CHAPTER 7
MATERIALS AND METHODS – MOLECULAR PHYLOGENETIC ANALYSIS

Taxa

Six species of *Curcuma* were selected to be obtained DNA sequences (table 6). They scatter to all subgenera and sections. Morphological characters are the main criteria of selection (table 8). Sequence of seven species of *Curcuma* and seven genera, one species of each, are taken from Genbank. *Caulokaempferia saxicola* and *Hedychium villosum* were selected as outgroup. These two genera belong to tribe *Zingibereae* and are sister group of *Curcuma* clade (Kress *et al*.,2002; Ngamriabsakul *et al*., 2004).

Materials

1. Plant materials in silica gel in zipped plastic bag
2. DNA extraction kit (DNeasy Plant Mini Kit, Qiagen)
3. PCR kit (Taq PCR Master Mix Kit, Qiagen)
4. Primer – ITS4 and ITS5
5. Autopipette and tips
6. 2 ml tubes and 200 µm tubes
7. PCR machine (GeneAmp® PCR System 9700)
8. Sequencer (ABI Prism 377)
9. Alignment programs: Bioedit and ClustralW
10. Phylogenetic analysis program: PAUP* 4.0b

Table 6 *Curcuma* species selected to be obtained DNA and voucher.

Taxa	Voucher
Curcuma caesia Roxb.	Maknoi 542
Curcuma cochinchinensis Gagnep.	Maknoi 521
Curcuma flaviflora S.Q. Tong	Maknoi 356
Curcuma longa L.	Maknoi 544
Curcuma parviflora Wall.	Maknoi 342
Curcuma sp. (Pitsanulok)	Maknoi 541

Table 7 Taxa obtained sequence from Genbank.

Taxa	Acc. No.	References
Curcuma alismatifolia Gagnep.	AY424751	Ngamriabsakul et al. (2004)
Curcuma amada Roxb.	AY424752	Ngamriabsakul et al. (2004)
Curcuma bicolor Mood and K. Larsen	AF478737	Kress et al. (2002)
Curcuma comosa Roxb.	AF478738	Kress et al. (2002)
Curcuma ecomata Craib	AY424753	Ngamriabsakul et al. (2004)
Curcuma harmandii Gagnep.	AY424754	Ngamriabsakul et al. (2004)
Curcuma petiolata Roxb.	AF202408	Wood et al. (2000)
Curcuma roscoeana Wall.	AF478739	Kress et al. (2002)
Curcuma rubescens Roxb.	AY424756	Ngamriabsakul et al. (2004)
Camptandra parvula Ridl.	AF478730	Kress et al. (2002)
Caulokaempferia saxicola K. Larsen	AF478732	Kress et al. (2002)
Hedychium villosum Wall.	AF478762	Kress et al. (2002)
Hitchenia glauca Wall.	AF202413	Kress et al. (2002)
Paracautleya bahtii R.M. Smith.	AY424766	Ngamriabsakul et al. (2004)
Smithatris supraneanea W.J. Kress & K. Larsen	AF478795	Kress et al. (2002)
Stahlianthus involucratus (King ex Baker) R.M. Smith	AF478796	Kress et al. (2002)

Table 8 Selected taxa and characters distribution.

Taxa	Leaf-base	Red patch	Infl.	Coma	Bract apex	Anther	Stylodes
Curcuma alismatifolia Gagnep.*	R	+-	T	+	A	0	-
Curcuma amada Roxb.*	C	-	T	+	A	1	+
Curcuma bicolor Mood and K. Larsen*	R	-	T	-	A	2	+
Curcuma caesia Roxb.	C	+	L	+	A	1	+
Curcuma cochinchinensis Gagnep.	R	+-	T	-	A	3	+
Curcuma comosa Roxb.*	C	+	L	+	A	1	+
Curcuma ecomata Craib*	R	+-	LT	-	A	2	+
Curcuma flaviflora S.Q. Tong	R	-	L	-	A	2	+
Curcuma harmandii Gagnep.*	R	-	T	-	A	0	-
Curcuma longa L.	C	-	T	+	A	1	+
Curcuma parviflora Wall.	R	+-	T	+	A	0	-
Curcuma petiolata Roxb.*	R	-	T	+	T	1	+
Curcuma roscoeana Wall.*	R	-	T	-	T	0	+
Curcuma rubescens Roxb.*	C	+	L	+	A	1	+
Curcuma sp. (Pitsanulok)	R	+-	T	-	T	0	-
Camptandra parvula Ridl.*	R	-	T	+	T	2	+
Caulokaempferia saxicola K. Larsen*	R	-	T	-	A	0	-
Hedychium villosum Wall.*	R	-	T	-	A	0	-
Hitchenia glauca Wall.*	R	-	T	-	A	0	-
Paracautleya bahtii R.M. Smith*	C	-	T	-	A	1	+
Smithatris supraneanea W.J. Kress & K. Larsen*	R	-	T	-	A	0	+
Stahlianthus involucratus R.M. Smith*	R	+	L	-	A	0	+

