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RAPD patterns as a useful tool to differentiate Thai *Piper* from morphologically alike Japanese *Piper*

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ABSTRACT Two species of *Piper* native to Japan, namely *P. kadsura* (Choisy) Ohwi and *P. retrofractum* Vahl were studied in comparison with *P. chaba* Hunt, a species found in Thailand. Relations among these three *Piper* species were compared using similarity indexes from morphological investigations and RAPD analysis. Based on morphology, the similarity indexes of the pairs: *P. chaba* Hunt and *P. retrofractum* Vahl, *P. chaba* Hunt and *P. kadsura* (Choisy) Ohwi, *P. retrofractum* Vahl and *P. kadsura* (Choisy) Ohwi. were 71.4%, 38.5% and 46%, respectively. RAPD was carried out using 14 different primers to examine DNA polymorphisms. When the amplified products were analyzed by agarose gel electrophoresis, it was found that *P. chaba* Hunt and *P. retrofractum* Vahl were very different in DNA patterns. Similarity indexes of the pairs, *P. chaba* Hunt and *P. retrofractum* Vahl, *P. chaba* Hunt and *P. kadsura* (Choisy) Ohwi, *P. retrofractum* Vahl and *P. kadsura* (Choisy)Ohwi were 9.4%, 9.3% and 22.2%, respectively. The results demonstrate a closer relation between *P. retrofractum* Vahl and *P. kadsura* (Choisy) Ohwi than between *P. chaba* Hunt and *P. retrofractum* Vahl.

KEYWORDS: RAPD, *Piper*, Similarity index.

INTRODUCTION

Piper is an important genus in the Piperaceae family since it has been used for traditional medicine and as a spice for a long time. In Thailand it has been expected to have 30 species. However, the number is uncertain because some are very similar in morphology. Moreover, some of these species are monoecious or dioecious, so that it is difficult to identify and classify by using only morphological characteristics. Other alternative methods, therefore, are needed for taxonomic study of plants in genus *Piper*. Molecular markers based on DNA polymorphisms may be another approach to serve the purpose since they have been successfully used for identification and classification of plants in many genera. Lifante and Aguinagalde[1] studied taxonomical relationships among three species of *Asphodelus* sect *Verinea* (Asphodelaceae): *A. fistulosus* L, *A. ayardii* Jahand & Mair e and *A. tenuifolius* Cav using Random Amplified Polymorphic DNA (RAPD) analysis. Haruki et al[2] indicated that RAPD could provide evidence for identifying the probable parent of some interspecific hybrid cultivars of Lily. Friesen et al[3] used the RAPD analysis of nuclear DNA in *Allium fistulosum* and *A. altaicum* and found 126 polymorphic fragments. Esselman et al[4] used RAPD markers to measure genetic diversity within and divergence among species of *Dendroseris* (Asteraceae: Lactuceae), a genus endemic to the Juan Fernandez, islands, Chile. Results were compared to the previous study employing allozymes. For five of the species, RAPD banding patterns distinguished all individuals examined. RAPD band diversities ranged from 0.003 to 0.022 within species: > 90% of total diversity was among species and < 10% within them. Relative levels of allozyme and RAPD diversity were similar for some species.

In this study, we performed RAPD analysis for DNA polymorphisms among three species of *Piper*. Two of them are native to Japan, namely *P. kadsura* (Choisy) Ohwi and *P. retrofractum* Vahl. The other is *P. chaba* Hunt, a species found in Thailand. *P. kadsura* (Choisy) Ohwi has unique morphology while *P. retrofractum* Vahl is morphologically similar to *P. chaba* Hunt. Firstly, morphology of these three

species are described and then RAPD patterns as well as similarity indexes are shown. The results indicated the potential of RAPD to distinguish the morphologically alike *Piper*.

