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Short Communication: Phylogenetic analysis and molecular identification of Canar (*Smilax* spp.) in Java, Indonesia Based on DNA Barcoding Analysis

LULUT DWI SULISTYANINGSIH[1,2,♥], ABINAWANTO[1,♥♥], MARLINA ARDIYANI[2,] ANDI SALAMAH[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Kampus UI Gedung E Level 2, Jl. Lingkar Kampus Raya, Pondok Cina, Beji, Depok 16424, West Java, Indonesia. Tel./Fax. +62-21-7270163, ♥♥email: abinawanto.ms@sci.ui.ac.id

[2]Herbarium Bogoriense, Botany Division, Research Center for Biologi, Indonesian Institut of Sciences. Cibinong Science Centre, Jl. Raya Jakarta-Bogor Km. 46, Cibinong-Bogor 16911, West Java, Indonesia, ♥email: lulutjv@gmail.com

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Abstract. *Sulistyaningsih LD, Abinawanto, Ardiyani M, Salamah A. 2018. Short Communication: Phylogenetic analysis and molecular identification of Canar (Smilax spp.) in Java, Indonesia Based on DNA Barcoding Analysis. Biodiversitas 19: 364-368. Smilax* spp. (Smilacaceae) has long been used as medicinal herbs especially in East Asia and North America as they were known to be rich in steroidal saponin. Pharmacological study has been carried out in Indonesia. This genus is widespread in Indonesia and fairly abundant in Java and has been known either as edible fruit or medicinal plants. Characteristics of *Smilax* as a dioecious plant with high morphological variations make it thorny in species identification. Various molecular approaches have been devised to overcome identification problems such as DNA barcoding. This study, therefore was conducted to analyze the DNA barcoding application for phylogenetic and identification of *Smilax* in Java. A total of 31 samples were used in this study including 19 accession numbers from NCBI GeneBank. The genus *Ripogonum* was used as the out-group in phylogenetic reconstruction. Samples were successfully extracted by CTAB method with some modifications. *rbc*L region was used as the DNA barcode showed sufficient variation and conserved flanks. Two unidentified specimens have high similarity with *S. leucophyla* and lies in the same clade. The phylogenetic tree constructed by Maximum Likelihood analysis. The result showed that the monophyletic of Smilacaceae consisted of four clades. The genus *Heterosmilax* nested with *Smilax* though with low bootstraps value. It supports the monogeneric status of Smilacaceae.

Keywords: DNA barcode, Java, *Smilax*, *rbc*L

INTRODUCTION

As a member of mega biodiversity countries, Indonesia has bio resources that have comparative advantages as food, health, and energy resources (Sumardja 1998). It was supported by the traditional knowledge of the local people which cause Indonesia to become one of the destination countries for bio-prospecting agents (Suwena 2006). Bio-prospecting is the exploration of biological material for commercially valuable genetic and biochemical properties (Posey 1997). One of the targets taxa of bio-prospecting is *Smilax* species (Suwena 2006).

The genus *Smilax* L. belong to the family Smilacaceae Vent. which are included in the order Liliales (Takhtajan 1997, Heywood et al. 2007, APG IV 2016). The genus *Smilax* produces rhizomes that are utilize in traditional medicine and beer brewing, while the stems are used in crafts. The roots were exported widely from the Neo-tropics for use in the treatment of syphilis. It was not clear which species of *Smilax* contain the active components since the phenotypic plasticity of the species in the Neo-tropics (Ferrufino-Acosta 2010).

The genus *Smilax* comprising *ca*. 300 species of mostly tropical and sub-tropical distribution. Backer and Bakhuizen v.d. Brink (1968), as well as Ungson and Sastrapradja (1976), recorded six *Smilax* housed in Java, i.e., *S. leucophylla*, *S. macrocarpa*, *S. modesta*, *S. odoratissima*, *S. perfoliata* and *S. zeylanica.* Whereas, World Checklist Smilacaceae of Java (Govaert et al. 2017) recorded 12 species, i.e. *S. blumei*, *S. gigantocarpa*, *S. javensis*, *S. klotzschii*, *S. leucophylla*, *S. luzonensis*, *S. macrocarpa*, *S. micrantha*, *S. modesta*, *S. nageliana*, *S. odoratissima* and *S. zeylanica*. *Smilax macrocarpa* knew as "canar" or "anggur hutan" bears; edible fruits which can be eaten fresh and tastier when sweetened or pickled known as "asinan". The young shoots of *S. leucophylla* are consumed by the local people (personal communication). The other *Smilax* species play a role as a remedy of gonorrhoea, syphilis, rheumatism, abscesses and also in the manufacture of steroidal hormones (Teo 1999).

