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Genetic Relationship in Strawberry Cultivars and Their Progenies Analyzed by Isozyme and RAPD

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ABSTRACT Isozyme pattern and RAPD technique were used to study the relationship of new hybrids in strawberry breeding in Thailand. The selected progenies from alternate crosses and their parental lines were investigated and the phylogenetic trees were constructed. Some polymorphic relations were observed when the four enzyme systems; LAP, MDH, ME and DIA were used, however they could not clearly distinct hybrid lines uniquely at either 90 or 95% similarity levels. RAPD technique, using 10 random primers, could effectively show most of the hybrid lines uniquely at 95% similarity level. Moreover, RAPD differentiated strawberry cultivars effectively. Data from this study on the hybrid line relationship could be useful for parental selection in strawberry improvement program.

KEYWORDS: strawberry isozymes, RAPD, breeding, identification.

INTRODUCTION

Strawberry has been introduced and planted in Thailand for more than 3 decades but it has not really adapted to the short day length, high humid and hot climate like the normal tropical Thai environments. Therefore, the yield of strawberry in Thailand is lower than that found in original habitats like Europe or America. Breeding program has been set up recently in order to overcome those limiting factors in strawberry growing, which has been a bottleneck of the commercial strawberry production. It is thus necessary to get most of the information on the breeding lines and their parents as well as the new hybrids. Detailed morphological study has been reported,[1] but could not clearly demonstrate the relationship between the breeding stocks and their progenies.

Molecular markers have been used in many plant breeding programs, especially in the hybrid and cultivar identification as well as mapping linked to important traits. In the case of strawberry, molecular markers were an important device of cultivar identification. Isozyme markers with the combination of 3 enzyme systems were used to classify the University of California released cultivars[2] and to identify 30 groups of 34 strawberry cultivars.[3] The other technique based on the analysis of the Randomly Amplified Polymorphic DNAs (RAPD) using a set of 10 primers, was sufficient to distinguish the cultivars uniquely.[4-8] RAPD markers have been applied in classification and demonstration of genetic relationship between 75 strawberry cultivars and breeding lines.[9] Furthermore, the combination of isozyme systems and RAPD have proved to be effective in the studies of diversity and genetic relationship in garlic,[10] Chaenomeles[6] and *Hydrilla verticillata*.[11] This paper aimed to elaborate on the application of suitable enzyme systems and the RAPD technique in the identification of hybrid clones and to determine the genetic relationship between the parent lines and their progenies which would be useful for the strawberry breeding in Thailand.

MATERIALS AND METHODS

The alternate crosses were made from four parental lines, namely Prarajchathan # 50 (B), Nyoho (N) and Questa (Q), and their progenies were selected and maintained in a greenhouse at Chiang Mai University for all investigations.

Isozyme Study

Enzyme Extraction

Young leaves were collected and kept at 4°C before 100 mg of leaf sample was taken from each line and placed in sterile mortar. Subsequently, the sample was homogenized in liquid nitrogen, after that 10 ml of the extraction buffer[2] was added. The homogenate was transferred to Eppendorf tube and centrifuged for 20 min at 14,000 rpm at 4°C. The supernatant was collected and kept in the ice bath for the further use. Polyacrylamide gel (7% stacking gel, 10% separating gel) was run with the discontinuous buffer system.[12]

Enzyme Systems and Staining

Four enzyme systems, LAP (leucine amino-peptidase), ME (malic enzyme), MDH (malate dehydrogenase), DIA (diaphorase) were selected in this experiment. Enzyme staining was performed according to the procedures described previously.[13-14]

Randomly Amplified Polymorphic DNA (RAPD)

DNA Extraction

Midiprep method modified by Pich and Schubert[15] was applied for DNA isolation. Leaves from the *in vitro* grown plantlets, ca 100 mg, were quickly frozen in liquid nitrogen, powdered with mortar and pestle and transferred into 3 vol. (w / v) of extraction buffer (500 ml NaCl, 50 mM Tris / HCl pH 8.0, 50 mM EDTA, with 1% (v/v) mercaptoethanal added immediately before use). The mixture was thawed and ice-cold 20% stock solution polyvinyl pyrolidone (PVP) was added to a final concentration of 6%. Solid SDS was added to a final concentration of 2% (w / v). The extract was mixed briefly and incubated in a 65°C water bath for 10 min. Then 1/10 vol of 5 M potassium acetate was added, followed by 30 minutes incubation on ice and centrifugation at 13,000 rpm for10 min at 4°C. The supernatant was transferred into a new tube, mixed with 0.6 vol isopropanol by inverting the tube three times and incubated on ice for 10 min. After the subsequent centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was completely discarded. The pellet was dried under vacuum, dissolved in 500 ml 1x TE (pH 8.0) and extracted once with 1 vol. of phenol - chloroform - isoamylalcohol (25 : 24 : 1). Following the centrifugation at 13,000 rpm for 10 min at 20°C, the aqueous phase was transferred to a new tube and nucleic acid was precipitated in isopropanol at 20°C for 5 min. During this period, the tubes were gently inverted at least five times. Finally, the tubes were centrifuged at 13,000 rpm for 10 min at 4°C and the pellets were washed in 70% ethanol, dried under vacuum and dissolved in 50 ml 1x TE (pH 8.0).