MATERIALS AND METHODS

Plant materials:

P. kadsura (Choisy) Ohwi from Okinawa, Japan
P. retrofractum Vahl from Okinawa, Japan
P. chaba Hunt from Khon Kaen, Thailand

Total DNA isolation

Small scale DNA isolation, modified from Rogers and Bendich[5] was used in this experiment. Fresh leaves 0.1 g or dried leaves 0.02-0.03 g were ground with sea sand to a fine powder, and 100 µl of β-mercaptoethanol and 500 µl of 2X CTAB solution (2% CTAB, 0.1 M Tris-HCl, 1.4 M NaCl, 1% PVP) were added. The mixture was incubated at 70 °C for 20 min with occasional inversion and then cooled at room temperature and further incubated at 55 °C for 15 min. A 500 µl of chloroform: isoamyl alcohol (24:1 v/v) was added with a brief inversion, incubated at room temperature for 30 min and then shaken for 10 min. After centrifugation at 15,000 rpm (Tomy Model MX-160) at 4 °C for 15 min, the upper phase was collected and repeated chloroform: isoamyl alcohol extraction. The upper phase was subsequently mixed with 0.1 volume of 10% CTAB solution and equal volume of ppt buffer (CTAB 1 g, 1 M Tris-HCl 5 ml, 0.5 M EDTA 2 ml adjust volume by distilled water to 100 ml), then the DNA-CTAB complex was allowed to form at room temperature for 15 min. DNA precipitates might be seen in this step if DNA is abundant, but mostly they were not seen. The mixture was centrifuged at 15,000 rpm, 4 °C for 15 min and the supernatant discarded. DNA pellet was gently mixed with 500 µl of 1 M NaCl-TE (1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 M EDTA) and incubated at 55 °C for more than 10 min or until DNA dissolved. A 500 µl of chloroform : isoamyl alcohol (24:1 v/v) was added and mixed by inversion for 3 min. The mixture was centrifuged at 15,000 rpm, 4 °C for 15 min. The upper phase was transfered to a new tube and mixed by inversion with 1 volume of isopropanol, DNA was spun down by centrifugation at 15,000 rpm, 4 °C for 15 min. DNA pellet was washed once with 100 ml of 70% EtOH and then let dry *in vacuo*. DNA was dissolved in 50 ml of TE and kept at –20 °C until used. The quality of DNA was checked by agarose gel electrophoresis (0.8%).

Polymerase chain reaction

The 10-mer primers (TOYOBO Ltd, Japan) used in this study are listed in Table 1. Polymorphism using a single arbitrary primer was conducted. PCR reaction in 25.75 µl final volume contained 4.5 µl of distilled water, 1.5 µl of primers (final concentration 3.86 µM), 1.25 µl of DNA template, 18.5 µl of master-mixed solution [188 µl of distilled water, 64 µl of dNTPs solution (final concentration 0.4 mM), 40 µl of 10X reaction buffer, 4 µl of Tth polymerase: 5 units/µl]. Each reaction was layered with mineral oil and subjected to Thermo Cycler (Astec Program Temp Control System PC-700). DNA was predenatured at 94 °C for 30 sec following by subsequent 45 repeats of PCR cycles: denaturation at 94 °C 30 sec, annealing at 40 °C for 2 min, primer extension at 72 °C for 3 min. After the final cycle, samples were incubated for further 7 min at 72 °C to ensure complete extension along the entire length of the target sequences.

PCR products were kept at 4 °C, then electrophoresed in 1% agarose gel, stained with ethidium bromide and visualised by UV illumination. Band patterns were photographed and only reproducible bands were scored. Similarity index was made on the basis of RAPD data.

Similarity index

Cluster analysis or pair analysis can be employed principally on morphological data, anatomical data or biochemical data.[6-8] In this study, we used pair analysis to calculate similarity index between a pair species of *Piper*. Parameters subjected to the analysis were from morphological data as well as RAPD

Table 1. Sequences of PCR 10-mer arbitrary primers used in this experiment.

