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IDENTIFICATION OF *PANAX* SPP. IN THE NORTHERN VIETNAM BASED ON ITS-rDNA SEQUENCE ANALYSIS

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SUMMARY

In order to accurately identify ginseng species (*Panax* spp.) that grow naturally in some Northern provinces of Vietnam, we collected 30 natural ginseng samples and used nuclear genetic region (ITS-rDNA) for current analysis. The success rate for nuclear genomic region PCR (ITS-rDNA) amplification is 100%. The bidirectional sequence read success rate obtained from the PCR product was 100%, with a nucleotide sequence length of 588 bp. Based on the analysis of ITS-rDNA region results, the samples of ginseng species from Tuyen Quang and Cao Bang provinces have a close relationship with *Panax notoginseng* (MLBS = 100%), while the samples of ginseng species from Ha Giang and Yen Bai provinces have a close relationship with *P. stipuleanatus* (MLBS = 99%). As the results, the ginseng samples from Tuyen Quang and Cao Bang provinces were identified as *Panax notoginseng* and the ginseng samples from Ha Giang and Yen Bai provinces were identified as *Panax stipuleanatus*. The obtained result also recorded new distribution areas of *P. notoginseng* in Tuyen Quang and Cao Bang provinces. Furthermore, we successfully registered the nuclear nucleotide sequences of these two *Panax* species on GenBank with 24 codes (from OM190408 to OM190410, from OM213014 to OM213030, and from OK376138 to OK376141).

Keywords: Ginseng, internal transcribed spacer (ITS), nucleotide sequence, *Panax*, *P. notoginseng*, *P. stipuleanatus*

INTRODUCTION

The genus *Panax* L. (Araliaceae) includes important and valuable medicinal plants which species mainly distribute in North America, East and Southeast Asia (Shu, 2007; Mabberley, 2008; Phan Ke Long *et al.*, 2014a; Zhang *et al*., 2015; Taram *et al*., 2018). While 19 species of the genus were recorded around the world (Pankey and Ali, 2012; Taram *et al.*, 2018), three species were found in the high mountains of Vietnam including *Panax vietnamensis* Ha et Grushv. (Vietnamese ginseng), *P. stipuleanatus* H.T.Tsai & K.M.Feng, and *P. bipinnatifidus* Seem. (Pham Hoang Ho, 2000; Tap, 2005). All the three species are rare and threatened in the national level and were listed in the Vietnam Red Data Book (2007) and Decree 84/2021/NĐ-CP

of the Vietnamese Government (Amending certain provisions of Decree 06/2019/NĐ-CP on the management of endangered, precious and rare wild fauna and flora, and the implementation of the CITES). Among them, *P. vietnamensis*, also known as Vietnamese ginseng or Ngoc Linh ginseng that is an endemic species occurring in Ngoc Linh mountain belonging to Kon Tum and Quang Nam provinces (Pham Hoang Ho, 2000; Tap, 2005). Recently, two varieties of the ginseng species were newly recorded for Vietnam. The first one, *Panax vietnamensis* var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai, which was found in Lai Chau province (Muong Te, Tam Duong, and Sin Ho districts), and the second one, *P. vietnamensis* var. *langbianensis* N.V.Duy, V.T.Tran & L.N.Trieu, which was found in Lam Dong province (Lang Biang mountain range) (Phan Ke Long *et al*., 2014a; Zhang *et al*., 2015; Duy *et al*., 2016).