DNA Amplification Condition and Gel Electrophoresis

DNA concentration ca. 5 ng/ml was amplified using 10 - base primers (Operon Technologies, Inc) (Table 1) and Ready - To - Go PCR beads (Amersham Pharmacia Biotch) in a 25 ml reaction. The thermo-cycle profile[8] was preceded with an initial denaturation soak for 5 min at 94°C. DNA was amplified for 47 cycles in a Perkin Elmer (model 9600) thermal cycle programmed for 1 min at 94°C, 1 min at 40°C, and 2 min at 72°C, followed by a 5 min soak at 72°C. Amplified products were separated by electrophoresis in 1.5% agarose gels, and visualized under UV light.

Table 1. The nucleotide sequences of the primers used in the experiments (primer names were according to manufacturer's Operon Technologies).

Primer Name	(Code)	Nucleotide Sequence (5'-3')
OPA - 01	(A1)	CAGGCCCTTC
OPA - 05	(A5)	AGGGGTCTTG
OPA - 13	(A13)	CAGCACCCAC
OPB - 07	(B7)	GGTGACGCAG
OPB - 11	(B11)	GTAGACCCGT
OPB - 14	(B14)	TCCGCTCTGG
OPC - 06	(C6)	GAACGGACTC
OPC - 16	(C16)	CACACTCCAG
OPD - 07	(D7)	TTGGCACGGG
OPD - 11	(D11)	AGCGCCATTG

Data Analysis

The bands from all enzyme systems and RAPD fragments were coded as present (1) or absent (0). A dendrogram was constructed based on the genetic distance matrix data by cluster analysis (SPSS for window release 9.0 for isozyme patterns and Bio1D+ Profile Image analysis software for RAPD).

Results and Discussion

Isozyme Patterns Analysis

Consistent banding patterns were obtained with all 4 enzymes studies. Seventeen patterns of enzyme polymorphism were observed from the 4 enzyme systems; 5 each for ME and DIA, 4 for MDH and 3 for LAP (Table 2). LAP gave three different banding patterns. N and B cultivars showed pattern A, and Q cultivar showed pattern B. All progenies of the NQ-QN family set showed pattern A, while some progenies of NB-BN family set showed pattern C that was different from the parents. The investigated progenies were chosen in a fewer numbers on the basis of their field performance groups, so the differences in enzymatic phenotypes might appear depend on enzyme types and their genes. Five banding patterns of LAP were found in 34 strawberry cultivars[5], and four different banding patterns were observed among 22 cultivars.[2] ME, DIA and MDH systems were reported here as the first for strawberry investigation. They have previously shown discrete polymorphism and were useful in distinguishing *Dendrobium* species.[12] The analysis of Jaccard's coefficient based on 4 enzyme banding patterns was performed in the classification and estimating genetic relationship among hybrids and their parents (Figures 1 and 2). In the NB-BN family set, the proximity matrix value ranged from 0.588 to 1.0, such that the members in this group could be separated into 10 clusters, namely N and B cultivars and 8 hybrids. BN 43 and BN 70 were the only pair that had the same 4 enzyme banding patterns, showing 100% similarity

(Fig 1). The NQ-QN family, comprised of 5 hybrids and N and Q parent lines, could be separated into 7 clusters based on the proximity matrix. In this group, NQ 14 and NQ 5 were identified as highly closed hybrids (86% similarity, Fig 2); nonetheless they were different in 2 enzyme banding patterns, namely LAP and ME (Table 2). The classification based on isozyme system should be adopted principally by the pattern of banding. Data from this study indicated that the selected isozyme systems were not adequate to uniquely identify all strawberry cultivars and their hybrids. This might be due to the genetic background of strawberry which is an octaploidy crop, that could make the selected enzyme patterns more complex compared with those of other crops. However, a combination of PGI, PGM and LAP could be used to separate some pairs of cultivar, which are not closely related.[3] Thus, another enzymes should be further investigated if one would like to apply the enzyme system for the strawberry identification.

