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Short communication

Molecular Phylogenetic Screening of *Withania somnifera* Relative From Indonesia Based on Internal Transcribed Spacer Region

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ABSTRACT

Withania somnifera (family *Solanaceae*), known commonly as Ashwaganda, is one of the important medicinal plants, and recent studies reported that Withanone, one of the chemical components in this plant, has ability to kill cancer cell. Because of endemic state of this plant to South Asia, exploring plant species under the same family which grow well in Indonesia has been of interest. The purpose of this study was to screen the Indonesian plant which has strong phylogenetic relationship with Ashwaganda. Thus, phylogenetic analysis using DNA sequences of internal transcribed spacer (ITS) region was conducted. Thus, 19 species of *Solanaceae* and two species of *Convolvulaceae* as outgroup were examined. Five ITS regions of Ashwaganda retrieved from GenBank were included in the phylogenetic analysis. Parsimony analysis showed that Indonesia *Solanaceae* comprises seven groups which is consistent with the global Solanaceae relationship as previously reported. Furthermore, our study revealed that two species, *Physalis angulata* and *Physalis peruviana,* are relative to *W. somnifera.* Morphologically, they share characters of flower and fruit. This result indicated that these two species are potential to have similar chemical properties as Ashwaganda, thus we can have new variants of Withanone originated from Indonesia with similar effect.

1. Introduction

The use of plant as a medicine has been done many years ago by people around the world. It is likely because the direct use of plant is believed to be more effective and has little side effect than those of chemical drugs (Singh *et al.* 2010). Ashwaganda (*Withania somnifera*) which belongs to family *Solanaceae* is one of the important medicinal plants in the region of South Asia. Recent studies reported that Withanone, one of the metabolites in this plant, has anticancer activity shown by some evidence in which it can inhibit cancer cell proliferation and increases the sensitivity of cancer cell to radiotherapy and/or chemotherapy (Widodo *et al.* 2007, 2008).

Widodo *et al.* (2010) reported that the leaf extract of Ashwaganda could inhibit the growth of a variety of human cancer cells as Withanone serves as the cancer inhibitory factor (i-Factor) and not harmful to normal human cell. Withanone induces signaling pathway that involves in the cell cycle progression, such as p53 and p21 expression in cancer cells. The p53 plays an important role to regulate cell cycle progression by p21 as downstream in this process. Widodo *et al.* (2009, 2010) and Priyandoko *et al.* (2011) also found that i-Factor protected the normal human cells against the oxidative damage caused by ultraviolet and hydrogen peroxide. However, this plant is not available around the globe as its distribution is limited to India, Bangladesh, and Pakistan. Therefore, exploration is needed to find Ashwaganda relatives in other regions.

In Southeast Asia, especially Indonesia, cancer remains a major cause of mortality and morbidity (Moore 2014). So far, existing medicines have not provided satisfactory results. In this study, in accordance with the invention of Withanone, molecular phylogenetic analysis based on DNA sequences of internal transcribed spacer (ITS) region was conducted to screen the Indonesian plant which has strong phylogenetic relationship with Ashwaganda.

Molecular phylogenetic serves as a “tool” to understand and describe the diversity of organism and reconstruct evolutionary relationship using molecular data such as DNA, RNA, and protein. In this approach, a group of organisms that share many identical characters are considered to be closely related, deriving from a

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common ancestor (monophyletic) and are assumed to have similar genetic patterns and biochemical properties (Hidayat and Pancoro 2008). Thus, molecular phylogenetic provides a basic information to which a new variants of Withanone extracted from Indonesia plants might be established.

The ITS region is part of nuclear ribosomal DNA, consisting of ITS-1, 5.8S gene, and ITS-2. The region has been widely used in molecular phylogenetic analysis of plant because of its small size (±600 base pair), highly conserved flanks, high copy number, high mutation rate of ITS-1 and ITS-2, and rapid concerted evolution (Baldwin *et al.* 1995), making it easy to handle.

