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Classification of Pepper (*Capsicum annuum* L.) Accessions by RAPD Analysis

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Abstract: Ten pepper (*Capsicum annuum* L.) germplasm accessions from Thailand were screened using random amplified polymorphic DNA (RAPD) markers. Twelve dodecamer oligonucleotide primers, singly and in combination were used. The similarity values among the studied genotypes range from 0.209 to 0.891. The resulting dendrogram divided the accessions into two major groups. The first group which include 8 accessions with white corolla was further divided into two subgroups. The first subgroup included 4 long-fruited (CA849, CA958, CA1107 and CA1118) and 1 medium-fruited (CA365). The second subgroup included 1 medium-fruited (CA367) and 1 short-fruited (CA398). However, the medium-fruited CA020 could not be clustered into any subgroup. The second group included a short-fruited with 1 white having green margin corolla (CA024) and a yellow green with green-yellow spots corolla (CA034). The obtained clustering based on RAPD markers was consistent with morphological characteristics of the different pepper accessions.

Key words: *Capsicum annuum* L., classification, combination, pepper, RAPD analysis

INTRODUCTION

Pepper (*Capsicum* sp.) which belongs to the family Solanaceae is historically associated with the voyage of Columbus[1]. Columbus is given credit for introducing pepper to Europe and subsequently to Africa and Asia. In Asia, it was quickly incorporated into native cuisines. It grows in the tropical, subtropical as well as temperate regions. Pepper, rich in vitamins A, C and E[2], is being used as a food flavoring, a coloring agent, a pharmaceutical ingredient and in other innovative ways.

The use of the numerous cultivars within the five domesticated species has grown exponentially[3]. These domesticated species are *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens* Ruiz and Pavon[4]. The most important species is *Capsicum annuum* L. which contains both pungent (hot) and sweet (mide) fruits. *Capsicum annuum* L. shows much variability, particularly in regard to fruits than those of any other species of *Capsicum*[5].

In Thailand, pepper is an important vegetable and major spice crop with considerable economic importance. It is available in the market in various types. The commercial varieties particularly the hybrid varieties of pepper have narrower genetic variation. Consequently, distinguishing these varieties from each other becomes more difficult.

Identification of *Capsicum* sp. usually depended on description of morphological characteristics at different growth stages. More recently, chromosome morphology[6], isozymes[7] and electrophoresis of soluble proteins[8] have been used to classify the diversity within the genus *Capsicum*. Many reports have described the value of molecular markers like restriction fragment length polymorphism (RFLP)[9], amplified fragment length polymorphism (AFLP)[10] and randomly amplified polymorphic DNA (RAPD)[10, 11] to study genetic diversity of *Capsicum*. The advantages of the RAPD technique are its simplicity, reduced running time and low cost.

Moreover, it does not make use of radioactive probes, not requiring previous knowledge of the DNA sequence to design the primers and requires only small amounts of DNA. RAPD markers can provide a robust

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Table 1: Morphological characteristics of pepper (*Capsicum annuum* L.) accessions used in this study

		Corolla		Fruit				
No.	Accession	Color[a]	Spot[b]	Position[c]	Shape[d]	Length[e] (size)	Width (cm)	Weight (g)
1	CA020	WH	AB	E	EL	M	0.66	1.69
2	CA024	WGM	AB	E, I, D	C	SH	1.50	2.25
3	CA034	YG	GR	E, I, D	EL	SH	0.73	0.64
4	CA365	WH	AB	D	EL	M	1.42	7.79
5	CA367	WH	AB	E	EL	M	1.05	2.91
6	CA398	WH	AB	E	EL	SH	0.74	1.15
7	CA849	WH	AB	D	EL	L	1.83	19.20
8	CA958	WH	AB	D	EL	L	3.01	42.05
9	CA1107	WH	AB	D	EL	L	1.63	15.36
10	CA1118	WH	AB	D	EL	L	1.55	19.67