RAPD Analysis

RAPD reactions were performed on DNA samples from hybrids and parent cultivars. The application of ten-primer system resulted in amplification of scorable polymorphic fragments that are depended on clones and primers. The highest number of 17 bands could be obtained when the C6-primer was used, and the lowest number 7 bands was obtained from the D7-primer (Table 3). The similarity values ranged from 85 to 94%, with the values closer to 100% indicating greater similarity. The BN-NB family set, could be separated

Table 2. Isozyme phenotypes of strawberry and their progenies.

Clone	Isozyme			
	LAP	ME	MDH	DIA
B	A	A	B	C
N	A	B	A	A
Q	B	A	B	B
NB 5	C	B	C	C
NB 24	C	A	D	E
NB 38	A	A	A	D
BN 36	C	B	A	C
BN 43	A	D	A	A
BN 60	C	E	A	C
BN 63	A	E	C	C
BN 70	A	D	A	A
NQ 4	A	B	D	C
NQ 5	A	B	C	D
NQ 14	A	B	B	C
NQ 15	A	C	B	D
QN 6	A	C	D	B
Number of patterns	3	5	4	5

Table 3. Number of different fragments observed among strawberry clones using 10 random primers.

Clone	Primer									
	Number of fragments observed									
	A1	A5	A13	B7	B11	B14	C6	C16	D7	D11
B	10	11	14	14	10	12	14	10	6	6
N	9	9	13	13	8	13	11	11	3	7
Q	9	13	8	14	9	8	12	6	6	5
NB 5	11	11	11	13	6	11	12	12	4	7
NB 24	11	11	11	9	8	12	11	13	5	7
NB 38	11	11	13	11	6	14	10	10	4	9
BN 36	8	8	10	12	9	14	12	11	5	10
BN 43	9	9	10	14	8	13	12	11	5	6
BN 60	11	11	9	10	9	13	10	13	5	7
BN 63	9	9	12	12	9	12	12	11	5	8
BN 70	8	8	12	13	9	13	12	13	6	8
NQ 4	7	11	11	10	9	12	12	4	4	9
NQ 5	8	12	9	14	9	13	9	10	5	7
NQ 14	7	12	9	14	8	12	9	11	5	8
NQ 15	9	12	10	13	8	10	12	6	6	9
QN 6	7	7	8	9	7	10	12	8	4	6
Total of differnt fragments	13	4	14	15	10	16	17	14	7	11

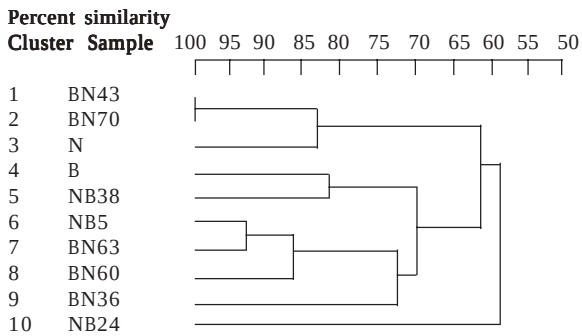

Fig 1. Dendrogram of Jaccard's coefficient of similarity representing the relationship of BN-NB family set based on 4 isozyme patterns family set based on 4 isozyme patterns.

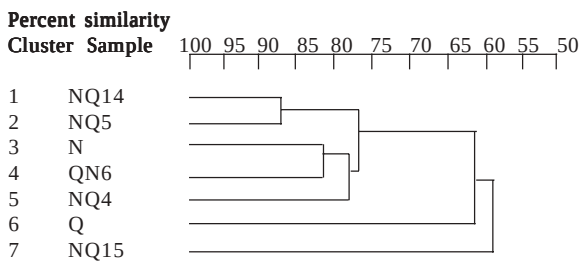

Fig 2. Dendrogram of Jaccard's coefficient of similarity representing the relationship of NQ-QN family set based on 4 isozyme patterns family set based on 4 isozyme patterns.