Taxonomically, *Smilax* is difficult genus as the plants are dioecious, show wide phenotypic variation and many herbarium specimens were lack flowers or fruits that make them difficult to identify. Ungson and Sastrapradja (1976) conducted study to determine morphological, phytochemical and cytological variations *Smilax* species of Java. They found variation in morphological characters, phenolic and saponin spots numbers and chromosome length but not in chromosome number. Chen et al. (2006) conducted a study of the pollen morphology of *Smilax* but did not find sufficient variation to discriminate between species. Therefore, the development of DNA-based markers has been important for the authentication of plants especially for medicinal plants, one of which is DNA

barcoding (Techen et al. 2014).

DNA barcoding is a novel technique of identifying biological specimens, which uses short DNA sequences from either nuclear or organelle genome (Hebert et al. 2003, Techen et al. 2014). This technique has been successful in animal identification at the species level and has been used in determining species boundaries, identifying new species and species discrimination (Hebert et al. 2003, 2004; Techen et al. 2014). DNA barcoding widely applied in plant studies immediately. The plastid locus most commonly sequenced by plant systematist for phylogenetic purposes is *rbc*L (1,5-bisphosphate carboxylase/oxygenase) (Shaw et al. 2005). The *rbc*L region has been suggested as a candidate for plant barcoding, even though it has been used to determine evolutionary relationships at the generic level and above (Kress et al. 2005). Therefore, the aims of this study were to determine the phylogenetic relationship between species by *rbc*L region and to evaluate *rbc*L region as molecular identification in *Smilax* spp. in Java.

MATERIALS AND METHODS

Materials

A total of 25 *Smilax*, two *Heterosmilax* and four *Ripogonum* samples were used in this study. The tissue material for DNA extraction was obtained from field studies in Mt. Halimun-Salak National Park, Mt. Gede-Pangrango National Park, Cidaun-Cianjur, West Java and also from Purwodadi Botanic Garden (as Culta), East Java, Indonesia. A total of 20 samples were obtained from NCBI GeneBank (Table 1). Sample's names were checked for synonyms and possible misidentifications, which then led to new names for four samples obtained from NCBI GeneBank and one sample probably misidentification (Table 2).

The 25 *Smilax* samples represented 10 species and five unidentified samples, which majority collected from Java, Indonesia. The two *Heterosmilax,* consist of one sample collected from field study in Cidaun-Cianjur, West Java, Indonesia and another one was obtained from NCBI GeneBank. Four *Ripogonum* samples represented two species were included as out-group for the phylogenetic analysis.

Procedures

DNA extraction and amplification

Total DNA was extracted from silica-gel dried leaves by modification of CTAB method (Doyle & Doyle 1987). Amplification of *rbc*L regions performed using 96-well thermal cycler. The PCR process was started with heat shock at 95^{o}C for 3 minutes. Three steps amplification process were done for 35 cycles. DNA was denaturized at 95^{o}C for 30 seconds. Annealing at 55^{o}C for 30 seconds. DNA extension was done at 72^{o}C for 60-90 seconds. The final extension was performed at 72^{o}C for 7 minutes. After 35 cycles, the profile was linked on hold at 4^{0}C. The PCR products were electrophoresed using 1% agarose gel to check the presence or absence of bands.

