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Application of DNA Barcoding to Authentic *Panax Vietnamensis*

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Abstract

Panax L. genus consists of 11 species and sub- species. It distribute in North America and in eastern Asia (mostly northeast China, Korea, Bhutan, eastern Siberia), typically in cooler climates. In Vietnam, up to now, currently five species of the genus *Panax* and one sub-species have been identified including *Panax bipinnatifidus* Seem., *P. stipuleanatus* Feng Tsai et, *P. vietnamensis* Ha et Grushv., *P. pseudoginseng* Wall., *P. ginseng* Meyer. and *Panax vietnamensis* var. *fuscidiscus. Panax vietnamensis* is endemic species in Vietnam that only distribute around Ngoc Linh mountain with the altitude from 1500m to 2400m, in limited geograpgical coordinates from $14^0 55'$ to $15^0 07'$ north latitude and from $107^0 51'$ to $108^0 05'$ east longitude. This species is unique *Panax* species that distributes to 15^0 north latitude and it is considered as the most valuable medicinal plants in Vietnam. But *Panax vietnamensis* and *Panax vietnamensis* var. *fuscidiscus* share many similar characteristics and make people often confused. In this research, we used of DNA barcoding to authentic *Panax vietnamensis*. We sequenced 4 chloroplast DNA regions includes MatK, rbcL, rpoB and 1 nuclear DNA regions ITS for comparison and choose the best one for identification of the *Panax* species. Our result showed that ITS-rDNA is the best marker for authentic *Panax* species. MatK is good for identify at species level but rpoB good for identify at subspecies level. The sequence of MatK, rbcL, rpoB, rpoC, ITS of *Panax vietnamensis* and *Panax vietnamensis* var. *fuscidiscus* were submitted to Genebank with accessory number as KJ 418201, KJ 418206, KT 154685, KT 194325, KT154583, KT 194326, KJ 418194, KJ 418193 respectively.

Key words: Panax vietnamensis ; ginseng ; DNA barcoding ; Panax vietnamensis var fuscidiscus.