Code	5' to 3'
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-07	GAAACGGGTG
OPA-09	GGGTAACGCC
OPA-11	CAATCGCCGT
OPA-13	CAGCACCCAC
OPA-15	TTCCGAACCC
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC
OPM-02	ACAACGCCTC
OPM-04	GGCGGTTGTC
OPM-05	GGGAACGTGT

patterns. The morphological data involved the characteristics of leaves, flowers and fruits. A few characteristics, which are poorly discriminated between the compared species, for example the colour of spike, were omitted. For analysis from RAPD, clearly visible RAPD bands were scored manually for presence (1) or absence (0) from the photographic results. Differing band intensities were not taken into account to avoid errors introduced by competition among priming sites during the initial rounds of PCR. Only bands reproducible in two dependent amplification reactions were included in the data analysis.[3]

Similarity index was calculated from $\frac{2\,n_{xy}}{n_x + n_y}$, when n_{xy} is the number of the common morphology or common DNA bands in x and y plants, n_x and n_y are the total morphology studied or DNA bands.[8]

Results and Discussion

Morphological data of three *Piper* species are described in Table 2. The characteristics used for similarity index calculation by pair analysis as mentioned in "Materials and Methods" are summarized in Table 3. Similarity index of morphology for *P. chaba* Hunt and *P. retrofractum* Vahl, *P. chaba* Hunt and *P. kadsura* (Choisy) Ohwi, and *P. retrofractum* Vahl and *P. kadsura* (Choisy) Ohwi are 0.714 (71.4%), 0.385 (38.5%), and 0.46 (46%) respectively. Examination of RAPD polymorphisms by the use of arbitrary primers was accomplished with 14 different primers (Table 1). The results indicated that RAPD patterns of the three species were absolutely different (Fig 1). The common DNA bands and the total bands were then scored for calculation of similarity indexes (Table 4). The mean of similarity indexes of *P. chaba* Hunt and *P. retrofractum* Vahl, *P. chaba* Hunt and *P. kadsura* (Choisy) Ohwi, and *P. retrofractum* Vahl and *P. kadsura* (Choisy) Ohwi are 0.094 (9.4%), 0.093 (9.3%), and 0.222 (22.2%) respectively. Although morphology of *P. chaba* Hunt is very similar to *P. retrofractum* Vahl, the similarity index lying on molecular data of these two species is only 9.4%.

Normally taxonomists distinguish *P. chaba* from *P. retrofractum* Vahl. by using reproductive organ. However, the identification has been obscured by the very similar out-looked feature of flowers. It is also difficult to observe the existence of stamens. Therefore, it is confusing to discriminate *P. chaba* from *P. retrofractum* Vahl by morphological approach. RAPD polymorphisms showed advantages for this purpose and classification could be easily elucidated with accuracy. Additionally, a higher similarity index of *P. kadsura* (Choisy) Ohwi and *P. retrofractum* Vahl may indicate a closer relation between these two species since they may evolve from the same ancestor in Japan.

Table 2. Morphology of three species of *Piper*.

plant characteristics	*P. kadsura* (Choisy) Ohwi	*P. retrofractum* Vahl	*P. chaba* Hunt
stem	scanden shrub, often radicant	climber with root at nodes	stout climbing and rooting
leaves	long petiole, ovatelanceolate or lanceolate, 5-13 cm long, 3-6 cm wide, long acuminate, obliquely at base, entire, subcoriaceous, glabrous above, often softhairy beneath, 5-nerved, 2-nerved from the base, 2-nerved from midrib near the base.	short petiole 0.5-0.75 cm lanceolate, 11-14 cm long, 4-6 cm wide, base asymmetric, acuminate, entire, membranous, glabrous, 2-nerved from the base, 4-nerved from midrib near the base, pennineved above it.	Very short petiole 0.3-0.5 cm, lanceolate, 10-17 cm long, 4-6 cm wide, base asymmetric, acuminate, entire, coriaceous, glabrous, 2-nerved from the base, 4-nerved from midrib near the base, pennineved above it.
spike	yellowish white, 3-8 cm long, nodding to pendulus, flower small, dioecious, bracts peltate, subsessile.	white, 2-10 cm long, peduncled 1-1.2 cm ,dioecious, male spike 2.5-8.5 cm, stamens 2 rarely 3 very short, female spike 1.75-3 cm, stigma 3, bract circular, spiral arrangement	white 5-5.5 cm long, peduncled 1-1.2 cm long, stigma 3, bracts circular, stamens 2.
fruiting spike	Cylindric	stoutly, conico-cylindric	stoutly, conico-cylindric
berries	globose, red when mature	globose, red when mature	globose, red when mature

