CHAPTER III

MATERIALS AND METHODS

Instruments

1. Autoclave
2. Automatic pipette P2, P20, P100 (Gilson Medical Electronics S.A., France)
3. Electrophoresis apparatus (Gelmate™ model 102)
4. –20 °C freezer
5. –80 °C freezer
6. High speed microcentrifuge
7. Controlling microcentrifuge
8. Vortex genic
9. Water bath
10. Electronic balance
11. Microwave oven
12. Spectrophotometer
13. Mortar
14. Liquid nitrogen tank
15. Thermal Cycler: GeneAmp PCR system (MJ Research model PTC 100)
16. UV transilluminator (Syngene)
17. Power supplies

Inventory supplies

1. 1.5 ml microcentrifuge tubes
2. 0.2 ml thin-wall microcentrifuge tubes
3. Bottles
4. Pipette tips
5. Glove

Chemical reagents

1. Agarose
2. Ethyl alcohol 70%
3. Ethylenediaminetetra acetic acid (EDTA)
4. Magnesium chloride ($MgCl_2$)
5. Sodium chloride (NaCl)
6. Sodium dodecyl sulfate (SDS)
7. Tris Acetate buffer (TAE buffer)
8. Tris Ethylenediaminetetra acetic acid buffer (TE buffer)
9. Tris-HCl
10. Ethidium bromide
11. Loading buffer
12. Isopropanol
13. *Taq* DNA polymerase (QIAGEN, Germany)
14. PCR buffer (QIAGEN, Germany)
15. dNTPs (QIAGEN, Germany)
16. Primer (QIAGEN; Operon Technology company, USA.)
17. 100 bp DNA Step Ladder (Promega, USA.)
18. Liquid Nitrogen

Oligonucleotide primers

Oligonucleotides used for PCR were purchased from QIAGEN Group of Companies: QIAGEN®, Operon®. The primer sequences are shown in Table 3.1 (Chiangda, 1998; Klangsinsirikul, 2000; Tunla, 2000; Popluechai, 2001; Pomkuntikaew, 2001; and Arunyawat, 1997).

Table 3.1 The sequences of all arbitrary primers in this study for *Alpinia* spp.

No.	Primer	Sequence 5′ ⟶ 3′
1	OPA11	CAATCGCCGT
2	OPA15	TTCCGAACCC
3	OPA 20	GTTGCGATCC
4	OPB18	CCACAGCAGT
5	OPC09	CTCACCGTCC
6	OPD02	GGACCCAACC
7	OPD11	AGCGCCATTG
8	OPG13	CTCTCCGCCA
9	OPG14	GGATGAGACC
10	OPK12	TGGCCCTCAC
11	OPK16	GAGCGTCGAA
12	OPV08	GGACGGCGTT
13	OPAB03	TGGCGCACAC
14	OPAB04	GGCACGCGTT
15	OPAB05	CCCGAAGCGA
16	OPAB08	GTTACGGACC
17	OPAK10	CAAGCGTCAC
18	OPAQ06	ACGGATCCCC
19	OPAQ12	CAGCTCCTGT
20	OPAS10	CCCGTCTACC
21	OPAX02	GGGAGGCAAA
22	OPAX17	TGGGCTCTGG

Sample collections

Thirty-seven giant galanga samples were collected from different areas of Thailand (Table 3.2). Young leaves were individually placed in the bottle and immediately transferred to a tank containing liquid nitrogen until further required.

Table3.2 Accessions of giant galanga varieties used in the variation study.

