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Original Article

Short *ITS* DNA barcode effectively distinguishes the medicinal plants *Cyclea barbata*

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Abstract

Cyclea barbata has been reported as traditional food and medicinal plant. Although its morphology resembles *Cissampelos pareira*, *Cyclea polypetala* and *Stephania japonica* very closely, chemical compositions of them are significantly different. Here we showed the capability of a short *ITS* region (277 nucleotides) for *C. barbata* identification. The partial *ITS* and *rbc*L regions from 14 samples, collected in many areas of Thailand, were amplified. The results after DNA blast on NCBI showed that all short *ITS* sequences closely related to *C. barbata*. The phylogenic analysis from the *ITS* and combined ITS/rbcL data by maximum parsimony and maximum likelihood methods showed that all samples was recognized into *C. barbata*. The results of DNA analysis were consistent with morphological identification. Our findings indicated that this short *ITS* region was able to distinguish *C. barbata* from their adulterants. This in turn can reduce cost and time for *C. barbata* identification using DNA barcode.

Keywords: moonseed family, phylogenic tree, DNA barcode, morphology, *Cyclea barbata*

1. Introduction

Cyclea barbata is recognized in Menispermaceae family (moon seed family) that globally distributes in tropical countries (Thanikaimoni, 1986). This plant is popular for providing several medicinal properties, such as antitumor, antipyretic, and antimalarial properties (Saxena, Pant, Jain, & Bhakuni, 2003), and antihypertensive activity (Jia-Qing, 2002), which many pharmacologic activities of their leaves are considered as result of the bis-benzyl-isoquinoline alkaloids. Its leaves contain major components such as bis-benzyl-isoquinoline alkaloids namely berbamine, chondocurine, alpha and beta cyclanoline, fangchinoline, homoaromoline, isochondocurine, isotetrandrine, lemacine, tetrandrine, minerals (calcium and phosphorus), and vitamins (A and B) (Tantisewie & Ruchirawat, 1992). Moreover, its leaves consist of pectin, viscous and elastic fiber, which is used as gelling agents in developing healthy drinks and foods (Chunthanom, Somboon, Sakulkoo, & Sihamala, 2013). However, *C. barbata* morphology is close to *Cissampelos pareira*, *Cyclea polypetala* and *Stephania japonica*, but pharmacological activities of these plants are significantly different. For example, the methanol root extract of *C. pareira* shows for antipyretic activity, while other do not perform antipyretic activity (Hullatti & Sharada, 2010). The bioactive components and medicinal properties of herbal products may be varied by several factors such as environment, ages, and parts of plants. Therefore, correct identification of the herbal plants is an important prerequisite to confirm quality, reproducibility, safety and effectiveness of the plants.

The morphological differentiation of *C. barbata*, *C. polypetala*, *C. pareira* and *S. japonica* can be differentiated by their male and female inflorescences (Manilal & Sabu, 1985). The aperture type of *Cyclea sp.* and *Cissampelos sp.* is

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tricolporate, while *Stephania sp.* is triporate. Additionally, *C. pareira* contains at least six stamens, and *Cyclea* sp. contains less than six stamens. The number of sepals in male flowers of *C. barbata* contains 4–5 sepals, while *C. polypetala* contains four sepals (Wang, Wang, & Chen, 2007).

However, morphological variation from effects of environment and habitats can provide trouble in classification of any organisms in natural habitats. Therefore, a DNA marker is a suitable tool, used as a nucleotide sequence, for identification of any organisms. A DNA barcode is a tool for rapid species identification based on DNA sequence that consists of a standardized short DNA sequence (400–800 bp) in principle of easy generation and species characterization, which aims to use the information of genes to identify all species of organisms (Kress & Erickson, 2005; Savolainen, Cowan, Vogler, Roderick, & Lane, 2005). Recently, the Plant Working Group of the Consortium for the Barcode of Life suggests a two-locus combination of organellar DNA, *rbc*L and *mat*K, as a plant barcode (Yao *et al.*, 2010), and several many studies have been reported that more data can be obtained from the use of nuclear genes (Chase & Fay, 2009) or chloroplast DNAs (Ngamriabsakul & Techaprasan, 2006). Moreover, *ITS* sequence and chloroplast DNA (i.e. *matK* and *trnL-F*) have been studied in Menispermeae family, which these regions are used for phylogenic tree construction of 20 species in 9 genera (Ortiz Rdel, Kellogg, & Werff, 2007), and a nuclear *ITS* data has been studied in the Menispermeae family and 9 taxa from other tribes (18 genera and 31 species) (Wang *et al.*, 2007). Phylogenies of the combined chloroplast *atpB* and *rbcL* data is analyzed by using both maximum parsimony and likelihood-based methods (Hoot, Zautke, Harris, Crane, & Neves, 2009). Previous reports show that *trn*L-F, *mat*K and *ITS* were used for phylogeny studies of Menispermaceae, which *C. barbata* and *C. polypetala* are clearly separated. The length of the sequence alignment of *mat*K, *trn*L-F, combined *mat*K -*trn*L-F and *ITS* are 1266, 1144, 2410 and 714 nucleotides, respectively (Wang *et al.*, 2007).

