Original Article

A Molecular Phylogeny of Panax L. Genus (Araliaceae) Based on ITS-rDNA and matK Support for Identification of Panax Species in Vietnam

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Abstract: Panax L. is a small genus in the Araliaceae family. In Vietnam, this genus is distributed in the high mountains in the North and in some high mountains in the Central Highlands. In traditional medicine, roots and rhizomes of all Panax species are of high utility. Recently, the finding of new distributions of some Panax species in Vietnam has caused much controversy and confusion. This study investigates 27 fresh specimens of 6 taxa of Panax genus that have been collected from 6 provinces of Vietnam. Based on the combined ITS-rDNA and matK dataset, a well-resolved phylogeny of Panax species/varieties distributed in Vietnam was constructed. Thereby, the study suggests identifying Tam that la xe as Panax stipuleanatus H.T.Tsai et K.M.Feng and Sam puxailaileng as Panax vietnamensis var. fuscidiscus K.Komatsu, S.Zhu & S.Q.Cai, which contributes a new distribution point of this variety in Vietnam. The study also shows that ITS-rDNA and matK genes are highly potential in identifying and distinguishing the taxa of Panax genus.

Keywords: Panax, ginseng, molecular phylogeny, taxonomy, ITS-rDNA, matK.

1. Introduction

Panax is a small genus of Araliaceae family, comprise about 16-18 species worldwide, distribution with a concentration in the Eastern Asia, Central to Eastern Himalayan region, China, South-East Asia and North America [1].

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In Vietnam, there are three species and two varieties of *Panax* have been recorded, consist of: Tam that hoang - *P. stipuleanatus* H. T. Tsai et K. M. Feng; Sam ngoc linh - *P. vietnamensis* Ha et Grushv. with two varieties Sam lai chau - *P. vietnamensis* var. *fuscidiscus* K.Komatsu, S. Zhu & S.Q.Cai, Sam langbian - *P. vietnamensis* var. *langbianensis* N.V. Duy, V.T. Tran & L.N. Trieu, and Tam that - *P. notoginseng* (Burkill) F.H.Chen. (cultivated species). *Panax* species have narrow distribution, concentrated in some high mountain ranges in the Northern (Tay Con Linh, Hoang Lien Son, Pu Si Lung, Pu Sam Cap moutain), in the Central (Pu Xa Lai Leng and Ngoc Linh mountain) and Central Highland (Langbian moutain) [2-5]. All of the *Panax* species are high value medicinal plants with various chemical composition [6]. Thus, authentication these species are very important for conservation and development these genetic resources.

Along with morphological method, the using of DNA barcode to classify *Panax* species are more and more popular over the world. Recently, a lot of study on phylogenetic relationship of *Panax* genus using the combination of DNA barcodes belong to chromosome and chloroplast genome were performed [7-12].

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**Figure 1.** Representation of the samples in this study.  
(A) Tam that hoang - *Panax stipuleanatus* Tsai & Feng; (B) Tam that la xe - *Panax* sp.1; (C) Sam ngoc linh - *Panax vietnamensis* Ha &Grushvitzky; (D) Sam lai chau - *Panax vietnamensis* var. *fuscidiscus* K.Komatsu, S.Zhu &S.Q.Cai; (E) Tam that bac - *Panax notoginseng* (Burkill) F.H.Chen; (F) Sam puxailaileng – *Panax* sp.2, with (G) flower and (H) flat disk flower base on longitudinal section of ovary. (Photo A-H taken by Pham Thi Ngoc; A-B: 7/2015; C: 7/2015; D: 7/2017; E: 5/2017; F: 9/2017 G-H: 7/2017).
In Vietnam, there were some studies based on molecular markers to study the phylogenetic relationship of *Panax* species aim to identify [5,13-16] or record new taxon [4,17]. The molecular markers used for analysis consist of DNA fragments in the chromosome and chloroplast genome as 18S rRNA, ITS, *psbA-trnH*, *matK*, *trnL*, *rbcL*, *rpoB*,... Recently, Manzanilla et al. (2018) have used a full plastome phylogenomic framework to resolve relationships in *Panax* and to identify molecular markers for species discrimination [12]. However, these studies had been carried out on one or few species/varieties instead of all the *Panax* species/varieties recorded in Vietnam. In particular, it has not paid attention to the samples of Tam that la xe which previously identified as *P. bipinnatifidus* Seem. by Wu Cheng Yi but laterly considered to be more similar to *P. stipuleanatus* by Pham Thi Ngoc et al. (2017) [18]. Tam that la xe possess most of the same morphological characteristics to *P. stipuleanatus* (bamboo-like rhizome, concave flower disk,...) and differ to this species only in pinnate leaflet. In addition, the samples of Sam puxailaileng (Pu Xai Lai Leng mountain, Ky Son dist., Nghe An prov.) were identified as *P. vietnamensis* based on ITS-rDNA sequence [5] need further studies with more DNA markers.

