ITSF FINAL REPORT

Development of Random Amplified Polymorphic DNA Method for Identity Assessment of *Piper betle* L., as a Starting Material for Herbal Medicine Product

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CHAPTER I
INTRODUCTION

Herbal medicine had long been used for disease prevention and therapy in Indonesia and are becoming increasingly popular in the world. Correct identification and quality assurance of the starting material is an essential prerequisite in herbal medicine to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy. Identification at species-, strain- and locality-levels, therefore is required for quality assurance/control of herbal medicine (Joshi *et al*., 2004; Yip *et al*., 2007; Yadav and Dixit, 2008).

Locality-level identification is great importance to ensure highest therapeutic effectiveness. Herbal medicine materials cultivated in different localities might differ in therapeutic effectiveness. Samples from the same localities are probably of the same strains, therefore, origin identification helps select the best strains of herbal medicine materials (Yip and Kwan, 2006; Yip *et al*., 2007).

Identification of plants at the species level is traditionally achieved by careful examination of the specimen's macroscopic and microscopic morphology. However, morphological identification is often not possible when the original plant material has been processed (Sucher and Carles, 2008).

One of the most reliable methods for identification of herbal medicine materials is by analyzing the DNA. DNA markers are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological conditions as well as environmental factors. DNA analysis methods can be classified into three types, namely polymerase chain reaction (PCR)-based, hybridization-based and sequencing-based (Joshi *et al*., 2004; Yip *et al*., 2007).

Techniques based on the PCR concept include random amplified polymorphic DNA (RAPD), which usually uses a 10 bp arbitrary primer at constant low annealing temperature (generally 34 - 37ºC). The resulting PCR products are generally resolved on 1.5 – 2.0 % agarose gels and stained with ethidium bromide (EtBr). RAPD is a quick and easy method to screen a large number of loci for DNA polymorphisms in a single PCR. Polymorphic markers can be generated rapidly without sequence information.(Weising *et al*., 2005; Semagn *et al*., 2006).

This method had been used to identify herbal medicines' geographical origins, such as *Hordeum spontaneum* (Volis *et al*., 2001), *Glycine max* (Chowdhury *et al*., 2002), *Pinellia ternata* (Chung *et al*., 2002), *Piper sp*. (Wadt *et al*., 2004), *Costus speciosus* Koen ex. Retz. (Mandal *et al*., 2007).

In this research project, we will perform development of RAPD method for DNA polymorphisms among many varieties and cultivars of betelvine (*Piper betle* L.) The source of DNA template is not from the DNA genome, but from the DNA fragment which had been resulted from amplification of DNA genome by Internal Transcribed Spacer (ITS) primer for *Piper sp*. Such DNA template can be more specific and not too long and random so the RAPD primers can amplify more specific and limited sequence of DNA genome. This method development can increase the specifity and effectivity of amplification process so we can get the more specific molecular marker for identifying herbal medicine, specially *Piper betle* L.

The purpose of this research is mainly to develop the method for mapping the DNA fingerprint of herbal medicine (betelvine, *Piper betle* L.) using ITS region of betelvine nrDNA as DNA template by Random Amplified Polymorphic DNA, at locality levels. Therefore the developed method will be used for discriminating several cultivars of betelvine at Surabaya, Purwodadi and Batu.

This assessment method is not only to complement conventional parameters in creating the passport data of medicinal raw drugs, but also can be used for routine quality control in the herbal medicine industry and in commercial and government testing laboratories (National Food and Drug Control System). Routine use of DNA technology as a quality control tool will not be more expensive than conventional HPTLC/HPLC methods, once the protocol and markers are available.

In this research we use *betelvine*, as a model for representation of the relatively high market value of herbal medicine which is frequently used in industries of traditional medicine at Indonesia.

Betelvine (*Piper betle* L., Piperaceae) or betel leaf vine, betel leaf pepper, betel pepper, is a slender creeper with adventitious roos. Stems glabrous, sulcate, thickened at the nodes. Leaves alternate, heart-shaped, palmately nerved, glabrous and shining on both sides. Inflorescence in drooping, dense axillary spike, consisting of male and female flowers. Berry globose. All parts of the plant have a special aroma (WHO, 1990).