Previous studies have recorded *Panax stipuleanatus* and *P. bipinnatifidus* from Ha Giang province (Pham Thanh Huyen *et al*., 2016) without evidence of molecular data. In addition, Bon *et al*. (2019) recorded a species of *Panax* occurring in Tuyen Quang province with the unknown species name. The accurate determination of species in the ginseng genus is very important for the selection of parent plants for further propagation, use, and conservation of this precious genetic resource. Meanwhile, the traditional method of taxonomy to identify species in the genus *Panax* is mainly based on morphological features whose characteristics are influenced by environmental factors (Jo *et al*., 2013). This identification, if the reproductive parts such as flowers or fruits are missing, will cause misleading results because the leaf morphology of the *Panax* species is quite similar and variable (Chen *et al*., 2013). In order to fill this gap, this study collected *Panax* spp. in four areas including Tuyen Quang, Ha Giang, Cao Bang, and Yen Bai provinces, then used the analysis method of the nucleotide sequence of the nuclear genetic region (ITS-rDNA) for identification of scientific names accurately. In plant, some gene regions chloroplasts (matK, rbcL, psbA-trnH...) and nuclear genomic regions (ITS-rDNA) are being applied widely used in relationship studies phylogeny, taxonomy and species identity. There are many publications on species identification of Ginseng on the basis of nucleotide sequence analysis ITS-rDNA (Phan Ke Long *et al*., 2014a; Le Thi Thu Hien *et al*., 2016; Pham Quang Tuyen *et al*., 2018.)

The sequencing results of collected ginseng samples in these four areas are the scientific basis to accurately identify scientific names of the studied samples. In addition, the study also contributes to building a molecular database of ginseng species in Vietnam. Moreover, the results with new distribution areas will contribute valuable information for the conservation activities of *Panax* species in Vietnam.

MATERIALS AND METHODS

Plant materials: During 2019 to 2021, total of 30 leaf samples of 30 individuals of *Panax* spp. were collected from the wild in Tuyen Quang (TQ), Ha Giang (HG), Cao Bang (CB), and Yen Bai (MC) provinces. Each sample was noted on location, geographic coordinates, elevation, leaf characters, and preliminary identification enclosed with labels.

Method of DNA extraction and purification: Total DNA was extracted using the Plant DNA isolation Kit (Norgenbiotek, Canada).

PCR amplification: The ITS-rDNA regions were amplified using PCR technique according to Phan Ke Long *et al*., (2014a) with primer pairs PaITSF 5'- CAC TGA ACC TTA TCA TTT AGA G -3' and PaITSR 5'-CTT ATT GAT ATG CTT AAA CTC AG-3'. The composition of each PCR reaction has a volume of 25 µl with the following components: 7 µl H_2O deion; 12.5 µl PCR Master mix kit (2X); 1.25 µl forward primer (10 pmol/µl); 1.25 µl reverse primer (10 pmol/µl); 3 µl DNA (10-20 ng). The reaction was performed on a model 9700 PCR machine (GeneAmp PCR System 9700, USA). Thermal cycle of PCR reaction includes: 94^{o}C for 3 min

followed by 35 consecutive cycles with steps: 94ºC for 45 seconds, 49ºC for 45 seconds, and 72ºC for 45 seconds; end the gene multiplication reaction at 72ºC for 10 minutes, keeping the product at 4ºC.

Sequencing and sequence correction: The nucleotide sequencing was performed at the Institute of Biotechnology - Vietnam Academy of Science and Technology. The DNA sequence after sequencing was corrected and regions of noise were removed using ChromasPro2.1.6 software. Nucleotide sequences of Ginseng samples were compared with those available on Genbank (using the BLAST tool in NCBI - http:www.ncbi.nlm.nih.gov/BLAST).
Analytical sequences were aligned using Bioedit software Ver.7.0.5.2 (Hall, 1999). Areas that cannot be sorted are removed prior to analysis.

Building phylogenetic tree: The phylogenetic tree was built based on the Maximum Likelihood (ML), performed with 1,000 replicates to determine the support value (bootstrap) in the ML (MLBS). The genetic distance (*P*) between species in the genus was calculated using Mega 11 (Tamura *et al*., 2021).

Table 1. Sample list of *Panax* spp. plants were collected from the wild in four areas.