Table 3. Characteristics used to calculate similarity index.

	characteristics	*P. kadsura* (Choisy) Ohwi.	*P. retrofractum* Vahl.	*P. chaba* Hunt.
leaves	leave shape	ovate-lanceolate	lanceolate	lanceolate
	petioles	long	short	very short
	base	oblique	oblique	oblique
	apex	acuminate	acuminate	acuminate
	margin	entire	entire	entire
	texture	subcoriaceous	membranous	coriaceous
		often soft hairy beneath	glabrous	glabrous
	nerves	5 nerved, 2 nerved from the base, 2 nerved from midrib	7 nerved, 2 nerved from the base, 4 nerved from midrib	7 nerved, 2 nerved from the base, 4 nerved from midrib
flowers	spike	yellow wish white	white	white
		dioecious	dioecious	perfect flower
		bract peltate	bract circular	bract circular
		stamen 2	stamen 2-3	stamen 2
fruits	fruiting spike	cylindrical	stoutly, conico-cylindrical	stoutly, conico-cylindrical
	berries	globose, red when mature	globose, red when mature	globose, red when mature

Table 4. The number of DNA bands and total DNA bands of three species of *Piper* used for calculation of similarity index.

primers	*P. chaba* Hunt (x) *P. retrofractum* vahl (y)			*P. chaba* Hunt (x) *P. kadsura* (Choisy) Ohwi (y)			*P. retrofractum* vahl (x) *P. kadsura* (Choisy) Ohwi (y)		
	Common bands	Total bands		Common bands	Total bands		Common bands	Total bands	
		X	Y		X	Y		X	Y
OPA-02	0	3	5	0	3	5	2	5	5
OPA-03	1	2	10	0	2	5	1	10	5
OPA-04	1	3	7	2	3	7	4	7	7
OPA-07	0	2	5	0	2	4	1	5	4
OPA-09	0	1	4	1	1	4	0	4	4
OPA-11	1	3	5	1	3	5	2	5	5
OPA-13	0	1	7	0	1	6	0	7	6
OPA-15	1	2	6	0	2	4	0	6	4
OPA-18	0	1	6	0	1	5	2	6	5
OPA-19	0	1	7	0	1	6	2	7	6
OPA-20	1	1	6	0	1	4	1	6	4
OPM-02	0	2	3	0	2	4	0	3	4
OPM-04	1	6	7	0	6	6	2	7	6
OPM-05	0	2	5	1	2	4	1	5	4