Table 1. List of samples included in this study

Taxon	Location	Habit	No of specimens
Smilax aspera	N/A	Climbing shrub	2
Smilax blumei	Cibodas, West Java, Indonesia	Climbing shrub	2
Smilax excelsa	Culta, Indonesia	Climbing shrub	1
Smilax lanceifolia	Vietnam	Climbing shrub	2
Smilax leucophylla	Java, Indonesia	Climbing shrub	4
Smilax leucophylla	N/A	Climbing shrub	2
Smilax macrocarpa	Java, Indonesia	Climbing shrub	1
Smilax megacarpa	Cibodas, Indonesia	Climbing shrub	1
Smilax ocreata	N/A	Climbing shrub	1
Smilax setosa	N/A	Climbing shrub	1
Smilax zeylanica	Cibodas, Java, Indonesia	Climbing shrub	1
Smilax zeylanica	N/A	Climbing shrub	2
Heterosmilax micrantha	Cidaun, Java, Indonesia	Climbing shrub	1
Heterosmilax japonica	Zhejiang, China	Climbing shrub	1
Ripogonum album	Australia	Climbing shrub	1
Ripogonum album	N/A	Climbing shrub	1
Ripogonum elseyanum	N/A	Climbing shrub	2

Table 2. List of samples obtained from NCBI GeneBank which have validated in this study

Accession number	Location	Species label	Species validation
JF9444427	Vietnam	*Smilax perfoliata*	*Smilax blumei*
JF9444422	Vietnam	*Smilax perfoliata*	*Smilax blumei*
JF944371	N/A	*Smilax glyciphylla*	*Smilax leucophylla*
KF4967685	N/A	*Smilax glyciphylla*	*Smilax leucophylla*
JF956433	Cibodas, Java, Indonesia	*Smilax megacarpa*	Missidentification?

DNA purification and sequencing

The purification and sequencing process was performed by a company, FirstBase-Singapore. Data sequences were edited by using ChromasPro programme (Technelysium Pty, Ltd).

Phylogenetic analysis

The phylogenetic analysis based on the *rbc*L sequences was performed using MEGA6 program (Kimura 1980, Tamura et al. 2013). The phylogenetic tree was constructed using the neighbor-joining (NJ) method with the Kimura-2-Parameter (K2P) model, pair wise deletion for gaps/ missing data. Clade support value was obtained by using bootstrap. Bootstrap support (BS) was categorized as strong (> 85%), moderate (70%-85%), weak (50%-69%) or poor (< 50%) (Kress et al. 2002).

RESULTS AND DISCUSSION

Nucleotide sequence of *rbc*L region and variation within species

The *rbc*L sequences region proven valuable information for phylogenetic relationship in Angiosperms since the *rbc*L gene which is present in the cpDNA of all photosynthetic eukaryotes has been sequenced extensively (Soltis et al. 1990, Chase et al. 1993). At this point, the variation on *rbc*L sequence regions was analyzed to determine the phylogenetic relationship of Smilacaceae in Java.

All samples were successfully sequenced with *rbc*L region. The length of *rbc*L region of *Smilax* used as in-group in this study was range from 615 to 814 bp, whereas the out-group sequences range from 552 to 1443 bp (Table 3). The *rbc*L region consists of the highly conserved region as much as 42,85%, the variable site 10,10% and the parsimony-informative only 4,54%. It was showed that *rbc*L region is conserved and can use as the universal primer for plant DNA barcoding.

Phylogenetic analyses and molecular identification

Sequence homology of the amplified sequences was detected using Basic Local Alignment Tool (BLAST). BLAST is a program which can detect sequence similarity between query sequence and sequences database. Sequence similarity searching is one of the first steps in any analysis of newly determined sequences (Pearson 2013). A total of 11 samples were searched for the sequence homologies and similarities. The sequence homologies of the unidentified specimens in this study were 99% (Table 4). All unidentified specimens have highly similarity with *S. aspera*. *Smilax aspera* is widely distributed from Micronesia, Mediterranean to Myanmar and South Tropical Africa but it has never been found in Indonesia.