The size of ideal DNA barcode should be short enough to amplify from degraded DNA for a single-pass sequencing (Chase *et al.*, 2007; Lahaye *et al.*, 2008; Spouge & Mariño-Ramírez, 2012). To develop DNA barcode, we used both morphological (male flower and leaves) and molecular data (*rbc*L and *ITS* sequences) to identify *C. barbata*. Both *rbc*L and *ITS* sequences were characterized to find appropriated DNA barcodes, including sequence analysis and phylogenic tree construction. The species similarity of the *rbc*L and *ITS* partial sequences were determined by comparing with the gene sequences of other species from Gene Bank database. Moreover, flower morphology was identified to confirm the species similarity.

2. Materials and Methods

2.1 Materials

Eight plant samples of *Cyclea* sp. were collected from 4 provinces in Thailand namely Nakhon Nayok (Nak), Petchaboon (Pech), Sakon Nakhon (Sak) and Sa Kaeo (Sake) provinces. The latitude and longitude of sampling areas were 14.06 N 100.58 E (Nak_3, Nak_2), 12.37 N 99.38 E (Pech_P1, Pech_P2), 16.95 N 103.99 E (Sak_S1, Sak_S3), and 13.99 N 102.42 E (Sake_SA1 and Sake_SA2). Additionally, other five Menispermaceae plant samples namely WH2, WRRC, ARRC, CRRC and KRRC.

The all specimens were labeled with full information. Additionally, young leaves and flowers were stored and transported at 0 to 4 °C in ice box to arrival at the laboratory. All plant leaves were stored at −80 °C until DNA extraction.

2.2 Morphological analysis, DNA extraction, PCR amplification and sequencing

External morphological characters including leaves and flowers of *C. barbata* was identified following Manilal and Sabu (1985). The sepal, stamen and anther samples were observed under stereo microscope, and were compared to previous reports of Wang *et al.* (2007). The fresh young leaves were used for DNA extraction that total DNA was extracted by using RBC Real Genomics™ DNA/RNA Purification kit (Taiwan). Then, the extracted DNA was used as DNA template in a mixed 20 µl PCR reaction that composed of plant DNA (1 µl), 10 ul of GoTaq® green master mix (GoTaq® DNA polymerase, 2X green GoTaq® reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$) and 2 µl of 50 µM primer. List of primer pairs for amplification of the DNA barcode regions were rbcLaF-M13/rbcLaR-M13, which produced a DNA fragments in range of 500-600 bp (CBOL Plant Working Group, 2009; Kress & Erickson, 2007; Yu, Xue, & Zhou, 2011), ITS5F (2F)/ITS2r produced a DNA fragment in range of 300-400 bp (Yu *et al.*, 2011; CBOL Plant Working Group, 2009; Ghorbani, Saeedi, & de Boer, 2017), matK4La /*mat*K1932Ra produced a DNA fragment in range of 600-700 bp (Kress, Wurdack, Zimmer, Weigt, & Janzen, 2015; Von Crautlein, Korpelainen, Pietilainen, & Rikkinen, 2011; Yu *et al.*, 2011) and trnTUGU2F (trnLF)/ strnLUAAR produced a DNA fragment in range of 600-700 bp (Prince, 2015). The PCR procedure was performed by a pre-denaturation at 94°C (1 min), and 35 cycle of denaturation at 94 °C (5 sec), annealing at 55 °C (30 sec) and extension at 72 °C (90 sec), then a final extension at 72 °C (5 min). The PCR products were purified according to protocol of QIA quick PCR Purification Kit. (QIAGEN Inc, U.S.A.). Sequencing of candidate DNA barcode regions were determined by Sanger sequencing method (Macrogen Inc., Korea).

2.3 Sequence analysis

The *rbc*L and *ITS* sequences were analyzed by using MEGA software version 7.0. Parameters namely aligned length (bp), coverage, a number of variable sites (%), number of conserved sites (%), number of parsimony-informative site (%), number of singleton sites (%), %T (STDV), %C (STDV), %A (STDV), %G (STDV), and genetic distance (SE) were measured. For genetic distance analysis, estimation of average evolutionary divergence through all sequence pairs was used. The number of base substitutions per site from averaging through all sequence pairs, standard error estimate(s), were obtained by 1,000 bootstrap replication. The DNA analysis was conducted using the Kimura 2-parameter model. The rate variation among sites was explained by a gamma distribution (shape parameter = 1). Codon positions were analyzed at 1st,

2nd, 3rd and non-coding sites, and gap or missing data on the position were eliminated.

2.4 Phylogenetic analysis

Phylogenetic trees were performed by using maximum parsimony (MP) and maximum likelihood (ML) by using MEGA7.0 software. The maximum parsimony was done by 1000 bootstrap replication (Felsenstein, 1985), complete gaps or missing data elimination, tree inference options with tree-bisection-reconnection (TBR), MP search method (-10), random addition, and MP search level 1. The maximum likelihood were performed with a likelihood bootstrap analysis of 1000 replication, nucleotide substitution model, Kimura 2-parameter model, 5 gamma distributed with invariant sites (G+I) for rates among sites, complete gaps or missing data elimination, tree inference options with ML heuristic method (nearest-neighbor-interchange, NNI), NJ/ BioNJ initial tree for ML, and a very strong branch swap filter (Hoot *et al.*, 2009; Wefferling, Hoot, & Neves, 2013).