[a]WGM: White with green margin; WH: White; YG: Yellow green, [b]AB: Absent; GR: Green yellow, [c]D: Declining; E: Erect; I: Intermediate
[d]C: Conical; EL: Elongated, [e]L: Long (>10-20 cm); M: Medium (>5-10 cm); SH: Short (1-5 cm)

classification criteria that could be useful in species separation and systematics. Therefore, the objective of this study was to distinguish the genetic relationships between 10 pepper accessions of *Capsicum annuum* L. collected from Germplasm Unit of Tropical Vegetable Research Center (TVRC) in Thailand using RAPD markers.

MATERIALS AND METHODS

Plant material: Seeds of ten pepper accessions were taken from Germplasm Unit of Tropical Vegetable Research Center, Kasetsart University (Table 1). The plants were grown in a greenhouse at Kagawa University from January to April 2003. Young leaves were collected, frozen in liquid nitrogen and stored at -85°C until extraction of DNA.

DNA extraction: Genomic DNA from ten accessions of pepper were isolated by the CTAB (Cetyl Trimethyl Ammonium Bromide) method[12] with slight modification. One gram of frozen young leaves was pulverized in liquid nitrogen using a cold mortar and pestle. The powder was mixed with 2 mL of preheated (60°C) 2x CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 1% PVP) and incubated at 60°C for 60 min with shaking. A 2 mL of chloroform-isoamyl alcohol (24:1 v/v) was added followed by incubation at room temperature with gentle shaking for 30 min. The mixture was then centrifuged at 3, 000 rpm for 15 min at room temperature; this procedure was done twice. The supernatant was added with 1/10 volume of 10% CTAB and mixed with an equal volume of precipitation buffer (1% CTAB, 5 mM Tris-HCl pH 8.0, 10 mM EDTA). The tube was gently shaked for 30 min. After centrifugation at 3, 000 rpm for 15 min, the precipitate was dissolved with 2 mL of NaCl-TE buffer (1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) at 55°C with shaking and 2 mL of isopropyl alcohol. The nucleic acid precipitate was washed with cold 70% ethanol, dried under vacuum for 5 min and resuspended in 300 μL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). A 1/10 volume of RNase solution (100 μg mL^{-1}) was added to DNA solution to get rid of the RNA and the mixture was allowed to react for 30 min at 55°C. The DNA length was determined in comparison with standard λ-DNA by electrophoresis in 1.2% agarose gel (Wako) in 1x TAE buffer while DNA concentration and purity were measured by spectrophotometry. The DNA solution was stored at -85°C until analysis.

Table 2: List of primers which produced amplified DNA fragment

Code	Sequence	Tm[a]	GC content[b]
C41	5'-AGCCTGTGGGCT-3'	40	66.7
C42	5'-CCAGATTTTCTG-3'	34	41.7
C43	5'-GGCGGCACAGGA-3'	42	75.0
C44	5'-CGCAGCCGAGAT-3'	40	66.7
C45	5'-GGACAAGTAATG-3'	34	41.7
C46	5'-GATGGTCCGTTT-3'	36	50.0
C47	5'-GCCGCTTCAGCT-3'	40	66.7
C48	5'-GGAGGATGGCCC-3'	42	75.0
C49	5'-ATCATCGTACGT-3'	34	41.7
C50	5'-GGCAACTGGCCA-3'	40	66.7
C51	5'-ATCAACGTACGT-3'	34	41.7
C52	5-GTCGACGGACGT-3'	40	66.7

[a]The denaturation temperature was calculated using the following equation: Tm (°C)= 4 x (G + C) + 2 x (A + T), [b]GC content was calculated by number of GC/ total base number

Oligonucleotide primers: RAPD primers were obtained from DNA Oligomer (12 mer) set C (Wako, Tokyo). The sequences of primers, GC content and Tm ratio are shown in Table 2.