Label	Location	Geographic Coordinates	Elevation (m)	Typical characters of leave	Preliminary identification
TQ1	Thuong Lam comm., Lam Binh Dist., Tuyen Quang Prov.	22°31'51N 105°21'29"E	1114	Leaflet margin slightly erose	*P. notoginseng*
TQ2	Thuong Lam comm., Lam Binh Dist., Tuyen Quang Prov.	22°32'9"N 105°21'6"E	1134	Leaflet margin slightly erose	*P. notoginseng*
TQ3	Sinh Long Dist., Na Hang Dist., Tuyen Quang Prov.	22°30'45"N 105°23'12"E	980	Leaflet margin slightly erose	*P. notoginseng*
TQ4	Sinh Long Dist., Na Hang Dist., Tuyen Quang Prov.	22°30'49"N 105°23'05"E	1014	Leaflet margin slightly erose	*P. notoginseng*
MC1	Cao Pha comm., Mu Cang Chai Dist., Yen Bai Prov.	21°44'48"N 104°14'14"E	1511	Leaflet margin slightly erose	*P. notoginseng*
MC2	Cao Pha comm., Mu Cang Chai Dist., Yen Bai Prov.	21°44'48"N 104°14'14"E	1511	Leaflet margin slightly erose	*P. notoginseng*
MC3	Cao Pha comm., Mu Cang Chai Dist., Yen Bai Prov.	21°44'48"N 104°14'14"E	1511	Leaflet margin slightly erose	*P. notoginseng*
MC4	Cao Pha comm., Mu Cang Chai Dist., Yen Bai Prov.	21°44'50"N 104°14'20"E	1512	Leaflet margin slightly erose	*P. notoginseng*
MC5	Cao Pha comm., Mu Cang Chai Dist., Yen Bai Prov.	21°44'50"N 104°14'24"E	1539	Leaflet margin slightly erose	*P. notoginseng*
MC6	Cao Pha comm., Mu Cang Chai Dist., Yen Bai Prov.	21°44'45"N 104°14'19"E	1564	Leaflet margin slightly erose	*P. notoginseng*
CB4	Ca Thanh comm., Nguyen Binh Dist., Cao Bang Prov.	22°46'54"N 105°50'51"E	1455	Leaflet margin slightly erose	*P. notoginseng*
CB12	Ca Thanh comm., Nguyen Binh Dist., Cao Bang Prov.	22°46'19"N 105°51'5"E	1315	Leaflet margin slightly erose	*P. notoginseng*
CB13	Ca Thanh comm., Nguyen Binh Dist., Cao Bang Prov.	22°46'43"N 105°51'45"E	1251	Leaflet margin slightly erose	*P. notoginseng*