3. Results and Discussion

3.1 Plant sample collection and morphological identification

The similarity in leaf shape, plant characters and habitats between *C. barbata* and *C. polypetala* causes traditionally naming them as green grass jelly plants. Although, *C. barbata* closely resembles *C. polypetala*, they are differentiated by male flowers, which *C. barbata* contains 4–5 sepals and *C. polypetala* contains four sepals (Wang, Wang, & Chen, 2007).

The morphology of Menispermaceae plants have been completely described by Forman (1991), therefore, the thirteen collected plant samples in this study were preliminary identified according to Forman (1991). The result showed that nine plant specimens are classified as *Cyclea sp.*, and the other four are *Stephania sp*. Among our nine *Cyclea sp.* samples, one was flowering and showed male flower. In similar to the result of Hoot *et al.* (2009), the our samples of *Cyclea sp.* showed morphological characters similar to *C. barbata* that leaves had hairy deltoid or ovate in shape, peltate with cordate to truncate at the base, slightly sinuate at the base with rounded angles, mucronate, and more or less hairy on the nerves and veins (Figure 1A-E). According to *C. barbata*, *C. polypetala* and *C. pareira* were dissimilar by quantity of stamens (*Cyclea sp*. stamens < 6 and *Cissampelos sp.* stamens ≥ 6) and the number of sepals in male flower (4-5 sepals for *C. barbata* and 4 sepals for *C. polypetala*) (Forman, 1991), which these characters were used for identification in this study. The flower samples (Nak_2) showed that male calyx was bell-shaped with four triangular lobes and hairy at outer surface (Figure 1A, B and C), and contained only one stamen (Figure 1D), peltate and exserted synandrium with four to six anthers in a circular fashion (Figure 1E). The flower morphology indicated that it was male flower of *C. barbata*. The identified *C. barbata* samples were further used for DNA analysis. DNA was extracted from each sample, and DNA barcode and sequences analysis were further studied.

3.2 Analysis of partial *rbc*L and *ITS* DNA sequences

Because *rbcL, ITS, matK*, and *trnL-trnF* were widely used as DNA barcodes to identify plants, we collected DNA data of *Cyclea sp*. from GenBank. It found that there were small DNA sequence data of *Cyclea sp*. in GenBank. Our result showed the DNA barcoding database as BOLD system for 13 sequences, while GenBank showed 23 sequences (Table 1). In addition, the order of available data quantity was *rbcL* >*ITS*>*matK*> *trnL-trnF* from BOLD system and GenBank. Nevertheless, *matK* and *trnL-trnF* were

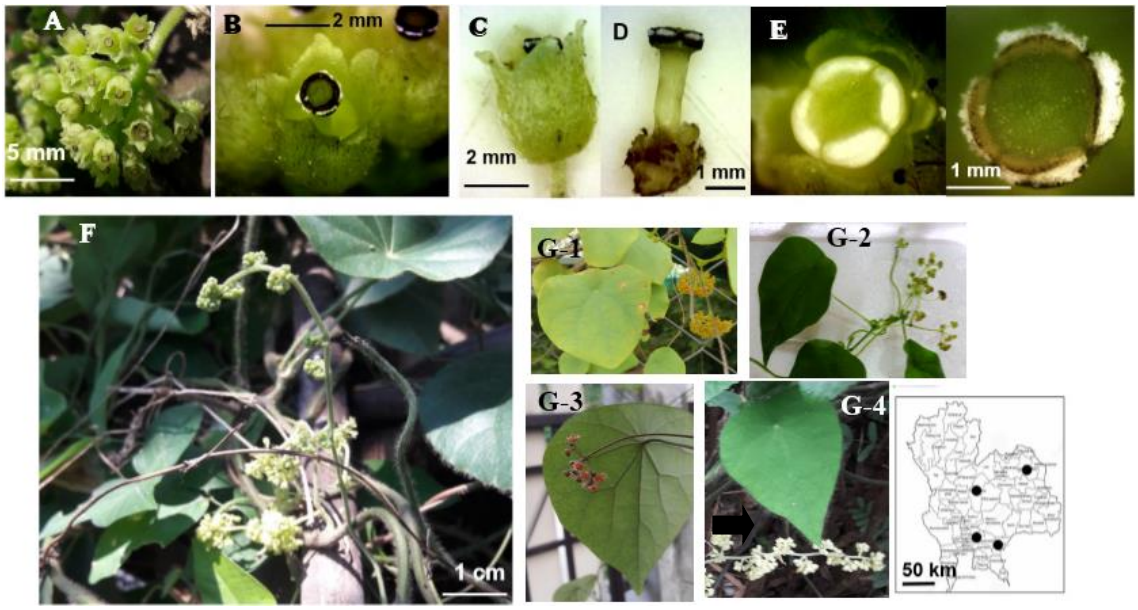

Figure 1. *Cyclea barbata* morphology. (A) male inflorescences, (B) 5 sepals in male flowers, (C) male flower, (D) one stamen, (E) anther, (F) climbing character, (G1-4) other species in moon seed family ; *Stephania cephalantha* G1, *Stephania hernandifolia* G2, *Stephania japonica* G3. Sampling locations shown on map in small box.