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1. Introduction

Panax L. is a small genus that belongs to Ariliaceae family. The genus *Panax* L. is known to be distributed in the northern hemisphere, extending from the mountain area bordering the east coast of North America including the northern United States and southwestern Canada (there are 2 species: *P. quinquefolius* (American ginseng) and *P. trifolius*) [10]. There are two species including *P. ginseng* and *P. japonica* distribute in Northeast Asia (including far eastern Russia, northeastern China, Korean Peninsula and Japan). The central distribution of the genus *Panax* L. may begin from southwestern China and spread to the north of Vietnam [9]. In fact, this area includes two adjacent border provinces; these are Yunnan (China) and Lao Cai (Vietnam). Here, there are seven species and subspecies growing completely naturally. Two cultivated species are *P. notoginseng*, which is imported from North America, and *P. pseudoginseng* (which is not found in the wild but it is assumed to have originated from nearby Himalayas as a result of a natural hybrid between two closely related species). This area can be reputed as the central distribution of the genus *Panax* L. in the world [5]. There are three species (*P. notoginseng; P. quinquefolius and P. trifoliatus*) in North America. At the end of the southern limit of the genus *Panax* L. is *Panax vietnamensis* (Vietnamese people refer to it as Ngoc Linh ginseng), which grows in central Vietnam, at $14^0$15' north latitude. Therefore the Vietnamese ginseng is considered a narrow endemic of central Vietnam. The Ngoc Linh Ginseng is a narrow endemic of central Vietnam with native distribution in Tu Mo Rong district, Dak Glei district (Kon Tum province), Nam Tra My district, Phuoc Son district, Hien district (Quang Nam province), on the Ngoc Linh Mountain, altitudes above 1.500m [5]. However, at present, this species is thought to be extinct in the wild because of exhaustive exploitation for many years and the slash and burn agriculture therefore narrowing their natural habitats. *Panax vietnamensis* has been put into red list of IUCN and red book in Vietnam [7, 8]. Up to now, this species is only cultivated in two conservation areas , one of which is Ginseng conservation place (Mang Ri commune, Tu Mo Rong district, Kon Tum Province) and the other one is Tra Linh medicine station (Tra Linh commune, Nam Tra My district, Quang Nam province) [10]. All species of the genus *Panax*.L have medicinal value [14]. In Vietnam, *Panax vietnamensis* is considered as the most value medicinal plant [12]. *Panax vietnamensis* var. *fuscidicus* is found first time in Phong Tho commune, Lai Chau province in Vietnam since 2012. This species has been found in southern Yunnan, China before and was named as *Panax vietnamensis* var. *fuscidicus* K.Komatsu, S.Zhu & S.Q. Cai in 2002. *Panax vietnamensis* var. *fuscidicus* share many similar characteristics with *Panax vietnamensis* such as leaves verticillate (3)-4 (5) at apex of stem, palmately compound, with 5 – (6-7) leaflets, membranous, petioles 7-12 cm, at base without stipule of stipule-like appendages petiolule 0.3-1 cm, at base with uncinate hairs, ca. 2mm long. The main and lateral veins of both surfaces sparsely bear incinate hair around 2mm long. Flowers greenish-yellowish, 3-4 mm in diam, pentamcrous, rarely hexamerous [10]…So, it is very difficult to authentic them based on morphology. One possible means to overcome this difficulty would be to establish a reliable DNA barcode. DNA barcodes have been developed using the sequences of specific regions of chloroplast DNA, and they have rapidly become an important tool for species identification. CBOL (2009) recommend 7 candidate plastid DNA regions include *atpF–atpH* spacer, *matK* gene, *rbcL* gene, *rpoB* gene, *rpoC1* gene, *psbK–psbI* spacer, and *trnH–psbA* spacer [1]. Conrad and his colleagues (2012) using 6 Nuclear DNA regions to test the ability of their identification and recommended ITS is the universal region DNA barcode marker for identification of Fungi [3], Chen (2010) also recommened ITS as DNA barcode for medicinal plant [2].

In this research, we used MatK rbcL, rpoB chloroplast DNA regions and ITS nuclear DNA regions to authentic *Panax* species.

2. Materials and Methods

2.1. Plant materials

The leaves of *P. Vietnamensis* were collectes at Tra Linh commune, Nam Tra My district, Quang nam province at the coordinates 15°08N-108°09E, altitude 1400m. The leaves of *P. vietnamensis* var. *Fuscidiscus* were collected at Phong Tho commune, Lai Chau province at the coordinates 22°20N, 102°32E, and altitude 1500m. Collected leaves are stocked in silicagen until used. The voucher specimens were stored in the Department of Molecular systematics and conservation genetics (IEBR-VAST).

2.2. DNA extraction

Total DNA was extracted from the samples using the modified CTAB method of Doyle and Doyle (1987) [4]. Liquid nitrogen was added to each sample (about 100 mg), which was then ground by hand. Total DNA yield and purity were assessed using a spectrophotometer and were then visualized on 1% agarose gels. Stock DNA was diluted to a concentration of 10 ng/μL.

2.3. DNA amplification and sequencing

PCR was performed in a 40-μL reaction volume containing 4 μL PCR 10X buffer, 1 μL 25 mM dNTP, 1 μL 20 μM of each primer, 1 μL 25 mM $MgCl_2$, 1 μL BSA, 1 μL 2.5 U Taq DNA polymerase and approximately 50 ng genomic DNA. The rbcL, matK, rpoB, rpoC, ITS genes from both *Panax* species samples were amplified using a standard protocol with universal primers (Table 1).

The amplification conditions were 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s, 54o for 30s, 56o for 1 min and 63o for 30s of rbcL, rpoB, ITS and MatK respectively and extension at 72°C for 1 min. The reaction was completed by a 10-min extension and hold at 4°C.