Label	Location	Geographic Coordinates	Elevation (m)	Typical characters of leave	Preliminary identification
HG1	Tan Thanh comm., Bac Quang Dist., Ha Giang Prov.	22°34'34.2"N 104°48'38.4"E	1416	Leaflet margin deeply lobed	*P. stipuleanatus*
HG2	Tan Thanh comm., Bac Quang Dist., Ha Giang Prov.	22°34'31.9"N 104°48'42.1"E	1328	Leaflet margin deeply lobed	*P. stipuleanatus*
HG3	Tan Thanh comm., Bac Quang Dist., Ha Giang Prov.	22°34'13.9"N 104°48'44.2"E	1233	Leaflet margin deeply lobed	*P. stipuleanatus*
HG4	Nam Ty comm., Hoang Su Phi Dist., Ha Giang Prov.	22°36'45.3"N 104°46'21.5"E	1548	Leaflet margin slightly erose	*P. stipuleanatus*
HG5	Nam Ty comm., Hoang Su Phi Dist., Ha Giang Prov.	22°36'25.2"N 104°46'14.6"E	1108	Leaflet margin deeply lobed	*P. stipuleanatus*
HG6	Cao Bo comm., Vi Xuyen Dist., Ha Giang Prov.	22°45'04.4"N 104°50'44.8"E	1436	Leaflet margin deeply lobed	*P. stipuleanatus*
HG7	Cao Bo comm., Vi Xuyen Dist., Ha Giang Prov.	22°45'01.9"N 104°50'20.8"E	1484	Leaflet margin deeply lobed	*P. stipuleanatus*
HG8	Cao Bo comm., Vi Xuyen Dist., Ha Giang Prov.	22°44'59.4"N 104°50'20.7"E	1552	Leaflet margin deeply lobed	*P. stipuleanatus*
HG9	Cao Bo comm., Vi Xuyen Dist., Ha Giang Prov.	22°44'55.8"N 104°49'39.7"E	1583	Leaflet margin deeply lobed	*P. stipuleanatus*
HG10	Cao Bo comm., Vi Xuyen Dist., Ha Giang Prov.	22°45'45.4"N 104°50'11.1"E	1365	Leaflet margin deeply lobed	*P. stipuleanatus*
HG11	Cao Bo comm., Vi Xuyen Dist., Ha Giang Prov.	22°45'45.4"N 104°50'11.1"E	1388	Leaflet margin deeply lobed	*P. stipuleanatus*
HG12	Ho Thau comm., Hoang Su Phi Dist., Ha Giang Prov.	22°37'41.4"N 104°35'47.1"E	1482	Leaflet margin deeply lobed	*P. stipuleanatus*
HG13	Ho Thau comm., Hoang Su Phi Dist., Ha Giang Prov.	22°37'41.4"N 104°35'47.1"E	1482	Leaflet margin deeply lobed	*P. stipuleanatus*
HG14	Ho Thau comm., Hoang Su Phi Dist., Ha Giang Prov.	22°37'36.8"N 104°36'00.1"E	1504	Leaflet margin deeply lobed	*P. stipuleanatus*
HG15	Ho Thau comm., Hoang Su Phi Dist., Ha Giang Prov.	22°37'36.8"N 104°36'00.1"E	1405	Leaflet margin slightly erose	*P. stipuleanatus*
HG16	Ho Thau comm., Hoang Su Phi Dist., Ha Giang Prov.	22°37'39.5"N 104°36'05.1"E	1364	Leaflet margin slightly erose	*P. stipuleanatus*
HG17	Ho Thau comm., Hoang Su Phi Dist., Ha Giang Prov.	22°37'38.8"N 104°36'29.1"E	1452	Leaflet margin slightly erose	*P. stipuleanatus*