general DNA barcode for plants, but the limited data in database did not help for our *Cyclea sp.* identification. The *matK* and *trnL-trnF* could not be successful amplified from all DNA samples, while the *rbcL* region could be amplified for 100% (13 samples) of 9 *Cyclea* sp. DNA samples (Nak 3, Sak_S3, Sake_SA1, Nak 2, Pech_P1, Sak_S1, Sake_SA2, Pech_P2 and CRRC), and four Menispermaceae plant DNAs (WH2, ARRC, KRRC and WRRC). In addition, the *ITS* region could be amplified for 61.54% (8 samples) namely seven *Cyclea* sp DNA (Nak 2, Sake_SA1, Sake_SA2, Pech _P2, Pech_P1, Sak_S1 and Sak_S3) and 1 Menispermaceae plant DNA (WH2) (Table 2 and 3). The PCR products showed a 600 bp size of partial *rbc*L gene and a 300 bp size of partial *ITS* gene (Figure 2). DNA fragments were extracted, purified and sequenced. These sequences were analyzed by alignment, blast and genetic distance. The position of aligned sequences showed that all DNA samples were partial sequences of full *rbc*L(1400 bp) and *ITS* gene (550 bp). Our partial DNA sequences of *rbc*L aligned with nucleotide position at 113 bp to 621 bp of full *rbc*L (JN051680.1), while partial DNA of *ITS* aligned with nucleotide position at 1 bp to 238 bp of full *rDNA* sequences (AY017405.1). The eight *rbc*L sequences of these *Cyclea* sp. samples obtained GenBank accession numbers namely MK011495, MK011496, MK011497, MK011498, MK011499, MK011500, MK011501, and MK011502 for Nak_3, Sak_S3, Sake_SA1, Nak_2, Pech_P1, Sak_S1, Sake_SA2 and Pech_P2 samples, respectively. Furthermore, seven *ITS* sequences obtained GenBank accession numbers namely MK011503, MK011504 MK011505, MK011506, MK011507, MK011508 and MK011509 for Nak_2, Sake_ SA1, Sake_SA2, Pech_P2, Pech_P1, Sak_S1 and Sak_S3 samples, respectively.

The BLAST sequence analysis showed that partial *rbc*L sequences were close to *C. polypetala*, while partial *ITS* sequence was close to *C. barbata*. The partial *rbc*L sequences provided for 83 to 98 % of query coverage, showed relatedness to *C. polypetala* with 338 to 917 nucleotides of a total blast score and with a 99 % sequence identity. However, partial *ITS* provided for 84 to 97 % of query coverage, showed that all sample were *C. barbata* with 641 to 911 nucleotides of a total blast score and sequence identity in range of 87 to 99 % (Table 2). Our short *rcb*L sequences (500 nucleotides) were so much conserved that they could be used for genus level, but they were not useful for species identification. While short *ITS* sequences indicated that all *Cyclea* DNA samples were *C. barbata*, these showed that short *ITS* sequences (277 nucleotides) could be used for identification of *C. barbata*, combined with morphological identification (Figure 1).

3.3 Sequences alignment and genetic distance analysis

Sequence alignment analysis was compared between *rbc*L and *ITS* sequences (Table 3). For short *rbc*L region, the sequences composed of 29.1 (1.6) %T (STDV), 21.1(0.5) %C (STDV), 26.5(0.9) % A (STDV) and 23.2 (1.4) %G (STDV). The aligned sequence showed 477 bp length, and the *rbc*L sequences were high conserved and low variable sites for 502 nucleotides (82.3%) and 98 nucleotides (16.1%), respectively. The number of parsimony-informative sites (%) and singleton sites were 26 nucleotides (4.26%) and 12 nucleotides (1.97%), respectively. Moreover, genetic distance, consistency index and retention index were 0.007 (0.002, SE), 0.80000 and 0.894737, respectively**.** All data showed that *rbc*L DNAs were much conserved with 82.3% conserved sites and the value of genetic distance was very close. Therefore, these plant DNA samples were difficult to separate the plant species from other.

For short *ITS* region, the sequences composed of 17.81 (3.6) %T (STDV), 25.91 (4.0) %C (STDV), 26.68 (5.7) %A (STDV) and 29.59 (4.2) %G (STDV). The aligned sequence showed 277 bp length, and showed less conserved and more variable site for 187 (27.1%) and 490 (71%), respectively. A number of parsimony-informative sites (%) and singleton sites were 282 nucleotides (40.9%) and 183 nucleotides (26.5%), respectively. Additionally, genetic

Table 1. BOLD system and GenBank of plant DNA barcode.