PCR amplification conditions: Amplification reactions were performed in a total volume of 25 μL containing 10x PCR buffer (100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl and 0.01% gelatin) (Boehringer Mannheim, Germany), 200 μM of each dNTP (Roche Diagnostics Mannheim, Germany), 200 nM of each primer (400 nM in case of single primer PCR), 0.1 U of Taq DNA polymerase (Gibco BRL, Gaithersburg, Md.) and 10 ng of genomic DNA. The mixture was overlaid with 24 μL of liquid wax (MJ Research, Inc.) to prevent evaporation. DNA

amplifications were performed in a thermal cycler (PC-700, Astec, Tokyo). The amplification profile consisted of an initial denaturation at 94°C for 5 min followed by 40 cycles of 30 sec at 94°C, 15 sec at 42°C, 1 min at 72°C, for DNA denaturing, annealing and extension, respectively and a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in 1.5% agarose gels in 1x TAE buffer. Molecular sizes of the amplification products were estimated using a λ-DNA digested by *Hind* III/ *Eco*RI. DNA bands were visualized by staining with ethidium bromide and photographed on a UV transilluminator with Polariod type 667 positive film. Each of the reactions was carried out twice, using different samples which were collected at different dates. Only bands that were bright and reproducible in both reactions were scored for further analysis.

Similarity and dendrogram analysis: Reproducible bands were scored as either 1 or 0 for presence or absence of bands, respectively. Data generated from the RAPD analysis were analyzed using Nei similarity index[13] which excludes common negative data on the basis of the following equation: Similarity $= 2N_{ab}/(N_a + N_b)$, where N_{ab} = number of scored amplified fragments with the same molecular weight shared between genotype a and b; N_a = number of scored amplified fragments in genotype a and N_b = number of scored amplified fragments in genotype b. A dendrogram was constructed on the basis of the similarity matrix data by unweighted pair group method with arithmetic average (UPGMA) cluster analysis using the software NTSYS-pc version 2.10t (Exeter Software, New York).

RESULTS

Screening of primers: Twelve primers were used singly and in pairs to generate 78 different PCR primers[14]. To prevent misextension of incorrect nucleotides and an annealed primer, the annealing temperature was raised at 42ºC. Of the 78 single and combined primers, 4% showed similar band patterns (monomorphic pattern) while 31% did not produce any band. The primers which did not produce band were as follows: single primer such as C41, C42, C49, C50 and C51 and the combined primers such as the combinations of C41 with either C42, C49, C50 and C51; C42 with either C45, C49, C50 and C51; C43 with either C49 and C51; C44 with either C45, C46, C49 and C51; C45 with either C50 and C51; C47 with C51; C49 with C50 and C50 with C51. The GC content is an important criterion for the selection of primers because it is associated with the Tm and related to the reproducibility of results[15]. High GC content (75%) of primer C43 had a larger number of amplified fragments and a higher fragment intensity than the primers with lower GC content. However, no band was produced in primers C41 and C50 even they showed the second highest GC content (Table 2). The presence and absence of the amplification products were constructed, followed by DNA typing (pattern). Several unique markers were detected (Table 3). C4145 (1.10 kb), C4647 (1.48 kb) and C4652 (1.25 kb) were specific to CA849; C4447 (0.80 kb), C4952 (0.80 kb) and C5052 (0.80 kb) were specific to CA958; C47 (1.13 kb) and C5052 (1.44 kb) were specific to CA1118 whereas C4144 (1.38 kb) was absent in CA365; C4147 (0.85 kb) and C52 (0.99 kb) were absent in CA398 and C4244 (0.75 kb) was absent in CA849 (Table 3).