Remark: * indicate taxa taken sequence from Genbank. + = present, - = absent, A = acute, C = cuneate, R = rounded, T = truncate, 0 = absent, 1 = anther spurs: sharp and downward-pointed, 2 = anther spurs: large and forwards-pointed, 3 = anther spurs: short filament.

Methods

1. Store pieces of leaf in silica gel to make it dry under -30˚C overnight.
2. DNA extraction – use extraction kit and follow the protocol of extraction kit (appendix 4). The method in brief is as follow:
    a. Grind leaf sample till become fine powder.
    b. Add enzyme (RNase) to digest cell, using extraction buffer as medium and heat at 60 ˚C.
    c. Remove cell debris.
    d. Precipitate protein, polysaccharide and other chemical substances by using chemical reagent at low temperature.
    e. Separate DNA from solution using filter and centrifuge then clean by alcohol.
    f. Precipitate DNA from filter.
    g. Perform gel electrophoresis to check the result. Using 1% agarose gel. Set electrophoresis machine at 400 Watt and 110 Voltz. Run the machine for 50 mintes.
    h. Store extracted DNA in refrigerator at -30˚C.
3. Amplification (PCR technique) – select the genome ITS1, 5.8S and ITS2 of nuclear ribosomal DNA. These genomes were used in molecular phylogenetic analysis in previous studies in phylogeny of *Zingiberaceae*. Sequences of some species of *Zingiberceae* are deposited in Genbank which can be used to confirm the results from this study and also in cladistic analysis. Use PCR kit (PCR master mix, Qiagen).
    a. Prepare PCR master mix, the preparing method is as in kit's handbook. It composes of four types of dNTP, PCR buffer, enzyme (*Taq* polymerase), $MgCl_2$ and distilled water. Template DNA (extracted DNA) and Primer (ITS4 and ITS5 - reverse and forward primer respectively) are added just before placing in PCR machine. The total volume of each reaction is 50 µl.
    b. PCR machine is set with initial denaturation (94°C) for 3 minutes, then set for 30 cycles of denaturation (94°C) for 1 minute, annealing (62°C) for 2 minute, extension (72°C) for 1.5 minute. After the last cycle, the

machine is set for final extension (72°C) for 10 minute. Keep the PCR products at 4°C until removing from the machine.

c. Perform gel electrophoresis to check the products using 2% agarose gel. The machine is set at 400 Watts and 110 Volts. Run the machine for 60 minutes.

4. Sequencing –Automate sequencer was used at the Scienentific Equipment Center of Prince of Songkla University. Sequence of seven species of *Curcuma* and seven genera, one species of each, were obtained from Genbank (table 6).
5. Alignment – Using program BioEdit together with ClustralW by import sequences from clipboard in fasta format. Perform multiple alignments by using ClustralW after that do manual alignment by using Bioedit. Export data in Nexus file to be used in analysis program.
6. Analysis – Using analysis program PAUP*4.0b. Adopt the parsimony criteria with Branch and Bound method. Characters were set to unordered and equal weighted. Branch and bound search was set to 100 replicates with random additional sequence. ACCTRAN (accelerate transformation) was chosen for character optimization. If the shortest tree were found more than one then adopt consensus method to find out the final relationship. *Caulokaempferia saxicol*a and *Hedychium villosum* were selected as outgroup, because they have been proved by Kress *et al*. (2002) and Ngamriabsakul *et al*. (2004). Finally, support of each branch was given a boostrap value. Bootstrap analysis was performed and set to heuristic search with 1000 replicates, TBR and 10 random additional sequences replicates per heuristic search. Clades with bootstrap value 50-74% represent week support, 75-84% moderate support and 85-100% strong support.