The identities of the PCR products were verified by electrophoresis on 0.8% agarose gels. All PCR products were purified using a QIA quick PCR purification kit (Qiagen, Germany). The purified PCR products were sequenced in both directions with the same amplification primers as for PCR using a Bigdye terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3100 capillary sequencer following the manufacturer instructions.

2.4. DNA analysis

Bidirectional DNA sequences of each fragment were assembled using the ChromasPro software (Technelysium) and then aligned by Mega 6.0 (Tamura and his colleagues 2013) [13]. The p-distance of rbcL, rpoB, matK and ITS was calculated using MEGA 6.0 in order to evaluate intra-specific and inter-specific divergence. Neighbor-

joining (NJ) trees based on p-distance were constructed using MEGA 6.0 to provide a graphical representation of genetic divergence among species.

Table 1: Sequence information of four used primers

DNA region name	Forward primer	Reverse primer	Tm (°C)	PCR product length	Reference
rbcL	TCTAGCACACGAAAGTCGA AGT	TTCGGCACAAAATACGA AACGATCTCTCCA	58	700	Hasebe and his colleagues (1994) [6]
rpoB	GCC ACC ATC GAA TAT CTG GT	ACA CGA TCT CGT CGC TAA CC	54	500	CBOL, 2009 [1]
MatK	CGATCTATTCATTCAATATT TC	TCTAGCACACGAAAGTCGA AGT	56	1500	Shaw and his colleagues (2005) [11]
ITS	TCC GTA GGT GAA CCT GCG G	GCT GCG TTC TTC ATC GAT GC	63	600	Conrad and his colleagues 2012 [3]

3. Results

3.1. PCR and sequencing efficiency

The efficiency of PCR amplification for matK, rbcL, rpoB and ITS was 96, 97,5, 89 and 95%, respectively for *Panax vietnamensis*, 99; 97; 90 and 92% for *Panax vietnamensis* var. *fuscidicus*. Bidirectional DNA sequences of each fragment were sequenced, their sequences then submit to the Genbank with the accession numbers KJ 418201.1, KT 154685, KT 154867 and KJ 418194.1 respectuvely for Panax vietnamensis, KJ 418206.1, KT 194325, KT 194323 and KJ 418193.1 respectively for *Panax vietnamensis* var. *fuscidicus* (Table 2).

Table 2 : The accession number of submitted sequences

	Genbank accession numbers			
Species	MatK	rbcL	rpoB	ITS
Panax vietnamensis	KJ418201.1	KT154685	KT154687	KJ418194.1
Panax vietnamensis var. *fuscidicus*	KJ418206.1	KT194325	KT194323	KJ418193.1

3.2. Alignment and variability

For the *Panax vietnamensis* and *Panax vietnamensis* var. *fuscidicus*, an aligned matK sequence of 1495 bp was obtained; this sequence contained 33 variable sites, of which 16 were informative parsimony sites. The aligned rbcL sequence was 640 bp with 26 variable sites, of which 7 showed informative parsimony. The aligned sequence of rpoB was 493 bp long with 12 variable sites, showed 6 informative parsimony sites. Specially, aligned ITS sequence of 588 bp contained 97 variable sites, in which 28 showed informative parsimony sites.

3.3. Comparison of identification ability of each DNA markers

Using p-distances value, we make the comparison of each DNA marker for identification ability for *Panax vietnamensis* with *Panax vietnamensis* var. *fuscidicus* and 7 other *Panax* species (data from Genbank)(Fig. 1). According that, ITS always give P-disdance value higher than matK, rbcL, rpoB between each species (Table 3). For 3 candidate chloroplast regions, rpoB region with 500 bp in length, showed 12 variable sites, 6 informative parsimony sites, is become more sensitive for identification *Panax* species than matK and rbcL. Our result also confirmed that *Panax vietnamensis* var.

fuscidicus is variety of *Panax vietnamensis* with genetic distance value is 0.001 (in matK), 0.005 (in rpoB), 0.007 (in rbcL) and 0.008 (in ITS) while average genetic distance of *Panax vietnamensis* with other *Panax* species is 0.014 (in MatK), 0.0139 (in rbcL), 0.015 (in rpoB) and 0.027 (in ITS) (Table 3). MatK region give the genetic distance value between *Panax vietnamensis* and *Panax vietnamensis* var. *fuscidicus* is only 0.001 but for other *Panax* spceis, it give genetic distance value quite good with the value always difference in each species, so the MatK region maybe good for identify in the species level but it is not really good for identify at sub-species level.