This plant was first cultivated at Malaysia and then its cultivation had been widespread to all tropic areas at Asia, include Indonesia (Rostiana *et al*., 1992).

A survey over several years indicated between 125 to 150 local cultivars of betelvines in India. On the basis of chemical constituent analysis of leaf essential oils, five prominent groups of betelvine cultivars, namely Bangla, Kapoori, Meetha, Sanchii and Desawari have been recognized. (Verma *et al*., 2004). According to Materia Medika Indonesia (1980) betelvine had four varieties, which were classified by morphological structure of plant.

Leaves of *Piper betle* are shown to possess antimicrobial, gastroprotective, wound healing, hepatoprotective, antioxidant, anti-fertility on male rats and antimotility effect on washed human spermatozoa. In Asian countries betel leaves are used for chewing and are credited with many medicinal properties (Arambewela *et al*., 2005).

The whole plant yields an essential oil containing eugenol, carvacrol, chavicol, allyl catechol, chavibetol, cineol, estragole, methyl – eugenol, p – cymene, caryophyllene and cadinene, tannins, sugars, carotene, thiamine, riboflavine, nicotinic acid, vitamin C and amino acids (WHO, 1990).

CHAPTER TWO
MATERIALS AND METHODS

2.1 Sampling Method

Betelvine (*Piper betle*) cultivars were collected from three locations with different geographic conditions (Surabaya, Purwodadi, Batu). Young leaf tissue was harvested, washed free of dirt, mopped dry and quickly frozen until used.

2.2 Total DNA Isolation

Genomic DNA isolation, modified from Rogers and Bendich which used by Chaveerach *et al*. (2002). Fresh leaves 1,0 g were ground and 500 µl of β-mercaptoethanol and 3500 µl of 2X CTAB solution (2% CTAB, 0.1 M Tris-HCl, 1.4 M NaCl) were added. The mixture was incubated at 55 ºC for 40 min with occasional inversion and then cooled at room temperature and further incubated at 55 ºC for 15 min. A 3500 µl of chloroform : isoamyl alcohol (24:1 v/v) was added, incubated at room temperature for 30 min and then shaken for 10 min. After centrifugation at 4500 rpm at 4 ºC for 45 min, 500 µl of the upper phase was subsequently mixed with 0.1 volume of 10 % CTAB solution and equal volume of ppt buffer, then the DNA-CTAB complex was formed at room temperature for 15 min. The mixture was centrifuged at 15,000 rpm at 4 ºC for 15min and DNA pellet was mixed with 500 µl of 1 M NaCl-TE and incubated at 55 ºC until DNA dissolved. Isopropanol 500 µl was added in this mixture. After centrifugation 15,000 rpm at 4 ºC for 15min, DNA pellet was washed once with 600 µl of 70 % ethanol and DNA was dissolved in 35 µl water free nuclease and kept at – 20 ºC until used.

2.3 PCR with ITS primers

The isolated DNA, was then be amplified with primer ITS Y-5 (5’ TAGAGGAAG GAGAAGTCGTAACAA 3’) and ITS Y-4 (5’ CCCGCCTGACCTGGGGTCGC 3’) using the method of Ito *et al*. (2000). The reaction was pre-denaturated at 95 ºC for 2 min, cycled 35 times at 95 ºC for 30 sec, 57 ºC for 1 min and 71 ºC for 2 min in a thermocycler. The final extension cycle allowed an additional incubation for 5 min at 71 ºC (Asmarayani and Pancoro, 2005)

2.4 PCR with RAPD primers

The amplification reaction, was modified from Verma *et al*. (2004), while the mixture was contained 12,5 µl GoTaq® Green Master Mix (Promega) which contain GoTaq® DNA polymerase supplied in 2X Green GoTaq® Reaction buffer (pH 8,5), 400 µM dATP. 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$.; 3,5 µl RAPD primer and 9 µl PCR result from PCR with ITS primers method ('betelvine-ITS' DNA template) in 25 µl reaction volume. The mixture was cycled 44 times at 94 ºC for 1 min, 35 ºC for 1,5 min and 72 ºC for 1,5 min in a thermocycler. The final extension cycle allowed an additional incubation for 5 min at 72 ºC.