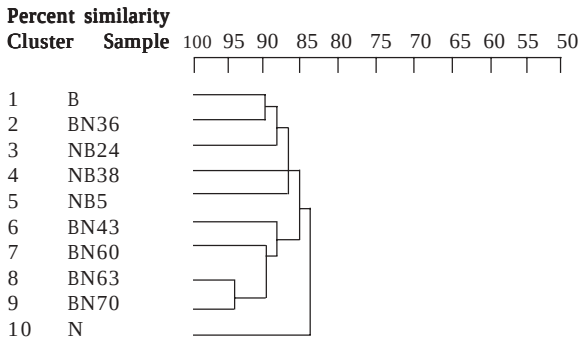

Fig 3. Dendrogram of Jaccard 's coefficient of similarity representing the relationship of BN-NB family set based on RAPD markers.

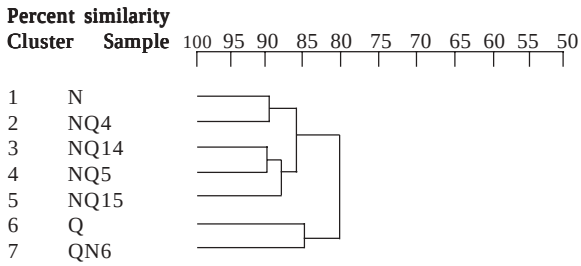

Fig 4. Dendrogram of Jaccard 's coefficient of similarity representing the relationship of NQ-QN family set based on RAPD markers.

into 10 clusters that were different from those obtained from the enzyme pattern (Fig 3 versus 1). Based on the RAPD technique, the BN 63 and BN 70 hybrids were grouped in the same category with 94% similarity. N was separated from others and had 85% similarity to the rest of the group (Fig 3). Moreover, the N and B cultivars could be clearly distinguished. All of the NQ-QN family could be singly separated. The NQ 14 and NQ 5 were identified as 89% matching and the NQ 4 was 88% similar to the N where as the QN 6 was 84% similar to Q (Fig 4).

A field test was performed to select for the progenies that produce having good field performance and the attractive fruit quality such as the bright red fruit color of the B cultivar. According to results from the RAPD analysis, the NQ-QN family set could be separated into two groups at 80% similarity as follows; the first group comprised of N, NQ 4, NQ 14, NQ5 and NQ 15, and the second group were the Q and QN 6. Members of each group were alike in terms of over all morphology and fruit color. Thus, results from this experiment indicated that the clusters of RAPD dendrogram could be related to the fruit characters, which made this technique very useful in strawberry identification and breeding program. However, fruit color was a quantitative trait,[7] and thus the application of molecular markers to tag traits of interest was rather complicated. Marker Assisted Selection (MAS) using a large number of molecular markers and traits of interest combined with the statistic analysis, or Bulked segregant analysis (BAS) would be more practical. One example is the use of seven RAPD markers developed from four RAPD primers that were linked to the red stele resistance gene *Rpf* 1.[16]

The similarities between cultivar B and N, and B and Q were considerably low, which should be due to their different origins; N was a Japanese cultivar, whereas B and Q were of an America origin. Other have reported the use 12 RAPD primers in the investigation of intraspecific relationship between North American *F. chiloensis* which found variations within subspecies of different origin.[17] The geographical difference had certain effects on crop adaptation to the environment.

The results from this study revealed that the RAPD technique with the set of 10 primers was not sufficient to identify the uniqueness of very closely related strawberry clones. Similar result was previously reported that technique could not distinguish the somaclonal variants of strawberry clones derived from meristem tip culture.[18] Moreover, the pedigree data in some strawberry cultivars showed no relationship with the estimated genetic similarity from RAPD data.[4] Eight cultivars and advanced selected lines could be uniquely identified using 10 RAPD primers and could be proved by the correlation between the number of shared banding profiles and pairwise coefficients of co-ancestry.[8] RAPD data gave better relationship than amplified fragment length polymorphisms (AFLP) in their ability to produce fingerprints and predict relationship among 19 strawberry cultivars.[5] Thus RAPD should be more intensively examined in the subsequent study.

Results from this study suggested that RAPD markers were suitable for easily distinguishing the population within the breeding program. Information from the dendrogram could be use to decide the future crosses. In this breeding program, we expected to enhance particular traits from each cultivar to form the superior progenies. Backcross method might show inbreeding effect result in a decline in vigor and fertility. The modified backcross and threeway hybrid methods would be used to avoid the inbreeding depression. So there is a plan to produce a threeway hybrid by crossing the outstanding hybrid from the B-N family (such as BN 43) with the hybrid with a low similarity with N but a high similarity with Q (such as QN 6). It is expected that the progeny with good characters from the three cultivars will result. Furthermore, the RAPD investigation in progeny population will be studied in correlation with other important traits.

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