2. Materials and Methods

2.1. Plant materials

In total, 21 species (19 of family *Solanaceae* and 2 of *Convolvulaceae*) distributed in Bandung and its surrounding area were analyzed. Family *Convolvulaceae* was used as outgroup as this family has been recognized as sister group to *Solanaceae* (Olmstead *et al.* 2008). Detailed information on the plant species used can be seen in Table.

2.2. Amplification and sequencing

Total DNA was extracted from fresh materials using a GeneJET Plant Genomic Purification Mini Kit (Thermo Scientific, USA) following manufacturer's instructions. The amplification of ITS region was carried out using the primer pairs ITS-5 (5'-TAGAGGAAGGAGAAGTCGTAACAA-3') as forward and ITS-4 (5'-CCCGCCTGACCTGGGGTCGC-3') as reverse primer (Hidayat *et al.* 2008; Figure 1). The polymerase chain reaction profile consisted of an initial 2-minute premelt at 95°C and 35 cycles of 30 seconds at 95°C (denaturation), 2 minutes at 57°C (annealing), and 2 minutes at 72°C (extension), followed by a final 10 minute extension at 72°C. The polymerase chain reaction products were sent to Macrogen, South Korea, for DNA sequencing.

DNA sequences obtained were analyzed to test their homology using Basic Local Alignment Search Tool which is available online in GenBank Web site (www.ncbi.nlm.nih.gov/).

2.3. Phylogenetic analysis

DNA sequences of the ITS region obtained were aligned with Clustal X (Thompson *et al.* 1997) and were adjusted manually. In addition, the ITS sequences of five cultivars of *W. somnifera* retrieved from GenBank were included in the analysis. Phylogenetic tree reconstruction based on parsimony method was performed using PAUP* version 4.0b10 (Swofford 1998). Insertion and deletion were treated as missing data. All characters were equally weighted and unordered (Fitch 1971). The evaluation of the internal support of clades was conducted by bootstrap analysis (Felsenstein 1985) using 1,000 replicates. The number of steps, consistency indices, and retention indices were calculated using the TREE SCORE command in PAUP*.

Table. Plant materials examined in this study

Family	Plant species	Voucher	Local name
Solanaceae	*Physalis angulata*	TH01	*Ciplukan*
	Solanum nigrum	TH02	*Leunca/ Ranti*
	Solanum wrightii	TH03	*Karundung*
	Solanum lycopersicum	TH04	*Tomat*
	Solanum torvum	TH05	*Terung liar*
	Solanum melongena	TH06	*Terung ungu*
	Nicotiana tabacum	TH07	*Tembakau*
	Brugmansia suaveolens	TH08	*Kecubung jingga*
	Brugmansia candida	TH09	*Kecubung putih*
	Brunfelsia uniflora	TH10	*Melati air*
	Cestrum sp	TH11	*Arum dalu*
	Datura metel	TH12	*Kecubung ungu*
	Petunia grandiflora	TH13	*Petunia*
	Nicandra physalodes	TH14	*Nandina*
	Cestrum x cultum	TH15	*Sestrum*
	Capsicum annuum 01	TH16	*Cabai*
	Capsicum annuum 02	TH17	*Cabai*
	Physalis peruviana	TH18	*Ciplukan*
	Solanum tuberosum	TH19	*Kentang*
Convolvulaceae	*Ipomoea batatas*	TH20	*Ubi jalar*
	Ipomoea cairica	TH21	*Ubi kates*

3. Results

The Basic Local Alignment Search Tool analysis resulted in structure of ITS region from all species examined which match with family of flowering plant deposited in GenBank database. In addition, a conserved 14 bp motif (5'-GAATTGCAGAATCC-3') in the 5.8S gene was found. This motif is useful to differentiate between flowering plants and other plant groups such as fern, moses, fungus, and algae (Jobes and Thien 1997). The length of the ITS region of Indonesia *Solanaceae* ranged from 535 to 668 bp. The aligned ITS comprised 668 characters. Of these, 161 (24%) were constant, 80 (12%) were uninformative, and 427 (64%) were potentially informative. A total of two most parsimonious trees of the length 2869 steps were produced with consistency index of 0.541 and retention index of 0.595. Figure 2 shows one of the two most parsimonious trees.