2.5 Agarose Gel Electrophoresis

Amplification products were separated by electrophoresis through 2.0 % agarose gels in 0.5 X TBE buffer, visualized and imaged after staining with ethidium bromide.

CHAPTER III

RESULT AND DISCUSSION

3.1 Sampling

Betelvine cultivars were collected from some locations at East Java and the details are given in table 1.

Table 1. Sampling Condition of Betelvine from Several Locations

Parameter	ST	SB	SS	P	B
Temperature (ºC)	30.5 – 31.9	30.0– 30.1	24.1- 24.4	31.1– 32.9	30.5– 32.9
Humidity (%)	75 - 79	59 - 71	78 - 80	60 - 63	54 - 66
Colour of leaves	Yellowish green	Light – dark green	Light – dark green	Dark green	Yellowish green
Harvest frequency	infrequently	never	frequently	never	never
Specific character	Spadix (-)	Thick leaves	none	None	Yellow spot on the leaves
Height (m)	2	0,5	1	3	5
Fertilizer	none	none	none	compost	manure

ST= East Surabaya, SB = West Surabaya, SS = South Surabaya, P = Purwodadi, B = Batu

Samples from Surabaya city were obtained from three individual gardens but samples from Purwodadi and Batu were obtained from Botanical Garden, Purwodadi Botanical Garden - LIPI and Balai Materia Medika - Batu, respectively.

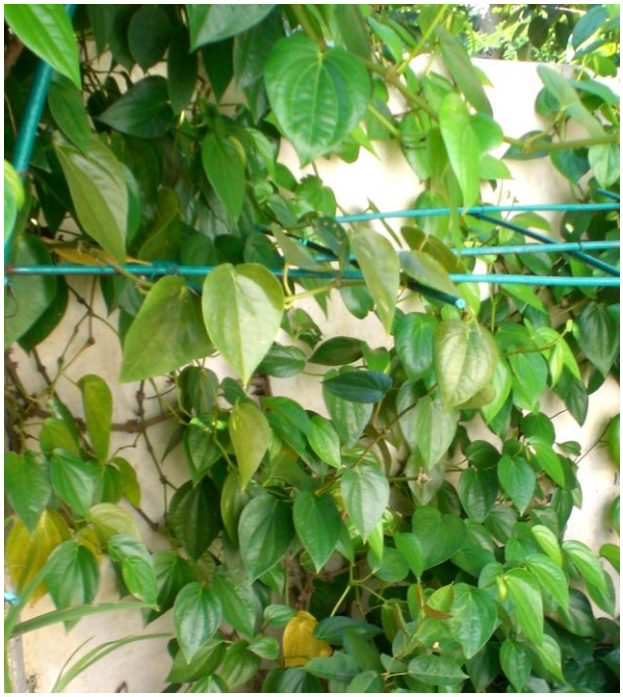

Figure 1. *Piper betle* at individual garden (East Surabaya)

Figure 2. *Piper betle* at Purwodadi Botanical Garden - LIPI

Figure 3. *Piper betle* at Balai Materia Medika, Batu

All plant samples were washed, sprayed with 70 % Ethanol, mopped dry and stored at -80 ºC during April – December 2009 while the color of all leaf samples had changed into light and dark brown because of the de-starch process happened during the storage. The change of color was not followed with the change of specific aroma of betelvine.

3.2 DNA Isolation

Genomic DNA from Betelvine cultivars was isolated using the Rogers and Bendich which was used by Chaveerach *et al*. (2002) for isolation the DNA of *Piper kadsura*, *Piper retrofractum* and *Piper chaba*. The modified method was performed in this research including the amount of fresh leaves and leaves stored at -80 ºC (0,2 – 1,0 g), incubation temperature (55 ºC), centrifugation rate, ethanol concentration and solvent for the isolated DNA.