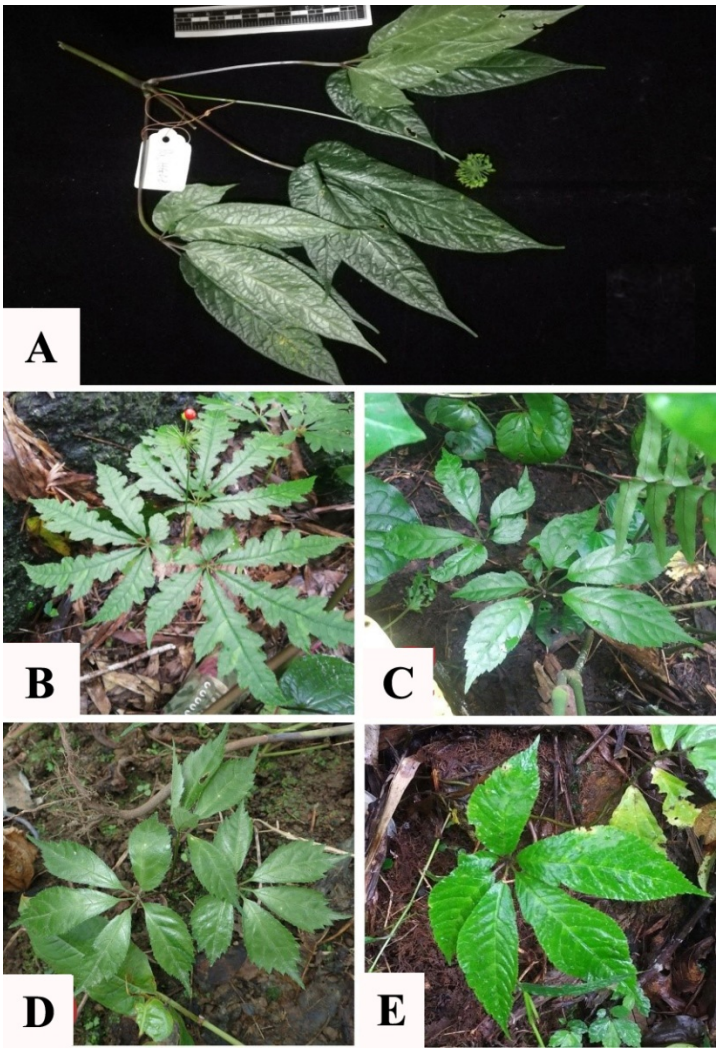

Figure 1. A, B – *Panax* sp. collected in Ha Giang with two types of leaf morphology (Leaflet margin slightly erose (A), Leaflet margin deeply lobed (B); C, D, E – *Panax* sp. collected in Tuyen Quang, Cao Bang, and Yen Bai provinces (Leaflet margin slightly erose). (A-B photos by Do Van Truong; C-E photos by Trinh Ngoc Bon).

RESULT AND DISCUSSION

After analysis of 30 samples of the natural ginseng by using the nuclear genetic region (ITS-rDNA), the nuclear nucleotide sequences of these *Panax* species had successfully registered on GenBank with 24 codes (from OM190408 to OM190410, from OM213014 to OM213030, and from OK376138 to OK376141).

The results of determining the kinship of the collected samples by the ML method have shown two distinct groups of *Panax* (Figure 2). The ginseng samples from Tuyen Quang (TQ) and Cao Bang (CB) provinces form a group closely related to *P. notoginseng* (MLBS 63%), while the ginseng samples from Ha Giang (HG) and Yen Bai (MC) provinces form a group closely related to *P. stipuleanatus* with high genetic similarity (MLBS 84%) (Figure 2). Based on the morphological characters of leaves, some ginseng samples from Yen Bai province (Table 1) were preliminary identified as *P. notoginseng*. However, their leaflet margins are slightly erose which is quite similar to that of *P. stipuleanatus*. Furthermore, our current analysis also showed that these ginseng samples nested within other samples of *P. stipuleanatus* (Figure 2).