In this study, we analyze the phylogenetic relationship based on the combined ITS-rDNA and *matK* sequences among all the *Panax* species/varieties in Vietnam for species/varieties discrimination.

### 2. Materials and methods

#### 2.1. Materials

Total of 27 fresh samples were collected and dried in sealed bags with silica-gel, stored in the laboratory, where each sample was preserved at a constant -20 °C for DNA analysis. The samples from four species/varieties and two unidentified *Panax* taxa (“Tam that la xe” - *Panax* sp.1 and “Sam Puxailaileng” - *Panax* sp.2) were collected in 6 provinces in Northern and Central of Vietnam: Lao Cai, Ha Giang, Lai Chau, Nghe An, Quang Nam, Kon Tum (*Table 1*). All specimens were stored in the herbarium of medicinal materials, National Institute of Medicinal Materials (NIMM).

**Table 1. Sampling information**

| Collection number | Vietnamese common name | Scientific name          | Collected location                  | Collection date | Collector          |
|-------------------|------------------------|--------------------------|------------------------------------|-----------------|--------------------|
| P.01              | Sam ngoc linh          | *P. vietnamensis* Ha et Grushv. | Tak Ngo, Tra Linh, Nam Tra My, Quang Nam | 06/07/2014      | Nguyen Xuan Nam    |
| P.02              | Sam ngoc linh          | *P. vietnamensis* Ha et Grushv. | Tak Ngo, Tra Linh, Nam Tra My, Quang Nam | 23/07/2014      | Nguyen Xuan Nam    |
| P.03              | Sam ngoc linh          | *P. vietnamensis* Ha et Grushv. | Mang Ruong 2, Ngoc Lay, Tu Mo Rong, Kon Tum | 27/07/2014      | Nguyen Xuan Nam    |
| P.04              | Sam ngoc linh          | *P. vietnamensis* Ha et Grushv. | Tra Linh, Nam Tra My, Quang Nam | 28/11/2014      | Nguyen Xuan Nam    |
| P.05              | Sam ngoc linh          | *P. vietnamensis* Ha et Grushv. | Mang Lung, Tra Linh, Nam Tra My, Quang Nam | 20/07/2015      | Nguyen Xuan Nam    |
| P.06              | Sam ngoc linh          | *P. vietnamensis* Ha et Grushv. | Mang Lung, Tra Linh, Nam Tra My, Quang Nam | 20/07/2015      | Nguyen Xuan Nam    |
| P.07              | Sam ngoc linh          | *P. vietnamensis* Ha et Grushv. | Mang Ruong 2, Ngoc Lay, Tu Mo Rong, Kon Tum | 23/07/2015      | Nguyen Xuan Nam    |
| Page  | Location                        | Plant Name                      | Person          | Date       |
|-------|--------------------------------|--------------------------------|-----------------|------------|
| P.08  | Sam ngoc linh                  | P. vietnamensis Ha et Grushv.   | Ko Xia II, Ngoc Lay, Tu Mo Rong, Kon Tum | 24/07/2015 |
| P.09  | Tam that la xe                | Panax sp.1                      | Ban Khoang, Sa Pa, Lao Cai | 13/07/2015 |
| P.10  | Tam that la xe                | Panax sp.1                      | Ho Thau, Hoang Su Phi, Ha Giang | 12/06/2016 |
| P.11  | Tam that la xe                | Panax sp.1                      | Ho Thau, Hoang Su Phi, Ha Giang | 12/06/2016 |
| P.12  | Tam that la xe                | Panax sp.1                      | Ho Thau, Hoang Su Phi, Ha Giang | 30/05/2017 |
| P.13  | Tam that la xe                | Panax sp.1                      | Ho Thau, Hoang Su Phi, Ha Giang | 30/05/2017 |
| P.14  | Tam that la xe                | Panax sp.1                      | Ta Phin, Sa Pa, Lao Cai | 08/08/2017 |
| P.15  | Tam that hoang                | P. stipuleanatus H.T.Tsai et K.M. Feng | Ban khoang, Sa Pa, Lao Cai | 13/07/2015 |
| P.16  | Tam that hoang                | P. stipuleanatus H.T.Tsai et K.M. Feng | Ho Thau, Hoang Su Phi, Ha Giang | 12/06/2016 |
| P.17  | Tam that hoang                | P. stipuleanatus H.T.Tsai et K.M. Feng | Ho Thau, Hoang Su Phi, Ha Giang | 12/06/2016 |
| P.18  | Tam that hoang                | P. stipuleanatus H.T.Tsai et K.M. Feng | Ho Thau, Hoang Su Phi, Ha Giang | 30/05/2017 |
| P.19  | Tam that hoang                | P. stipuleanatus H.T.Tsai et K.M. Feng | Ta Phin, Sa Pa, Lao Cai | 08/08/2017 |
| P.20  | Sam lai chau                   | P. vietnamensis Ha & Grushv. var. fuscidiscus K.Komatsu, S. Zhu & S.Q.Cai | Muong Te, Lai Chau | 24/04/2017 |
| P.21  | Sam lai chau                   | P. vietnamensis Ha & Grushv. var. fuscidiscus K.Komatsu, S. Zhu & S.Q.Cai | Sin Ho, Lai Chau | 27/07/2017 |
| P.22  | Sam lai chau                   | P. vietnamensis Ha & Grushv. var. fuscidiscus K.Komatsu, S. Zhu & S.Q.Cai | Sin Ho, Lai Chau | 27/07/2017 |
| P.23  | Sam lai chau                   | P. vietnamensis Ha & Grushv. var. fuscidiscus K.Komatsu, S. Zhu & S.Q.Cai | Sin Ho, Lai Chau | 27/07/2017 |
| P.24  | Sam lai chau                   | P. vietnamensis Ha & Grushv. var. fuscidiscus K.Komatsu, S. Zhu & S.Q.Cai | Muong Te, Lai Chau | 19/09/2017 |
| P.25  | Sam puxailaileng               | Panax sp.2                      | Muong Long, Ky Son, Nghe An | 14/07/2017 |
| P.26  | Sam puxailaileng               | Panax sp.2                      | Muong Long, Ky Son, Nghe An | 29/09/2017 |
| P.27  | Tam that bac                   | Panax notoginseng (Burkill) F.H.Chen | Hoang Su Phi, Ha Giang | 30/05/2017 |
2.2. Methods