The DNA purity and concentration of isolated DNA from fresh leaves and leaves stored at -80 ºC from many locations show many variations but amenable to PCR amplifications. In common, the isolated DNA from fresh leaves formed clearly pellet which could be suspended in the 70 % Ethanol in the washing steps, but the isolated DNA from leaves stored at -80 ºC, could not form the visible pellet. Drábková *et al*. (2002) showed that preparation effects of samples and template impurity were greater in plants stored that in fresh plants.

The isolated DNA of fresh leaves from ST, was used for optimization the amplification process with ITS primers and screening process of RAPD primers.

For amplification purpose, the amount of fresh leaves and leaves stored at -80 ºC for DNA isolation is about 1,0 g because this amount of plant materials would give large yields without decrease their DNA purity. Large yield is very important in RAPD method because this method will need many sample DNAs.

3.3 PCR with ITS Primers

ITS region of nrDNA is biparentally inheredited and it has proven to be a useful source of characters for phylogenetic studies in many angiosperm family because its enormous number of nrDNA repeat unit arranged in tandem repeats in plant genome. The presence of highly conserved sequences flanking each of two spacers, can make this region easy to be amplified (Alvarez and Wendel, 2003).

The isolated DNA of fresh leaves of betelvine, then was amplified with ITS primers by Polymerase Chain Reaction (PCR) Method. These primers amplify the entire ITS region

of betelvine and result one band (700 bp) on gel agarose after stained with ethidium bromide.

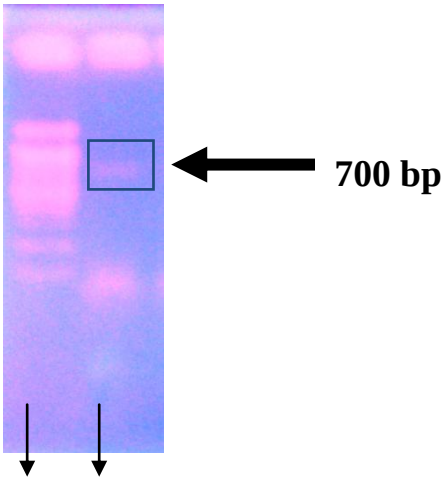

Figure 4. Ethidium bromide stained agarose gel of amplification result of betelvine genomic DNA with ITS primers (ITS Y-5 (5' TAGAGG AAGGAGAAGTCGTAACAA3') and ITS Y-4 (5'CCCGCCTGACCT GGGGTCGC 3'); M : Marker 100 bp ladder Intron Biotech. 7 µl; ITS-betel = amplification result of betelvine DNA with ITS primers

3.4 PCR – RAPD on ITS Region of Betelvine.

Betelvine DNA were tested with twenty decamer primers. Four primers (OPF-02, OPF-09,OPF-12, OPF-14) resulted clearly RAPD profiles from ITS region of betelvine, as shown on figure 2.

Examination of RAPD polymorphisms of the fresh leaf and leaves sample stored at -80 ºC, by the use of arbitrary primers, was accomplished by primer OPF-14. The results indicate that RAPD patterns among the samples were quite different.

There was one common DNA band (indicate with black-dashed arrow in figure 6.) which was assumed to be the specific DNA region for betelvine. For assuring this assumption, in the next research this band must be cut, purified and sequenced to obtain the specific DNA sequence.