Menispermaceae plants	DNA barcode			
	*rbc*L	*ITS*	*mat*K	trnL-trnF
Cyclea polypetala	GBVT926-13* GBVY172214* KF181462.1** JN051680.1**	JN051680.1** HG004782.1** AY017407.1**	GBVP2316-14* GBVP2316-14 * EF143863.1** HG004889.1**	EF143895.1**
Cyclea hypoglauca	GBVJ116-11* GBVJ117-11* GBVJ118-11* FJ626594.1** HQ260777.1** FJ026482.1**	HQ260777.1** KJ566124.1** AY017406.1**	GBVJ115-11 * EF143862.1**	EF143894.1** KJ566155.1**
Cyclea barbata	GBVJ114-11* a FJ026481.1a GENG1271-15*b	AY017405.1** AY017405.1** FJ026481.1a	GBVJ113-11* - EF143861.1**	EF143893.1**
Cissampelos pareira	GBVJ092-11* GQ436372.1**	ITSAJ4225-14* GQ434399.1**	-	-

Symnoname of *Cyclea barbata* as [a] *Cyclea burmanii* and [b] *Cyclea peltata*
* Sequence data from BOLD system
** Sequence data from NCBI's GenBank database

Table 2. Top 100 blast hits of partial *ITS* and *rbc*L gene.

Sample ID	gene	Homology	Accession No.	Query cover	Total score	%Identity
Nak_2[a]	*ITS*	*Cyclea barbata*	AY017405.1	83	911	92
	rbcL	*Cyclea polypetala*	JN051680.1	84	917	99
Pech_P1[a]	*ITS*	*Cyclea barbata*	AY017405.1	97	891	98
	rbcL	*Cyclea polypetala*	JN051680.1	83	889	99
Pech_P2[a]	*ITS*	*Cyclea barbata*	AY017405.1	97	898	93
	rbcL	*Cyclea polypetala*	JN051680.1	97	911	99
Sak_S1[a]	*ITS*	*Cyclea barbata*	AY017405.1	96	641	99
	rbcL	*Cyclea polypetala*	JN051680.1	97	891	99
Sak_S3[a]	*ITS*	*Cyclea barbata*	AY017405.1	96	889	98
	rbcL	*Cyclea polypetala*	JN051680.1	96	898	99
Sake_SA1[a]	*ITS*	*Cyclea barbata*	AY017405.1	84	911	93
	rbcL	*Cyclea polypetala*	JN051680.1	96	641	95
Sake_SA2[a]	*ITS*	*Cyclea barbata*	AY017405.1	84	889	99
	rbcL	*Cyclea polypetala*	JN051680.1	98	907	99
WH2[a]	*ITS*	*Stephania hernandifolia*	AY017398.1	97	891	87
	rbcL	*Stephania japonica*	KU204903.1	96	880	99
Nak_3[b]	*rbcL*	*Cyclea barbata*	AY017405.1	83	911	92
CRRC[b]	*rbcL*	*Cyclea polypetala*	JN051680.1	93	902	99
ARRC[b]	*rbcL*	*Stephania cephalantha*	JN051691.1	91	911	99
		Stephania brachyandra	JN051690.1	91	911	99
		Stephania rotunda	FJ026509.1	91	911	99
		Stephania venosa	EU526996.1	91	911	99
KRRC[b]	*rbcL*	*Stephania japonica*	KU204903.1	94	904	99
		Stephania abyssinica	JN051689.1	94	904	99
		Stephania longa	HQ260802.1	94	904	99
WRRC[b]	*rbcL*	*Stephania cephalantha*	JN051691.1	91	911	99
		Stephania brachyandra	JN051690.1	91	911	99
		Stephania rotunda	FJ026509.1	91	911	99
		Stephania venosa	EU526996.1	91	911	99

[a] DNA template from these samples could be amplified with *rbc*L and *ITS* primer sets. [b] DNA template from these samples could be amplified only *rbc*L primer set. Other 5 Menispermaceae plant samples namely WH2, CRRC, ARRC, KRRC and WRRC.

distance, consistency index and retention index were 0.108 (0.041, SE), 0.745, and 0.773, respectively. The number of variable sites (71%) and parsimony-informative sites (40.9%) from this study were similar to previous *ITS* study of Wang *et al.* (2007). It has been reported that aligned matrix of *ITS* regions has a length of 714 characters, which have 381 variable (53.36%) and 313 parsimony informative sites (43.83%) (Wang *et al.*, 2007). The consistency index (CI) of the *rbc*L sequences (0.800) was higher than *ITS* (0.745). It indicated that the *rbc*L sequences were homoplasy less than the *ITS* sequences (if CI=1, it means no homoplasy). Homoplasy was used to explain similarity in form and structure, but it did not have a common evolutionary origin (Diogo, 2007; Farris, 1989; Klingenberg & Gidaszewski, 2010).

3.4 Phylogenetic analysis

Recently, the maximum likelihood and maximum parsimony methods have been used to study of *C. polypetala* and *C. pariara* DNA (Wang *et al.*, 2007). Therefore, these methods were applied for DNA sequences analysis. Molecular phylogenetic analysis of the *rbc*L region by maximum likelihood method (Figure 3, ML) showed two major groups, *Cyclea* species and *Stephania* species. All collected samples were in *Cyclea* sp. (box) namely *C. polypetala*, *C. hypoglauca*, *C. babarta* and *C. pareira*. However, they could not distinguish in species level. The results from maximum likelihood method were similar to maximum parsimony analysis of taxa (Figure 3, MP). Maximum likelihood phylogenetic tree of the *ITS* region (Figure 4, ML) showed that the tree recognized into two major groups. The first group consisted of *Cyclea* sp., *S. hernandifolia*, *S. japonica*, and *S. longa*, and the second group consisted of *S. tetrandra*, *S. brachyandra* and *S. cephalantha*. The first group indicated that genetic distance of all collected samples was close to *C. barbata* (marked by asterisk). The results were confirmed with