The tree, as current circumscription, confirms that family *Solanaceae* is monophyletic group. The tree also demonstrated that Indonesia *Solanaceae* comprises seven groups that is consistent with the global *Solanaceae* relationship (Olmstead *et al.* 2008). Groups 1 to 7 correspond to tribe *Physaleae*, *Solaneae*, *Capsiceae*, *Datureae*, *Cestreae*, *Petunieae*, and *Nicotineae*, respectively (Figure 2). In addition, our study also supported classification system in the level of subfamily. While groups 1 to 4 belongs to subfamily *Solanoideae*, groups 5 and 6 are in *Cestroideae*, and group 7 falls in *Nicotianoideae*.

4. Discussion

Our results show that group 1 consists of two genera which are *Withania* and *Physalis*. This group shares similar character of enlarge persistent calyx (like balloon) that cover the fruit (Zhiyun *et al.* 1994). While groups 2, 5 and 7 house species of single genus *Solanum*, *Cestrum*, and *Nicotiana* respectively, group 3 comprises two genera *Capsicum* and *Nicandra*. Two genera which are *Datura* and *Brugmansia* are in group 4 that is supported by big trumpet like flower (Zhiyun *et al.* 1994; Olmstead *et al.* 2008). *Brunfelsia* and *Petunia* in group 6 share character of bowl like calyx (Zhiyun *et al.* 1994).

The phylogenetic tree obtained in this study revealed one interesting result. The position of tomato (*Solanum lycopersicum*) has been taxonomically debatable. Formerly, it was named scientifically as *Lycopersicum esculentum*, making genus *Solanum* as non-monophyletic group. Then, based upon their molecular phylogenetic analyses, Passarin *et al.* (2008) and Olmstead *et al.* (2008) proposed new name *Solanum lycopersicum* instead of *Lycopersicum esculentum* for tomato to maintain *Solanum* as monophyletic group. Again, this study supported their efforts.

Our results also show that the relative of *W. somnifera* (Ashwaganda) in Indonesia is *P. angulata* and *P. peruviana*, sharing morphological characters of their flower and fruit. According to the basic principle of molecular phylogenetic study mentioned earlier, these two species have the potential to be used as an alternative

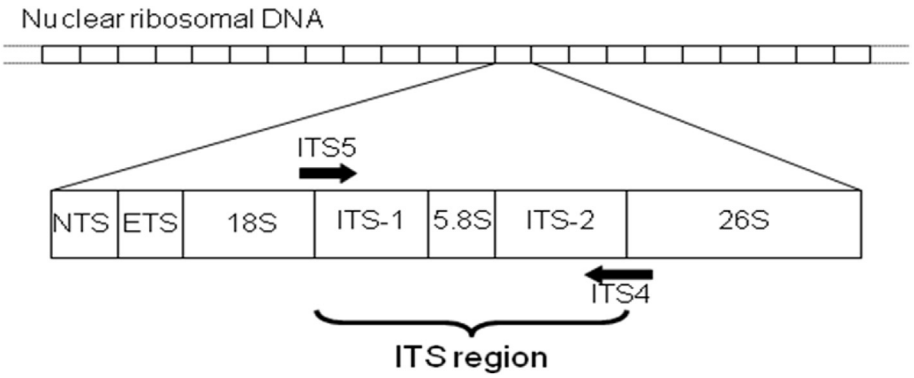

Figure 1. The internal transcribed spacer (ITS) region with location of primers used in this study.