Table 3: Specific distinguishable bands of 10 pepper (*Capsicum annuum* L.) accessions

Accession	Specific primer and measurement range (kb) of DNA
CA020	C4147-1.68[a]; C4243-0.58, 0.63, 0.97; C4246-0.78, 0.83; C4252-1.89; C45-0.65, 1.67; C4650-1.22; C47-0.76; C4951-0.78
CA024	C4144-1.17, 1.20; C4148-0.56; C4152-1.18; C4246-0.89; C4247-0.60, 1.16, 1.20, 1.89; C4248-0.56, 0.67; C4347-1.20; C4350-0.57, 1.30; C4352-1.15; C44-1.00, 1.20; C4447-1.00, 1.42; C4548-0.56; C47-0.86; C4952-1.39; C5052-1.15
CA034	C4145-0.98; C4248-0.69; C4647-1.60; C4652-0.72; C4952-1.15, 1.47
CA365	C4144-1.38; C44-0.71
CA367	C4146-0.78, 0.81, 1.18; C4252-1.29, 1.45
CA398	C4143-0.87; C4147-0.85; C52-0.99
CA849	C4145-1.10; C4244-0.75; C4647-1.48; C4652-1.25
CA958	C4447-0.80; C4952-0.80; C5052-0.80
CA1107	C4145-1.60; C4244-1.29; C4452-1.37
CA1118	C47-1.13; C5052-1.44

[a]Combination of C41 and C47, and nucleotide separated on agarose gel of 1.68 kb, Underlined showed only one absent band

Similarity and dendrogram analysis: The RAPD patterns obtained from 10 pepper accessions of *Capsicum annuum* L. using primer C5152 are shown in Fig. 1. The Nei estimate of similarity was used to construct a similarity matrix. The similarity values ranged from 0.209 for CA024 and CA020 to 0.891 for CA1107 and CA365 (Table 4).

The dendrogram, result showed that pepper accessions were separated into two major groups at a similarity value of 0.757 (Fig.2). The first group, which was further categorized into two subgroups included 8 accessions with white corolla. One subgroup included 4 long-fruited accessions, namely CA849, CA958, CA1107 and CA1118 and 1 medium-fruited accession, CA365. The second subgroup included medium-fruited, CA367 and short-fruited, CA398. A medium-fruited accession CA020 with white corolla, could not be clustered into any subgroup. The other major group included short-fruited having white with green margin corolla, CA024 and yellow green with green-yellow spots corolla, CA034.

Table 4: Similarity matrix of pepper (*Capsicum annuum* L.) accessions generated using Nei's estimate of similarity

	CA020	CA024	CA034	CA365	CA367	CA398	CA849	CA958	CA1107	CA1118
CA020	1.0000									
CA024	0.2090	1.0000								
CA034	0.3768	0.7600	1.0000							
CA365	0.7544	0.2857	0.4154	1.0000						
CA367	0.7069	0.2344	0.3636	0.8148	1.0000					
CA398	0.7304	0.2362	0.3817	0.8598	0.7706	1.0000				
CA849	0.7563	0.2901	0.4444	0.8649	0.8142	0.8214	1.0000			
CA958	0.7705	0.2985	0.4348	0.8596	0.8276	0.8000	0.8739	1.0000		
CA1107	0.7797	0.3077	0.4478	0.8909	0.8393	0.8288	0.8870	0.8814	1.0000	
CA1118	0.7967	0.2963	0.4317	0.8522	0.8034	0.8103	0.8500	0.8780	0.8908	1.0000

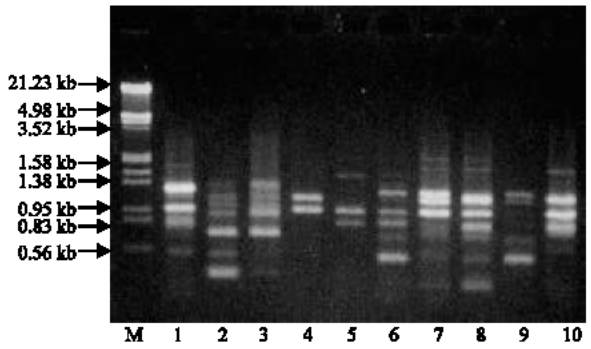

Fig 1: RAPD profile from pepper generated by C5152 primer. Lanes numbered 1 to 10 are DNA samples from 10 pepper accessions described in Table 1 M; λ-DNA (*Hind* III/ *EcoR* I digested)

