutangula*, *L. cylindrica* and *T. cucumerina* and belongs to tribe Sicyoceae. The rest of species were separated into two clades (IV and V) with *Benincasa hispida*, *Lagenaria siceraria*, *Citrullus lanatus subsp. lanatus* and *C. amarus* were placed in clade IV, while all accessions of genus Cucumis, *C. sativus*, *C. melo var. inodorus*, *var. reticulatus* and *var. flexosus* were placed in clade V. These two clades (IV and V) are members of tribe Benincaseae. Genetic similarities based on cross amplifications of SSR markers were calculated (Table 3). The minimum value of 12% was for *C. sativus* against *C. moschata*. The maximum value of 99.6% was for *C. melo* var. inodorus against *C. melo var. reticulatus*, respectively (Table 3).

**Table 3**: Genetic similarity matrix among 16 cucurbit species assessed by 515 cucumber SSR markers

| Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.000 | | | | | | | | | | | | | | | |
| 2 | 0.943 | 1.000 | | | | | | | | | | | | | | |
| 3 | 0.886 | 0.946 | 1.000 | | | | | | | | | | | | | |
| 4 | 0.928 | 0.932 | 0.932 | 1.000 | | | | | | | | | | | | |
| 5 | 0.430 | 0.448 | 0.448 | 0.452 | 1.000 | | | | | | | | | | | |
| 6 | 0.444 | 0.475 | 0.460 | 0.464 | 0.642 | 1.000 | | | | | | | | | | |
| 7 | 0.485 | 0.485 | 0.505 | 0.490 | 0.494 | 0.560 | 1.000 | | | | | | | | | |
| 8 | 0.460 | 0.442 | 0.462 | 0.466 | 0.503 | 0.533 | 0.752 | 1.000 | | | | | | | | |
| 9 | 0.419 | 0.442 | 0.440 | 0.444 | 0.500 | 0.563 | 0.899 | 0.791 | 1.000 | | | | | | | |
| 10 | 0.249 | 0.268 | 0.260 | 0.261 | 0.475 | 0.551 | 0.376 | 0.361 | 0.368 | 1.000 | | | | | | |
| 11 | 0.247 | 0.266 | 0.258 | 0.259 | 0.478 | 0.554 | 0.373 | 0.359 | 0.366 | 0.996 | 1.000 | | | | | |
| 12 | 0.251 | 0.270 | 0.262 | 0.263 | 0.478 | 0.560 | 0.378 | 0.350 | 0.364 | 0.987 | 0.987 | 1.000 | | | | |
| 13 | 0.444 | 0.462 | 0.477 | 0.465 | 0.619 | 0.656 | 0.591 | 0.500 | 0.570 | 0.523 | 0.526 | 0.526 | 1.000 | | | |
| 14 | 0.427 | 0.444 | 0.459 | 0.448 | 0.602 | 0.680 | 0.573 | 0.509 | 0.553 | 0.533 | 0.536 | 0.537 | 0.974 | 1.000 | | |
| 15 | 0.442 | 0.444 | 0.444 | 0.450 | 0.318 | 0.384 | 0.491 | 0.450 | 0.483 | 0.275 | 0.273 | 0.263 | 0.423 | 0.408 | 1.000 | |
| 16 | 0.120 | 0.134 | 0.134 | 0.131 | 0.292 | 0.331 | 0.227 | 0.230 | 0.245 | 0.621 | 0.625 | 0.617 | 0.306 | 0.320 | 0.157 | 1.000 |

Representative number of cucurbit species; *Cucurbita moschata* (1), *C. maxima* (2), *C. ficifolia* (3), *C. pepo* (4), *Benincasa hispida* (5), *Lagenaria siceraria* (6), *Luffa acutangula* (7), *Trichosanthes cucumerina* (8), *L. cylindrica*l (9), *Cucumis melo* var. inodorus (10), *C. melo var. reticulatus* (11), *C. melo var. flexosus* (l2), *Citrullus lanatus subsp. lanatus* (13), *C. amarus* (14), *Momordica charantia* (15) and *Cucumis sativus* (16), respectively.

## 4 Discussion

Among many types of recently developed molecular markers, SSRs have become one of the most widely used molecular markers in various genetic research. SSR transferability studies circumvent the difficulty and heavy investment in new marker development. Ren *et al*. reported that out of 995 cucumber genomic SSR markers, 48.9% could give amplification products in melon, 25.9% in watermelon and 22.2% in pumpkin [26]. Moreover, [27] reported that 10.9% of 995 cucumber SSR markers from [26] could amplify the bottle gourd genomic DNA. The transferability of 515 SSR markers from cucumber to 7 other cultivated cucurbit crops (13 species) showed decrease percentages from melon, bottle gourd, watermelon, wax gourd, luffa, bitter gourd and then pumpkin, respectively. The high transferability rates from cucumber to melon and to watermelon were similar to past reports [26], [27]. This research is the first one that examined the SSR marker transferability rate from cucumber to snake gourd (*T. cucumerina*).

Cross amplification of cucumber-derived SSR markers in other species demonstrated presence of sufficient homology between sequences flanking the SSR loci and subsequently can reveal genetic distance, gene content and order conservation between related species. Our transferability results were in good agreement with taxonomic classification of the Cucurbitaceae [35], [36] and also with discovery of well-preserved intergenomic homology between cucurbit species containing high number of collinear gene pairs [37]. Transferability from cucumber to melon was highest followed by bottle gourd and watermelon confirmed the report that after the occurrence of Cucurbit-common tetraploidy, then the watermelon (*Citrullus lanatus*) lineage split first leaving cucumber (*Cucumis sativus*) and melon (*C. melo*n) evolved as sister clades [37]. Transferability rates of cucumber-derived SSRs to crops within the same Benincaseae tribe (watermelon, melon, bottle gourd, wax gourd) were considerably high (17.09–45.44%), while lower rates (12.82–13.98%) were observed for species in the Sicyoeae tribe (Luffa), and for *M. charantia* (8.76%) of Momordiceae tribe, and the lowest rates (6.41–7.18%) were observed for species in the Cucurbiteae tribe (pumpkin). The relationship suggested by the transferability rates observed in our study differed from reported taxonomic classification of close relationship between tribe Benincaseae and Cucurbiteae. The differences may be explained by several factors including a) differences in germplasm sources of each species that were used in this study, b) the optimum annealing temperature of SSRs primers to cucumber genomic DNA used in this study (57°C) might be unsuitable for good amplification in other tested species. Moreover, some cucumber SSR markers could cross amplify and produced patterns suggesting presence of multiple sites of SSR in the tested species as had been reported before [27]. The presence of multiple cucumber-derived SSR binding sites in some tested crop species corroborates the occurrence of core-eudicot-common hexaploidization event in the ancestral plant prior to the speciation of Cucurbitaceae causing some cucurbit species to possess copies of same sequences [37].

This study described microsatellite primers showing acceptable amplification in minor cultivated cucurbit species. Our result demonstrated that many already-developed cucumber SSR markers could directly be used in future basic and applied research programs in other cultivated cucurbits without having to spend time and research funding on new marker development. These markers can be used in many varieties of applications such as germplasm classification and characterization, evaluation of inbreed line, testing F1 hybrid purity, study genetic diversity and other breeding applications in cucurbits.

## Acknowledgments

This research was supported by funding from National Science and Technology Development Agency (NSTDA)-University-Industry Research Collaboration or NUI-RC (Thailand) and the CHIA TAI CO., LTD. which gives an excellent opportunity and fund for this research.

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