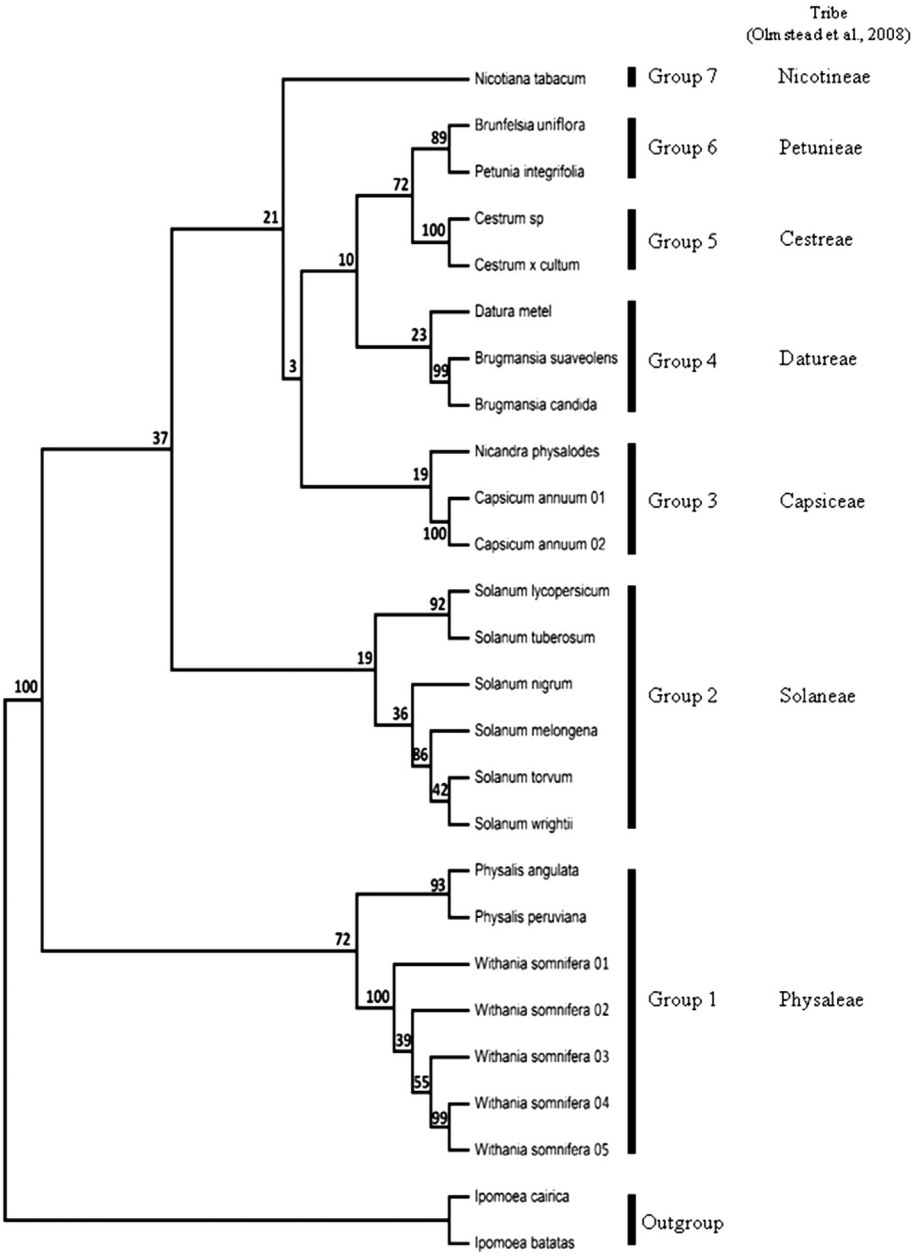

Figure 2. Phylogenetic tree of Indonesia *Solanaceae* based on the internal transcribed spacer region. Bootstrap values are shown above each branch.

medicinal plant of establishing new variant of Withanone from Indonesia which has anticancer activity.

Licodiedoff *et al.* (2013) reported that all parts of body of *P. angulata* and *P. peruviana* (local name: Ciplukan) contains saponin (in shoot), flavonoids (in leaf and shoot), phenols, physalin, tannin, cryptoxantine, ascorbic acid, sugar, Withangulatin A (in fruit), palmitate and stearat acid (in seed), alkaloid (in root), chlorogenic acid (in stem and leaf). With those chemical components, local people often use this plant traditionally in their daily lives to treat various diseases. Further studies dealing with the genetic variation of both plants originated from selected area in Indonesia, characterization of active chemical compound contained in these plants and bioassay are important in the future.

Conflict of interest statement

The authors do not have any conflict of interest regarding the publication.

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