Table 3: Genetic distance value of each DNA markers for *Panax vietnamensis* compare with *Panax vietnamensis* var. *fuscidicus* and 7 other *Panax* species

Species	matK	rbcL	rpoB	ITS
P.vietnamensis	0	0	0	0
P.vietnamensis var. fuscidicus	0.001	0.007	0.005	0.008
P.japonicus	0.012	0.012	0.012	0.015
P.notoginseng	0.013	0.012	0.012	0.024
P.pseudoginseng	0.014	0.015	0.019	0.019
P.quinquefolius	0.015	0.013	0.014	0.015
P.ginseng	0.016	0.014	0.012	0.016
P.stipuleannatus	0.019	0.015	0.021	0.037
P.trifolius	0.017	0.016	0.018	0.063

3.4. Phylogeny

Based on ITS and ropB sequences, 9 *Panax* species divided into 2 group, *Panax ginseng*, *Panax japonicas*,

Panax notoginseng that distribute in the east were in 1 group, *Panax quinquefolius*, *Panax pseudoginseng* that distribute in the North America were in other group and more closer. Our NJ phylogeny tree also confirmed that *Panax vietnamensis* var. *fuscidicus* closest with is *Panax vietnamensis* (Fig.2)

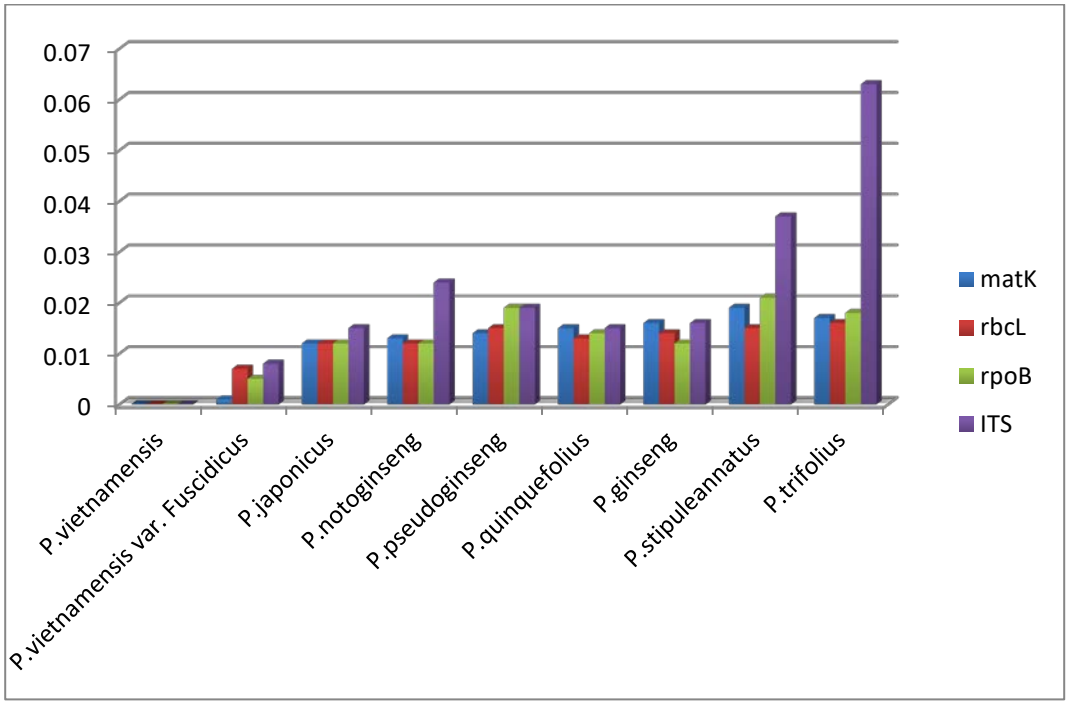
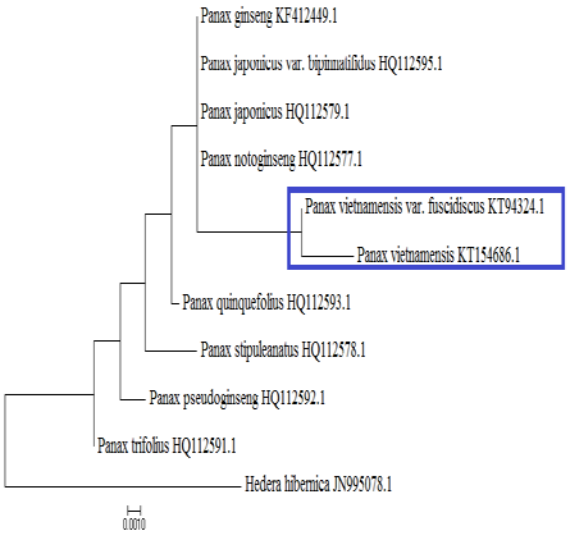
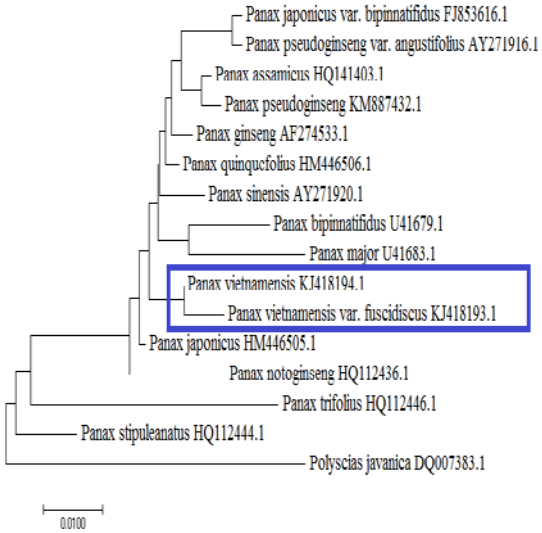