2.2.1. DNA extraction, PCR amplification, DNA purification and sequencing

Total DNA was extracted from dried leaves of those samples, using a Qiagen DNeasy plant extraction kit (Qiagen, Germany) with the provided protocol. Complete ITS-rDNA region gene and partial matK gene amplifications via the polymerase chain reaction (PCR) were performed using 5 µl (10-50 ng) total DNA as a template in 25 µl reaction mixture, containing 0.5 µM of each primer, 2.5 µl dNTP mix, 2.5 µl DreamTaq Buffer 10X, 0.625 U DreamTaq DNA polymerase (ThermoFisher Scientific, USA) and deionized water. For application of the ITS region amplification, the primers used were C26A (5'- GTT TCT TTT CCT CCG CT - 3') and Nnc18S10 (5'-AGG AGA AGT CGT AAC AAG- 3') [8]; thermal cycling conditions were as follows: a sample denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94°C for 50 sec, primer annealing at 54°C for 55 sec, primer extension at 72°C for 2 min, and final extension step at 72°C for 10 min. For partial matK gene amplification, the primers used were matK AF (5'- CTA TAT CCA CTT ATC TTT CAG GAG T-3') and matK 8R (5'- AAA GTT CTA GCA CAA GAA AGT CGA-3') (Ooi et al. 1995); thermal cycling conditions were as follows: a sample denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 2 min and primer annealing at 62°C for 1 min and primer extension at 72°C for 2 min, and final extension step at 72°C for 10 min. PCR products were then purified using QIAquick Gel Extraction Kit (Qiagen, Germany). Sanger sequencing was performed on an ABI 3730 Genetic Analyzer system using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

2.2.2. Phylogenetic analysis

The newly generated sequences were assembled by Geneious version 6.0.6 (http://www.geneious.com, Biomatters, 2013). Nucleotide sequences were aligned using MUSCLE 3.8.31 (Edgar, 2004). Sequences from the sampled taxa of the genus Panax collected in this study along with those from Genbank and of the outgroup taxa were examined for sequence comparisons and phylogenetic trees reconstruction based on two dendrogram building methods: Maximum Likehood (ML) in RaxML 8.2.8 (Stamakis, 2014) and Bayesian Inference (BI) in MrBayes v.3.2.6 (Ronquist và Huelsenbeck, 2003) on the CIPRES Science Gateway (Miller et al., 2010) under the GTR + G model with 1000 replications.