Table 3. Sequences alignment and analysis. The 13 *rbcL* sample sequences including 8 *Cyclea* sp. DNA (Nak 3, Sak_S3, Sake_SA1, Nak 2, Pech_P1, Sak_S1, Sake_SA2 and Pech_P2) and 5 Menispermaceae plant DNA (WH2, CRRC, ARRC, KRRC and WRRC), and the 8 ITS samples namely 7 *Cyclea* sp. DNA (Nak 2, Sake_SA1, Sake_SA2, Pech_P2, Pech_P1, Sak_S1 and Sak_S3) and 1 Menispermaceae plant DNA (WH2).

	DNA barcode		
	*rbc*L	*ITS*	*rbc*L/*ITS*
Number of individuals	13	8	-
PCR success (%)	100 (13/13)	61.54 (8/13)	-
PCR product size (bp)	550	400	-
Sequencing success (%)	100	100	-
Sequence length (bp)	500	300	800
Aligned length (bp)	477	277	602
[a] Number of variable sites (%)	98(16.1)	490(71)	524 (70)
[b] Number of conserved Sites (%)	502(82.3)	187(27.1)	673 (84.1)
[c] Number of parsimony-informative site (%)	26(4.26)	282(40.9)	347 (43.4)
[d] Number of singleton sites (%)	12(1.97)	183(26.5)	163 (20.4)
%T (STDV)	29.1(1.6)	17.81(3.6)	23.6 (1.7)
%C (STDV)	21.1(0.5)	25.91(4.0)	24.1 (1.8)
%A (STDV)	26.5(0.9)	26.68(5.7)	25.5 (2.2)
%G (STDV)	23.2(1.4)	29.59(4.2)	26.8 (1.8)
Genetic distance (SE)	0.007(0.002)	0.108(0.041)	0.062 (0.007)
Consistency index, CI*	0.80000	0.745283	0.779310
Retention index, RI*	0.894737	0.773109	0.850467
Consistency Ratio (CR) = *CI / RI*	0.8941	0.8699	0.91633

*parsimony model; [a] A variable site composed of at least two types of nucleotides or amino acids. Certain variable sites showed singleton or parsimony-informative, while a constant site did not show variable. [b] A site, consisted of the same nucleotide or amino acid, was a constant site. MEGA could recognize a site as a constant site in case of at least two sequences showing unambiguous nucleotides or amino acids. [c] A site was parsimony-informative in case of containing at least two nucleotide types (or amino acid), and at least two of them under a minimum frequency. [d] A singleton site consisted of at least two nucleotide types(or amino acid), which one occurred at most in multiple times. MEGA could recognize a site as a singleton site in case of at least three sequences showing unambiguous nucleotides or amino acids.

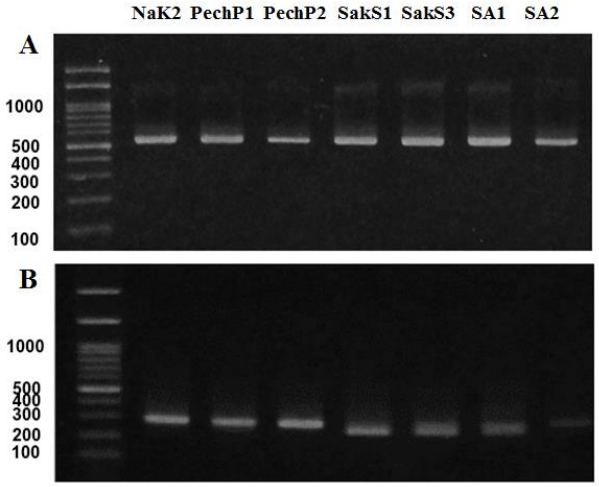

Figure 2. Sizes of PCR products. (A) *rbc*L fragments and (B) *ITS* fragments. The *ITS* region of the Nak3 sample could be not amplified.

maximum parsimony analysis of taxa (Figure 4, MP). Moreover, the sequences of *rbc*L and *ITS* were combined, and used for molecular phylogenetic analysis (Figure 5). Maximum likelihood and maximum parsimony analysis confirmed that genetic distance of all collected samples (box) were close to *C. barbata* (marked by asterisk). The bootstrap values was low for the chloroplast *rbc*L gene, 44% for maximum likelihood tree and 36-52% for maximum parsimony tree (Figure 3), while *ITS* showed high bootstrap values, 76-86% for maximum likelihood tree and 64-93% for maximum parsimony tree (Figure 4). The phylogenetic analysis of combined *rbc*L/ *ITS* showed high bootstrap values, 90-100% for maximum likelihood tree and 86-99% for maximum parsimony tree (Figure 5). Because Menispermaceae flowering