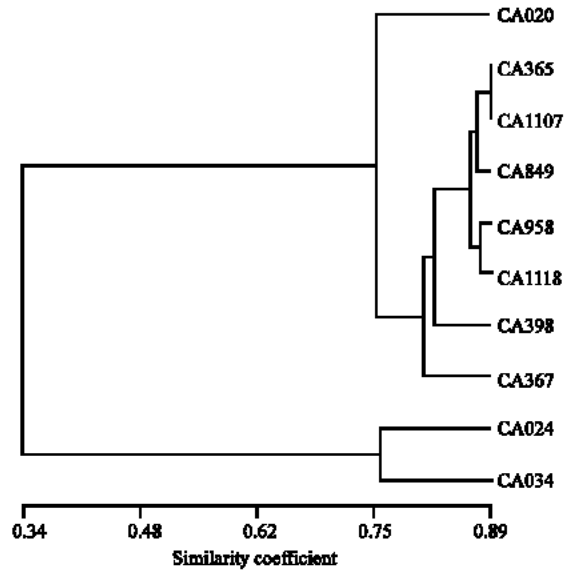

Fig 2: Dendrogram of 10 pepper accessions of *Capsicum annuum* L. by UPGMA cluster analysis

DISCUSSION

In this present study, we distinguished 10 accessions of *Capsicum annuum* L. collected from Germplasm Unit of TVRC in Thailand using RAPD technique. A total of 78 single and combined primers were used to screen polymorphism between accessions. Five of the twelve single primers could not be used as RAPD primers due to the absence of bands. The higher GC contents produced higher frequencies of RAPD because of increase in total frequency of amplified fragments[16]. The absence of band in primers C41 and C50, which has the second highest GC content could be attributed to a stem loop and hairpin loop formation[17]. Combination of two primers for RAPD analysis is effective for detecting polymorphic fragments which could be hardly detected using single primer. This result is in agreement with the findings of Klein-Lankhorst *et al.*[14] in tomato that the combination of two primers resulted in the appearance of new amplified DNA fragments that were not produced when each primer was used separately.

When the cluster analysis of RAPD patterns were associated with morphological characteristics of 10 pepper (*Capsicum annuum* L.) accessions, it was found that the accessions could be classified into two major groups. The first group included 8 accessions of white corolla with 4 long-fruited (CA849, CA958, CA1107 and CA1118), 3 medium-fruited (CA020, CA365 and CA367) and 1 short-fruited (CA398). The second group included 2 accessions of short-fruited with 1 white with green margin corolla (CA024) and 1 yellow green with green-yellow spots corolla (CA034). It indicates that the small-fruited accessions formed a more divergent group than the large-fruited accessions. This result supports the previous studies of Lefebvre *et al.*[9] and Paran *et al.*[10] that the small-fruited cultivars of *Capsicum annuum* formed a more divergent group than the large-fruited cultivars. Within the first group, long and medium-fruited were more closely related to each other due to their declining fruit position while the medium-fruited (CA367) and short-fruited (CA398) have erect fruit position. It is interesting that CA020 with medium and erect fruits was distantly related to CA367 and CA398. It is likely that this accession is genetically distinct from each others.

Although, CA024 and CA034 are short-fruited, they were further categorized into different groups because of

its difference in corolla and fruit position. CA024 has white with green margin corolla with conical fruit shape while CA034 has yellow green with green- yellow spots corolla. The presence of corolla with green-yellow spots in CA034 was suggested to be the wild form of *Capsicum annuum* L.[18].

This study showed that RAPD analysis provides a more specific technique and reliable method in classifying pepper (*Capsicum annuum* L.) accessions having closely related genetic make up and therefore a valuable tool for genetic and breeding studies as well.

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