Supporting information: Genbank accession numbers of 45 DNA sequences of ITS-rRNA and 45 sequences of matK belonging to 13 taxa that distributed in 6 countries and territories consist of American, China, Japan, Nepal, Taiwan and Vietnam.

3. Results and discussion

3.1. ITS-rDNA and matK sequences analysis

Obtained partial of ITS-rDNA and matK gene sequences of the samples were found to be 702 - 715 and 1061 - 1104 base pairs in length respectively. Sequences of the samples in the same taxon were similar suggesting the intraspecies stability of Panax species on ITS-rDNA and matK gene sequences. Comparison among eight taxa of Panax occurred in Vietnam found out the differences in 40 positions on ITS-rDNA and 27 positions on matK gene (Table 2 & 3). This results show that the ITS-rDNA and matK gene are high potential in distinguishing those taxa of Panax genus in Vietnam.

Comparing the ITS-rDNA and matK gene sequences between Tam that hoang (Panax stipuleanatus) and Tam that la xe (Panax sp.1) samples, we found that all the samples divided into two groups related to the two collected sites: Lao Cai and Ha Giang province. Samples in the same group were 100% identical in ITS-rDNA and matK gene sequences while samples in different groups are distinguished by nucleotide position 472 on ITS-rDNA gene and nucleotide position 462 on matK gene. Group 1 including all the samples collected in Lao Cai (Sa Pa)
Considered as genetic "Panax stipuleanatus" and unidentified Tam that la xe - "Panax stipuleanatus" (P16, P17, P18 and P11, P12, P13, P14). The substitutions of G by T at position 462 on the *matK* gene fragment and T by C at position 472 on ITS-rDNA could be considered as genetic diversity in different distribution populations.

Table 2. Comparison of ITS-rDNA gene sequence among the samples and eight taxa of *Panax* genus in Vietnam.

| Locus | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-------|---|---|---|---|---|---|---|---|---|----|
| *P. stipuleanatus* (1) | C | C | C | C | A | A | T | A | T | C |
| *P. stipuleanatus* (2) | . | . | . | . | . | . | . | . | . | . |
| *Panax* sp.1 (3) | . | . | . | . | . | . | . | . | . | . |
| *Panax* sp.1 (4) | . | . | . | . | . | . | . | . | . | . |
| *Panax* sp.2 (5) | . | T | G | G | G | C | G | C | T | C |
| *P. vietnamensis* (6) | T | T | G | G | G | C | G | C | T | C |
| *P. vietnamensis* var. fuscidiscus (7) | . | T | G | G | G | C | G | C | T | C |
| *P. notoginseng* (8) | . | A | T | T | G | T | G | C | G | C |
| *P. vietnamensis* var. langbianensis (9) | . | T | G | G | G | C | G | C | T | C |
| *P. bipinnatifidus* (10) | . | T | G | G | G | C | G | C | T | C |

**Noted:** Studied samples and genbank accession numbers (1): P15, P19; (2): P16-P18; (3): P09, P14; (4): P10-P13; (5): P25, P26; (6): P01-P08; (7): P20-P24; (8): P27; (9): KX768322 (N.V.Duy et al., 2016); (10): HQ112362, HQ112417, HQ112456 (Zuo Y. et al., 2011).

3.2. Phylogenetic analysis

The ML analyses for individual DNA regions were mostly congruent, but relationships among major clades were poorly resolved. So, the combined ITS-rDNA and *matK* data set retrieved a well-supported topology for *Panax*. Topology of the BI analysis was mostly congruent with the ML tree. Thus, the ML tree with bootstrap (BS) and Bayesian posterior probability (PP) values is presented in Figure 2. Our phylogenetic analyses recognized five well-supported clades in *Panax*. The two remaining taxa (*Aralia*) grouped in another well-supported lineage (BS = 93%, PP = 1.00; Figure 2).