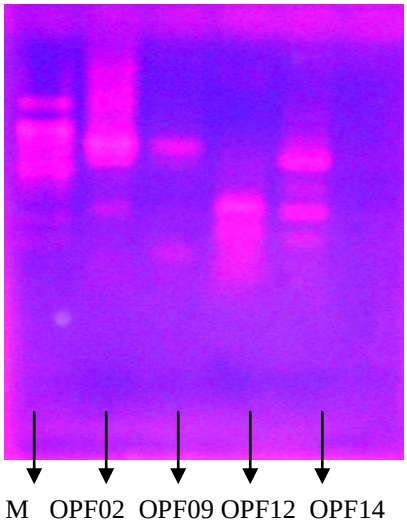

Figure 5. Ethidium bromide stained agarose gel of RAPD reaction with primer OPF-02, OPF-09, OPF-12, OPF-14 on ITS region of betelvine (*Piper betle* L.) DNA. M : Marker 100 bp ladder Intron Biotech. 7 µl; OPF-02 : 5' CAGGATCCCT 3'; OPF-09 : 5' CCAAGCTTCC 3'; OPF-12 : 5' ACGGTACCAG 3'; OPF-14 : 5' TGCTGCAGGT 3'

DNA pattern from PCR-RAPD on ITS region of betelvine show less amount of band than common DNA pattern from RAPD on DNA genome. This result could be happened because the template DNA which amplified was ITS region, with 700 bp, shorter than the length of the whole DNA genome.

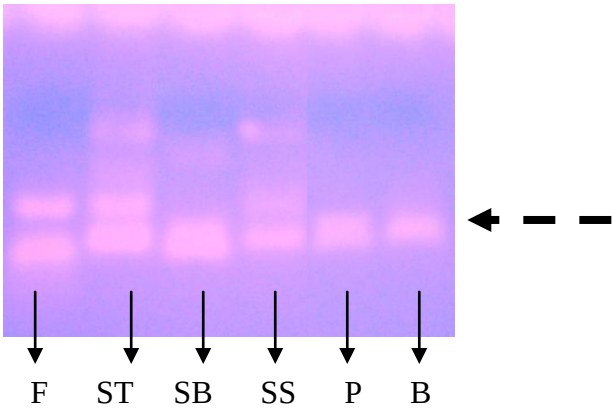

Figure 6. Ethidium bromide stained agarose gel of RAPD reaction with primer OPF-14 on ITS region of betelvine (*Piper betle* L.) DNA OPF-14 : 5' TGCTGCAGGT 3'; F = fresh leaves on East Surabaya (ST), SB = West Surabaya; SS = South Surabaya, P = Purwodadi, B = Batu

Our preliminary work had revealed that amplification the betelvine DNA with the RAPD primers directly often resulted the smear band (data not shown), but after amplification on the ITS region of betelvine, we could get the distinct and clear bands on

agarose gel after stained with ethidium bromide. We assumed that the betelvine DNA genome is too long and complex so that the RAPD primers could anneal to many sites at the DNA so that the yield of amplification is too low and it was shown with the smear band at agarose gel after electrophoresis method.

ST and SS had shown the similar bands than SB, this phenomenon was supported with the similar morphological conditions of ST and SS. P and B also had shown the similar bands each other but they had different DNA patterns if compared with ST, SS and SB (figure 3.)

We assume that betelvine is adapted to the location in different ways in response to geographical and climate features. This means that the same species can have genetic diversity in accordance with specific regions.

Asmarayani and Pancoro (2005) also showed that betelvine samples which were cultivated at Jogjakarta (Central Java) and Bogor (West Java), were linked each other with weak relationship based on ITS regions of nrDNA. This condition was assumed caused by widely domestication process in this species. Human interference in plant domestication results in botanical evolution diversity.

Furthermore, the distinctive bands obtained from RAPD reaction on ITS region of betelvine, will be possible to use as markers discriminating the geographic origin of betelvine.

CHAPTER IV
CONCLUSION

Quality control is one of the key issues in the modernization of Indonesian traditional medicine. The early investigation at this research is to find the accurate and fast system for controlling the quality of raw material on traditional medicine industry by developing RAPD method of betelvine from several locations.

This developed method using ITS region of betelvine nrDNA as a template could give specific and a few amount of DNA bands so these bands will be possible to use as markers discriminating several cultivars of betelvine at locality levels, between betelvines from Surabaya, Purwodadi and Batu.

For future development, it is necessary to compile a reference library of Indonesian medicines with genetic information, especially for endangered species and those with high market value.

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