Table 3. Sequence length variation of *rbc*L region within out-group and in-group

No		Species	No. Acc./ no. Coll.	Sequence length (bp)
Out-group	1	*Ripogonum album*	KF496404	552
	2	*Ripogonum album*	KU304150	1443
	3	*Ripogonum elseyanum*	AY465709	1310
	4	*Ripogonum elseyanum*	KC899483	745
In-Group	5	*Heterosmilax japonica*	JF976612	814
	6	*Heterosmilax micrantha*	DG2152	755
	7	*Smilax aspera*	KJ719863	716
	8	*Smilax aspera*	KJ719881	716
	9	*Smilax blumei*	JF944422	615
	10	*Smilax blumei*	JF944427	615
	11	*Smilax exelsa*	S1	753
	12	*Smilax lanceiofolia*	JF944382	615
	13	*Smilax lanceifolia*	JF944385	615
	14	*Smilax leucophylla*	DG2207	776
	15	*Smilax leucophylla*	DG2208	751
	16	*Smilax leucophylla*	DG2209	772
	17	*Smilax leucophylla*	LDS360	752
	18	*Smilax leucophylla*	JF944371	615
	19	*Smilax leucophylla*	KF496785	566
	20	*Smilax macrocarpa*	LDS305	779
	21	*Smilax ocreata*	KF181486	570
	22	*Smilax zeylanica*	JF944438	615
	23	*Smilax zeylanica*	JF944440	615
	24	*Smilax zeylanica*	JF956478	756
	25	*Smilax ovalifolia*	JF944420	615
	26	*Smilax setosa*	JF944312	615
	27	*Smilax* sp. 1	LDS355	752
	28	*Smilax* sp. 2	KBA4	770
	29	*Smilax* sp. 3	AK3065	772
	30	*Smilax* sp. 4	LDS200	754
	31	*Smilax* sp. 5	LDS359	768

Table 4. Statistical simulation of BLAST sequence homology of unidentified specimens with *rbc*L region

Unidentified specimen	ID species	BLAST similarity (%)	Sequence cover (%)	E-value (BLAST)
Smilax sp. 1	*Smilax aspera*	99	95	0
Smilax sp. 2	*Smilax aspera*	99	96	0
Smilax sp. 3	*Smilax aspera*	99	95	0
Smilax sp. 4	*Smilax aspera*	99	96	0
Smilax sp. 5	*Smilax aspera*	99	95	0