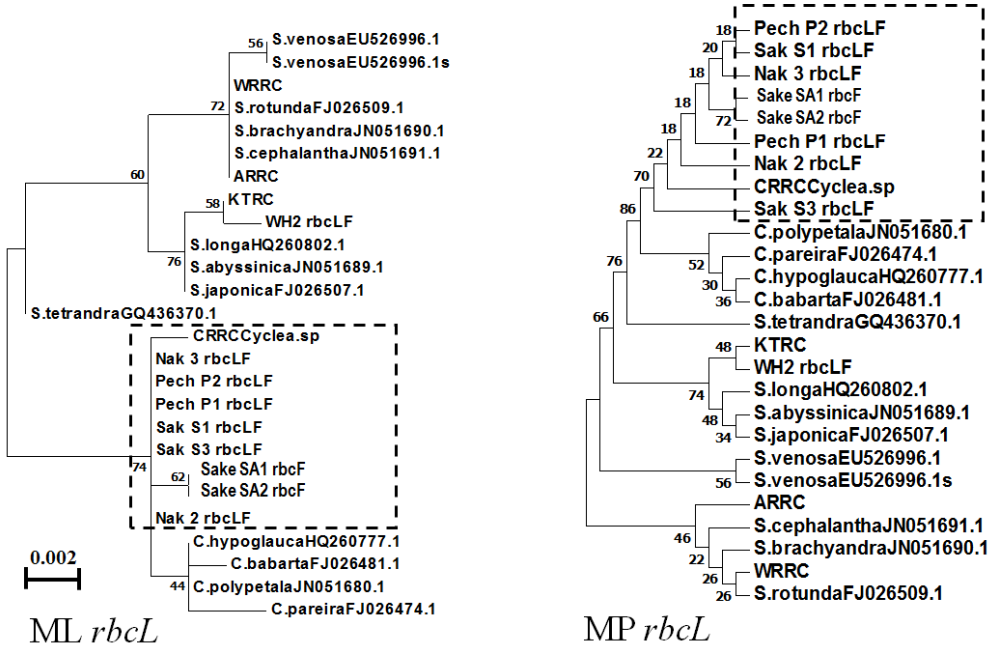

Figure 3. Molecular phylogenetic analysis of the *rbc*L region. The 13 *rbc*L sequences were obtained from all samples namely Nak_3, Nak_2, Pech_P1, Pech_P2, Sak_S1, Sak_S3, Sake_SA1, Sake_SA2, WH2, ARRC, WRRC, KTRC, and CRCC. The maximum likelihood tree (ML) from seven-locus DNA barcode alignment (*rbc*L) was performed with the highest likelihood (-775.44), and branch lengths were proportion of the number of substitutions per site. Maximum parsimony analysis of the parsimonious tree (length = 194) was performed. Asterisk was DNA of *Cyclea barbata* from GenBank.

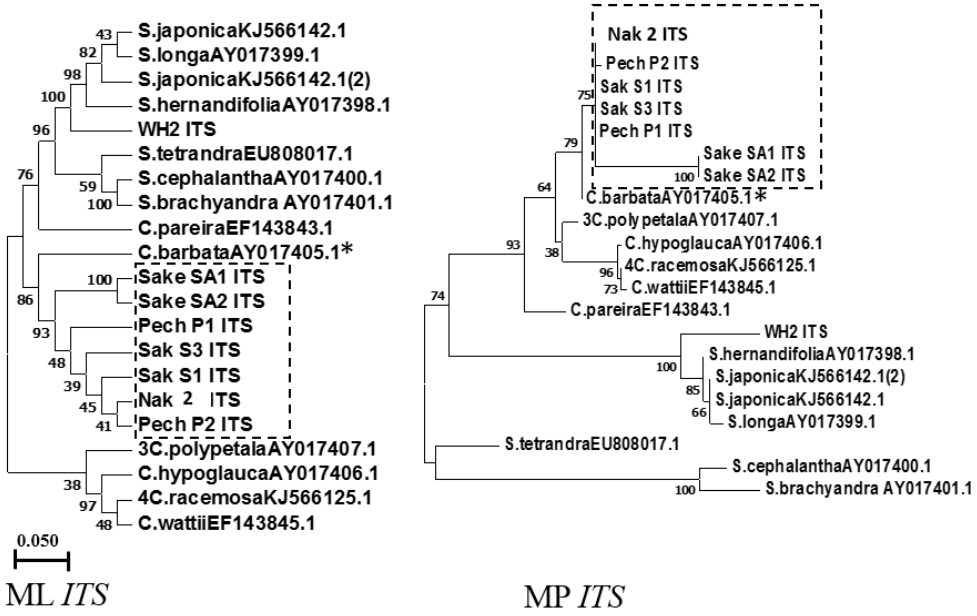

Figure 4. Molecular phylogenetic analysis of the *ITS* region. Because the *ITS* region could not be amplified by using WRRC, KTRC, CRCC and Nak_3 DNA templates, these samples did not provide *ITS* data. The *ITS* sequences were obtained from some samples namely Nak_2, Pech_P1, Pech_P2, Sak_S1, Sak_S3, Sake_SA1 and Sake_SA2. The maximum likelihood tree (ML) from seven-locus DNA barcode alignment (*ITS*) was performed with the highest likelihood (-1102.71), and branch length was proportion of the number of substitutions per site. Maximum parsimony analysis of the parsimonious tree (length = 163) was shown. Asterisk was DNA of *C. barbata* from GenBank.