	Name (color-size type)	Origin
1	KhaDang (red-medium cultivated)	Khugtaphao, Uttaradit
2	KhaYuek (white-large cultivated)	Khugtaphao, Uttaradit
3	KhaLeang (yellow-medium wild)	Khugtaphao, Uttaradit
4	KhaSaku (red-small wild)	Khugtaphao, Uttaradit
5	KhaYuek (white-large cultivated)	Banhuahad, Uttaradit
6	KhaDang (red-medium cultivated)	Banhuahad, Uttaradit
7	KhaPar (red-medium wild)	Banhiha, Uttaradit
8	KhaDang (red-medium cultivated)	Tron, Uttaradit
9	KhaDang (red-medium cultivated)	Kosumpee, Kamphaengphet
10	KhaYuek (white-medium cultivated)	Bansakaew3, Kamphaengphet
11	KhaYuek (white-large cutivated)	Bankang9, Nakhon Sawan
12	KhaYuek (white-large cutivated)	Bankang3, Nakhon Sawan
13	KhaYai (white-large cutivated)	Banyantar, Nakhon Sawan
14	KhaLing (red-medium wild)	Banyantar, Nakhon Sawan
15	KhaTadang (red-large cultivated)	Bankardan, Nakhon Sawan
16	KhaDang (red-medium cultivated)	Bansalaloi, Lop Buri
17	KhaLing (red-medium wild)	Khon Kaen
18	KhaYuek (white-medium cultivated)	Bansakaew, Kamphaengphet
19	KhaTadang (red-medium cultivated)	Banaoyklongthakhuy, Lop Buri
20	Kha (red-medium cultivated)	Takai, Chachoengsao
21	KhaKaw (red-medium cultivated)	Bankrokanya, Samut Prakan
22	KhaLeang (yellow-medium wild)	Bankrokanya, Samut Prakan
23	Kha (red-medium cultivated)	Barpar, Samut Prakan

Table 3.2 (continued)

	Name (color-size type)	Origin
24	Kha (red-large cultivated)	Barpar, Samut Prakan
25	KhaDang (red-medium cultivated)	Panumsarakam, Chachoengsao
26	KhaNoldang (red-large cultivated)	Nayararm, Rayong
27	Kha (red-medium cultivated)	Lapsing, Chanthaburi
28	KhaDang (red-medium cultivated)	Nongbon, Srakaew
29	KhaKut (white-large wild)	Bantayag, Srakaew
30	KhaLuang (white-small cultivated)	Taham, Prachin Buri
31	KhaTadang (red-medium cultivated)	Banna, Nakhon Nayok
32	KhaNol (red-medium cultivated)	Banna, Nakhon Nayok
33	KhaYai (white-large cultivated)	Banna, Nakhon Nayok
34	KhaTadang (red-medium cultivated)	Jombuo, Rachaburi
35	KhaTadang (red-medium cultivated)	Danmakhamthia, Kanchanaburi
36	KhaDang (red-medium cultivated)	Uthong, Suphan Buri
37	KhaDang (red-medium cultivated)	Pothong, Angthong

DNA extraction strategies

Samples were frozen in liquid nitrogen. Plant DNA was extracted from 50 mg of young leaf tissue and ground in a mortar to give a fine powder from. Total DNA was extracted from each sample using the SDS extraction procedure (Kuntapanom and Ikeda, 1998). After grinding, The powder was mixed with extraction buffer 600 µl and put into a microcentrifuge tube (1.5 ml). It was incubated at 65 °C for 15 minutes in water bath (shake gently every 5 minutes). Next, 200 µl of 3M sodium acetate (pH 5.2) was put into the mixture (shake strongly) and incubated on ice for 30 minutes. Then, this mixture was centrifuged at 14,000 rpm, 4 °C for 20 minutes. The liquid was transferred into a new tube. It was added with the equal amount of isopropanol (shake gently) and incubated at –20 °C for 10 minutes. The supernatant was discarded. Precipitate was washed with 500 µl of 75% ethanol and centrifuged at 12,000 rpm, 4 °C for 10 minutes. This liquid was carefully discarded. The precipitate was air-dried. After that, it was resuspended by 100 µl of sterile distilled water, and put 67 µl of 5 M NaCl and 2 times of 99 % ethanol. This mixture was incubated on ice for 10 minutes and centrifuged at 12,000 rpm, 4 °C for 5 minutes. Then, this

liquid was carefully discarded. The precipitated DNA was air-dried. Extracted DNA was resuspended in 100 µl of TE buffer and put on ice until dissolving. The DNA solution was stored at -20 °C and utilized as the template for PCR amplification. The concentration was estimated by electrophoresis on a 0.8 % agarose gel and DNA was quantified by spectrophotometer. This extracted DNA solution had been diluted to 100 ng/µl before PCR amplification.