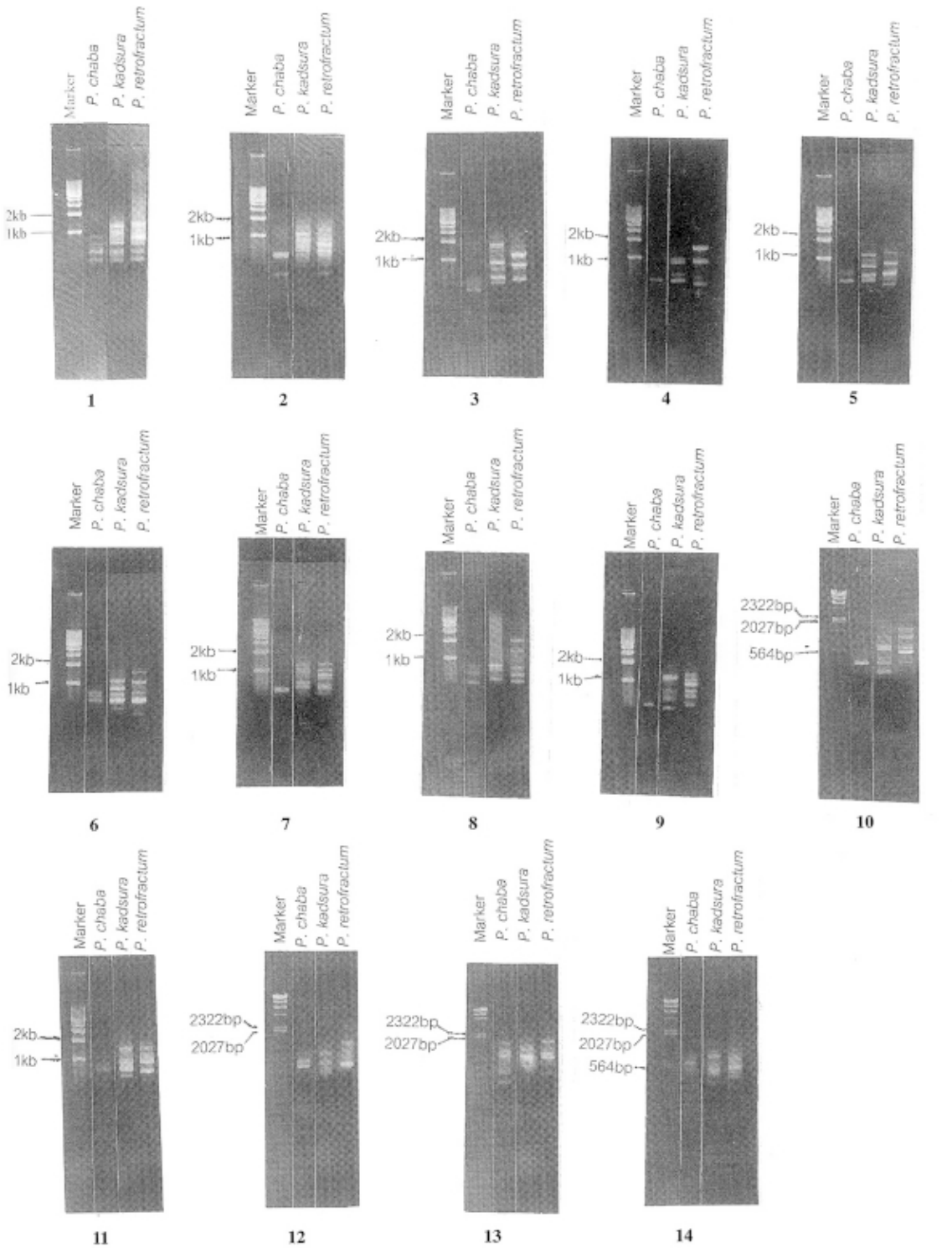

Fig 1. Ethidium bromide stained agarose gel of RAPD reaction with primer OPA-02 (1), OPA –03 (2), OPA-04 (3), OPA-07 (4), OPA-09 (5), OPA-11 (6), OPA-13 (7), OPA-15 (8), OPA-18 (9), OPA-19 (10), OPA-20 (11), OPM-02 (12), OPM-04 (13), OPM-05 (14) of *P. chaba* Hunt, *P. kadsura* (Choisy) Ohwi, *P. retrofractum* Vahl.

Our work shows usefulness of similarity index based on RAPD pattern. The index can distinguish *Piper* species independent from morphology. We are now conducting this technique to identify a number of plants in the genus *Piper* which are found in Thailand.

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