Figure 1: Comparison the identification ability of each DNA marker for *Panax* species



NJ tree based on rpoB sequence (A)



NJ tree based on ITS sequence (B)

Figure 2: Phylogentic trees for 9 *Panax* species based on rpoB and ITS sequences

4. Discussion

All species of the genus *Panax*.L are available as medicinal resource in traditional medicine. *Panax vietnamensis* was found in the Ngoc Linh Mountain in Vietnam and considered as an endemic species. This species also was defined as a precious medicinal plant of Vietnam, listed to one of the four most valuable ginseng species in the world because their's saponin, acid amin and mineral quality much higher than other *Panax* species, it was high evaluated on economic value, on health improvement and healing by specialized organizations [12]… but it was absent in the natural environment. Moreover, due to limited distribution and overexploited made *Panax vietnamensis* fall into the threatened group. At present, this species is only cultivated in several conservation areas on Ngoc Linh Mountain where belong to Quang Nam and Kontum provinces, Vietnam. *Panax vietnamensis* has been listed into red list of IUCN and red book in Vietnam. *Panax vietnamensis* var. *fuscidicus* ís a new medicinal plant that found in Lai Chau province, Vietnam since 2012 [10]. This species share many similar with *Panax vietnamensis* and make people often confused. We used three chloroplast DNAs include MatK, rbcL, rpoB and one nuclear ITS-rDNA to identify of *Panax* species as well as recognize the *Panax vietnamensis* and *Panax vietnamensis* var. *fuscidicus*. Our result showed that ITS-rDNA is the best marker to authentic *Panax* species. For chloroplast DNA markers, rpoB is useful to identify in the sub species level. MatK is the marker that useful for identification at species level *Panax* genus.

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