PCR amplification for RAPDs

PCR amplification was performed according to Arunyawat (1997) using primers synthesized by Operon Technologies (Alameda, USA). The reaction mixture (20 µl) consisted of 1x Reaction buffer (QIAGEN), 2 mM $MgCl_2$, 150 µM dNTPs, 40 ng of each primer, 1 U *Taq* DNA Polymerase (Promega), and 30 ng template DNA. PCR was carried out in a thermal cycler: GeneAmp PCR system (MJ Research model PTC 100) under the following conditions (Table 3.3).

Table 3.3 PCR condition.

Temperature (°C)	:	93	34	72	;	93	36	72	;	93	37	72
Time (minutes)	:	1	1	2	;	1	1	2	;	1	1	2
No. of cycle (cycle)	:		2		;		2		;		36	

Reaction was used total 40 cycle that final cycle (72°C) added 5 minutes to complete the final extension step. After finish, PCR produce was check by electrophoresis.

Approximately, 10 µl of the amplified RAPD products was loaded onto a 2 % agarose gel and separated by electrophoresis in TAE buffer at 100 volts. Gels were stained with ethidium bromide and products visualized by UV light. PCR products were photographed by UV transilluminator (Syngene) for data analysis. A 100 bp DNA Step Ladder (Promega, USA.) was included in all gels as a molecular weight standard.

Agarose gel electrophoresis

An appropriate amount of agarose was weighed out and mixed with TAE buffer to make the desired gel concentration (2 % agarose gel for detection of RAPD-PCR products). The dissolved agarose was heated until complete solubilization and cooled at room temperature to 50°C before poured into a gel mold in which a comb was already inserted. When the gel had solidified, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1x TAE buffer that cover the gel for approximately 0.5 cm.

An appropriate amount of RAPD-PCR amplified DNA was mixed with one-third volume of the loading dye buffer before carefully loaded into the well. A 100 bp DNA Step Ladder (Promega, USA.) was used as a DNA standard. Electrophoresis was operated at 100 volts until bromophenol blue moved to approximately 0.5 cm from the bottom of the gel. The electrophoresed gel was stained by immersed in a 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide in distilled water. DNA fragments were visualized under a UV transilluminator and photographed through a red filter.

Statistical analysis of genetic variation

The reproducible and well resolvable bands with the molecular length were scored from photographs of the gels according to the recorder in a binary matrix. Both weak bands with negligible intensity and smearing bands were excluded from final data analysis. The bands with the same molecular weight and mobility were treated as identical fragments. In the data matrices, the presence of a band was coded as 1, whereas the absence of the band was coded as 0. The data matrices were analyzed by the SIMQUAL (similarity for qualitative data) program of NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System for Personal Computers, Version 1.8), and similarities between accessions were estimated using the Jaccard coefficient, calculated as $J = a/(n-d)$ where a is the number of positive matches (i.e. the presence of a band in both samples), d is the number of negative matches (i.e. the absence of a band in both samples), and n is the total sample size including both the numbers of matches and unmatches (Rohlf, 1994). Dendrograms were produced from the resultant similarity matrices using the UPGMA (unweighted pair-group method with arithmetic averages) method.

Analysis of anti-fungal agent

The sample was extracted from rhizome of giant galanga to check the quantity of crude extract which related to anti-fungal agent (1′- acetoxychavicol acetate) as described by Jaradrattanapaiboon (2002).