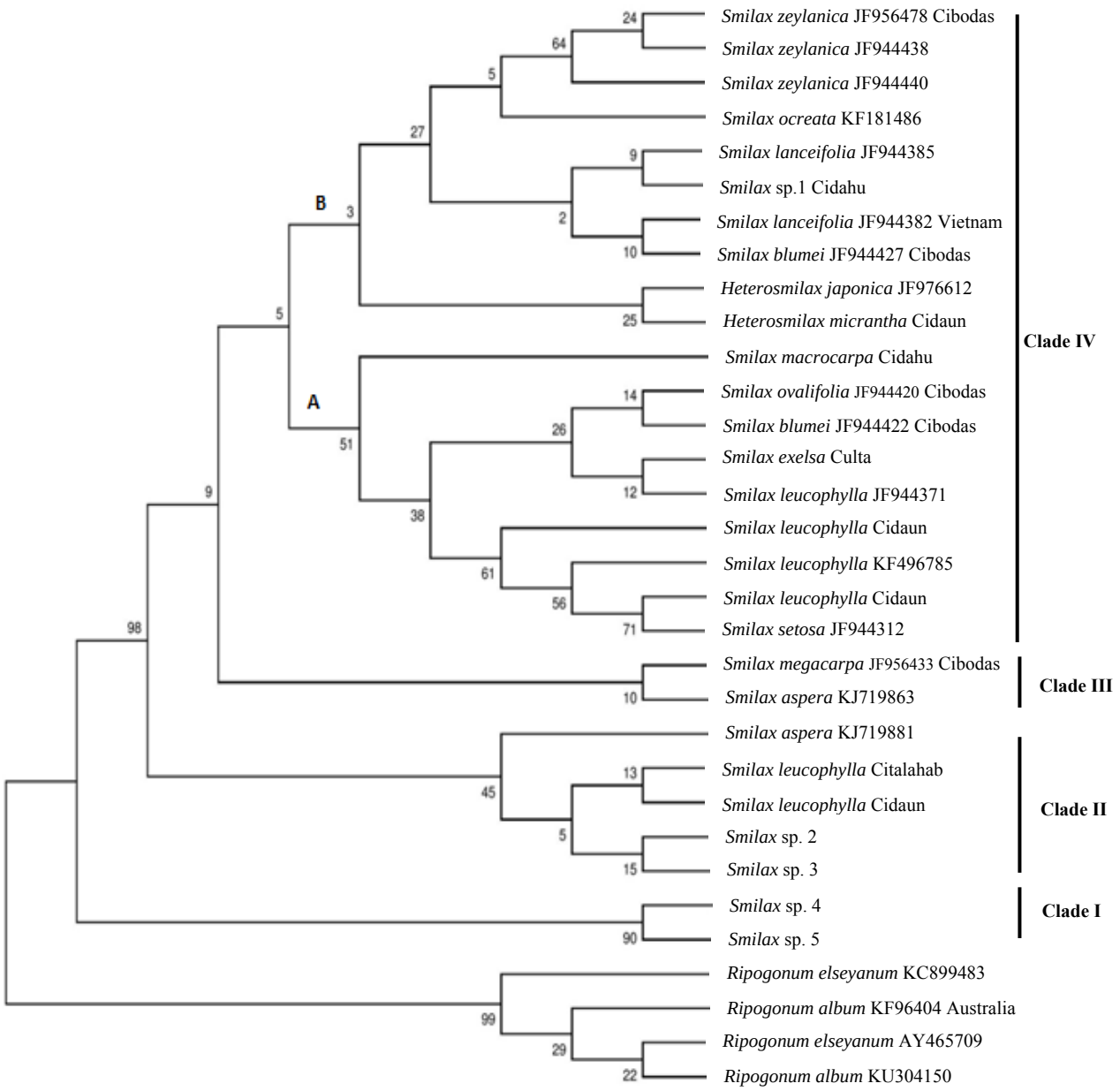

Figure 1. Neighbour-joining tree of *Smilax* based on *rbc*L barcode, including test *Smilax* sp. specimens

The phylogenetic trees was constructed using NJ method. The separation support of the in-group and out-group is strengthened (BS=99%). NJ analysis showed the in-group was separated into four clades. The two unidentified specimens, *Smilax* sp. 4 and *Smilax* sp. 5 were separated from the other taxa and placed in clade I with

strong bootstrap support (BS=90%). Clade II consisted of *S. aspera*, *S. leucophylla*, and the two others unidentified specimens (*Smilax* sp. 2 and *Smilax* sp. 3) with weak bootstrap support (45%). Clade III consisted of *S. aspera* and specimen obtained from NCBI GeneBank which identified as *S. megacarpa* collected from Cibodas. This specimen probably mislabel since *S. megacarpa* distributed in Assam to S. China and West & Central Malesia (WCSP 2017). Clade III act as the basal lineage to clade IV which consisted of two sub clade. *Smilax macrocarpa*, *S. ovalifolia* (probably mislabel), *S. blumei*, *S. exelsa*, *S. leucophylla* and *S. setosa* grouped in sub clade A. While, *S. zeylanica*, *S. ocreata*, *S. lanceifolia*, *Smilax* sp. 1, *S. blumei*, *Heterosmilax japonica* and *H. micrantha* grouped in sub clade B (Fig. 1).

The sequence variation from reference sequence and phylogenetic reconstruction is the basic principle for species identification in plants (Altschue et al. 1997). Based on the phylogenetic reconstruction, Smilacaceae is monophyletic with strong bootstrapping (BP = 99%). The genus *Heterosmilax* was nested with the genus *Smilax*. It supported the monogeneric of Smilacaceae (family containing only the genus *Smilax*), even though with low bootstrapping. The unidentified specimen, *Smilax* sp. 1 lies on clade IV subclade B in the midst of *S. lanceifolia* and *S. blumei* with low bootstrapping. Whereas *Smilax* sp. 2 and *Smilax* sp. 3 lies on clade II in the midst of *S. leucophylla* and *S. aspera*. The two last unidentified specimens, *Smilax* sp. 4 dan *Smilax* sp. 5 separated from the others on clade I with strong bootstrapping.

Our results suggests that the use of universal primer, *rbc*L for DNA barcoding is successful for amplification, identification and discrimination on genera level but not in the species level. Therefore the use of pair combination of DNA barcodes, such as *rbc*L + *mat*K were required for identification and discrimination on species level. The utilization of next-generation sequencing which is focusing on more conserved regions would be powerful for plant identification, especially for medicinal plants (Schindel & Miller 2005).

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