The phylogeny constructed from combined ITS-rDNA and *matK* data showed that Tam that hoang (*P. stipuleanatus*) and Tam that la xe (*Panax* sp.1) are 100% identical and belong to the same clades. Otherwise, the samples of them separated into two clades related to geographical isolation. Clade II.1 including samples collected in Ha Giang with ML bootstrap and Bayesian PP values are 96% and 0.93; Clade II.2 including samples collected in Lao Cai with ML bootstrap and Bayesian PP values are 100% and 1.00 respectively. The results of our recent morphological study also showed that Tam that hoang (*P. stipuleanatus*) and Tam that la xe (*Panax* sp.1) are similar in rhizomes, flowers, fruits, and seeds; they are only distinguished in leaflets shape (entire leaflets in Tam that hoang and pinnate-leaflets in Tam that la xe) [18].
Table 3. Comparison of *matK* gene sequence among the samples and eight taxa of *Panax* genus in Vietnam

| Taxon                                      | Locus          |
|--------------------------------------------|----------------|
| P. stipuleanatus (1)                       | C T T A A A A T A T T C T T A A C T C C A A A T T A T |
| P. stipuleanatus (2)                       |               |
| *Panax* sp.1 (3)                           |               |
| *Panax* sp.1 (4)                           |               |
| *Panax* sp.2 (5)                           | A G A G G C G C G G C C G T T G A C G G G G C |
| P. vietnamensis (6)                        | A G A G G C G G C G C T T G A G C G G G C C |
| P. vietnamensis var. fuscidiscus (7)       | A G A G G C G G C C G C G C G T T G A C G G G G C |
| P. notoginseng (8)                         | A G A . . C . G C G C C G C T T G A A A . C G G G C . |
| P. vietnamensis var. langbianensis (9)     | A G A G G C G G C C G C C G C T T G A G C G G G C A |
| P. bipinnatifidus (10)                     | A G A G G C G G C C . C G C T T T A A . C G G G G C |

**Noted:** Studied samples and genbank accession numbers (1): P15, P19; (2): P16-P18; (3): P09, P14; (4):P10-P13; (5): P25, P26; (6): P01-P08; (7): P20-P24; (8): P27; (9): KX768328 (N.V.Duy et al., 2016); (10): HQ113000, HQ113055, HQ113094 (Zuo Y. et al., 2011).

Therefore, by the analysis of ITS-rDNA and *matK* genes sequences and phylogenetic analysis of these two gene regions, we suggest that Tam that la xe and Tam that hoang are belong to the same species as *Panax stipuleanatus* H.T.Tsai et K.M.Feng. However, to determine whether Tam that la xe is a variety of *P. stipuleanatus*, it is necessary to carry out more studies on others molecular markers or even chemical composition.

Comparison between the samples of Sam puxailaileng with Sam lai chau (*P. vietnamensis var. fuscidiscus*) found that ITS-rDNA and *matK* gene sequences of them are 100% identical. The phylogenetic analysis on ML tree also showed the dissociation of Sam puxailaileng along with Sam lai chau are well-supported lineage (BS = 97% and PP = 1.00). On the other hand, in morphology, two Sam puxailaileng samples have identical characters with Sam lai chau such as: Rhizome horizontal with alternate scars, leaflet unlobed, without stipules, disk flower flat (Figure 1-F,G,H).

Thus, by the analysis of ITS-rDNA and *matK* gene sequences and phylogenetic analysis based on these two gene fragments, we suggest to identify Sam puxailaileng samples (P25, P26) collected at Pu Xai Lai Leng mountain (Ky Son district, Nghe An province) as *P. vietnamensis var. fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai. This results contribute a new location of *P. vietnamensis var. fuscidiscus* in Vietnam.

**4. Conclusions**

Base on the combined ITS-rDNA and *matK* dataset, this study retrieved a well-resolved phylogeny of *Panax* species/varieties distributed in Vietnam. Whereby, the results suggest to identify Tam that la xe, which is used to confusing with Sam vu diep (*Panax bipinnatifidus* Seem.) as *Panax stipuleanatus* H.T.Tsai et K.M.Feng. In addition, this results also support Sam puxailaileng as *Panax vietnamensis var. fuscidiscus* contributing a new distribution point of this variety in Vietnam. The study also showed that ITS-rDNA and *matK* genes are high potential in distinguishing and identifying the taxa of *Panax* genus.
Figure 2. Maximum likelihood (ML) tree for *Panax* genus based on the combined ITS-rDNA and *matK* data (ML bootstrap (BS) and Bayesian (PP-values) are indicated above branches as BS/PP form).
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