morphology is commonly used for species identification, time is required for sample collection and searching for flowering plants. In addition, commercial products of *Cyclea sp.* such as dry leaf or its powder cannot be identified. The analysis of the partial *rbc*L and *ITS* sequences showed that the 500 nucleotides of *rcb*L region were many conserved. Therefore,

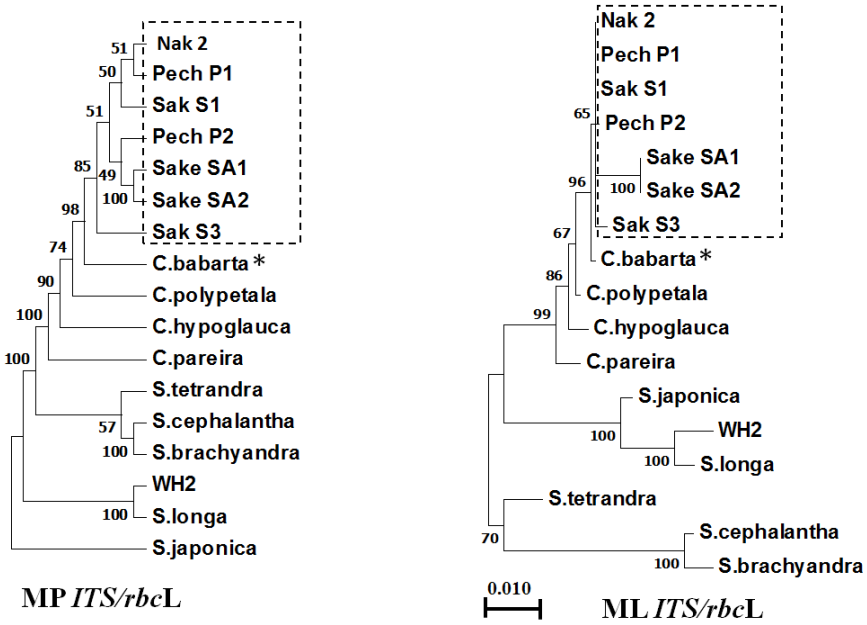

Figure 5. Molecular phylogenetic analysis of the *ITS* + *rbc*L region. Because the *ITS* region could not be amplified by using WRRC, KTRC, CRCC and Nak_3 DNA templates, these samples did not provide *ITS* data. The maximum likelihood tree (ML) from seven-locus DNA barcode alignment (*rbc*L+ *ITS*) was performed with the highest likelihood (-1884.52), and branch length was proportion of the number of substitutions per site. Maximum parsimony analysis of the parsimonious tree (length = 150) was shown. Asterisk was DNA of *C. barbata* from GenBank.

they could be used for genus level, but they were not useful for species identification. While the 277 nucleotides of the *ITS* region could indicate all *Cyclea* DNA as *C. barbata*. Then, the short *ITS* region could be used for identification of *C. barbata*, combined with morphological identification.

The phylogenetic studies have been reported that *rbc*L is best suitable to reconstruct relationship in genetic level, but it is not useful for species levels (Chase *et al.*, 2007). In addition, several reports have been reported some limited data of the *rbc*L gene for species differentiation of plants, the *rbc*L gene is more efficiency in lower than higher plants (Dong *et al.*, 2014), and cpDNA haplotype sharing is found among closely related species (Acosta & Premoli, 2010). However, the report of Hollingsworth, Graham, and Little (2011) shows that the ITS-2 region has efficiency in plant discrimination. The success rate of identification with the ITS region is 92.7% at species level (Hollingsworth *et al.*, 2011). Therefore, the *ITS* gene is the best suitable region for DNA barcoding application. Currently, the *ITS* region shows highly effective as a phylogenetic marker for both plants and animals (Chen *et al.*, 2010). For Menispermeae plants, previous reports show that the *ITS* data are consistent with morphology namely cotyledon, stylar scar, the type and shape of aperture, features of colpal membrane, and the number of sepals in male flowers (Wang *et al.*, 2007). Our studies showed that the short *ITS* sequences were similar to those of *C. barbata* with showing identity value at high level, and were consistent with morphological identification. These results were confirmed by genetic distance and phylogenetic analysis. For sequence alignment, the consistency index of the *rbc*L region (0.800) was higher than the value of the *ITS* region (0.745). It indicated that the *rbc*L sequence was homoplasy below those of the *ITS* sequence. In addition, phylogenetic analysis from the *mat*K gene and *trn*L-F regions, and the nuclear *ITS* region of Menispermeae plants have been reported by Wang *et al.* (2007). The monophyly of *Stephaniinae* and *Cissampelinae* in the Menispermeae plant is confirmed by cpDNA and *ITS* sequences, and by the morphological characters (Wang *et al.*, 2007).

Finally, our study showed that phylogenetic analysis of the *ITS* region related to morphological characteristics, therefore, the short *ITS* sequence (277 bp) could be used to distinguish the samples at species level and 8 samples were close to *C. barbata*. These results were consistent with morphological identification.

4. Conclusions

Plant morphology varies with its environment factors and habitats, therefore, the morphological identification may confuse. Importantly, same plant species may contain different pharmacological activities. Therefore, DNA barcode is an important tool for identification of several medicinal plants. Here we showed the useful of the short *ITS* region (277 nucleotides) that was able to identify *C. barbata*.

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