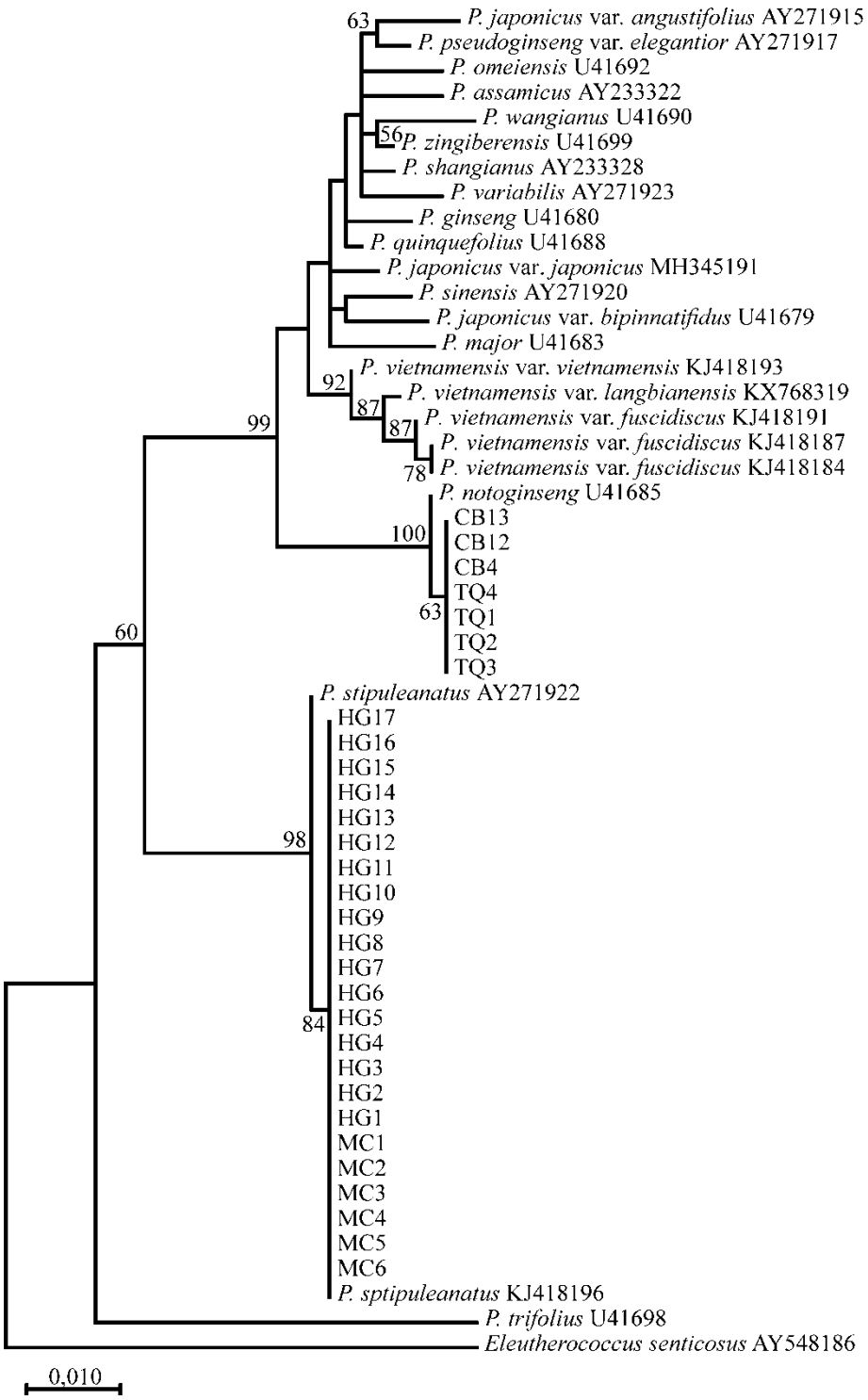

Figure 2. Relative relationship of the studied *Panax* samples with other species in the genus published in Genbank based on the analysis of nuclear nucleotide sequence (ITS-rDNA) by Maximum Likelihood (ML) analysis. Numbers on bootstrap index branches. *Eleutherococcus senticosus* (AY548186) is outgroup species.

The current results allowed the identification of the ginseng samples collected in Tuyen Quang (from TQ1 to TQ4) and Cao Bang provinces (CB4, CB12, CB13) as Tam That (*P. notoginseng*) and the ginseng samples collected in Ha Giang (from HG1 to HG17) and Yen Bai provinces (from MC1 to MC6) as Tam That Hoang (*P. stipuleanatus*) (Table 1 & Figure 2).

In terms of morphology, the samples of *Panax* specimens collected in Ha Giang and Yen Bai provinces had two types of leaflet blade shapes which are similar to those collected in Phu Xai Lai Leng mountain range in Nghe An province that were also identified as *Panax stipuleanatus* (Phan Ke Long *et al*., 2019), with two types of leaflet blades presented, leaflet margin with slightly erose (Fig. 1A) and deeply lobed (Fig. 1B).

When conducting ITS-DNA sequence analysis of Ha Giang ginseng samples, the results showed that there was no nucleotide difference. The samples had a close relationship with *P. stipuleanatus* which ginseng samples collected in Lai Chau province with values bootstrap up to 98% (AY271922, Fig. 2). On the other hand, the results of the analysis of the ginseng samples collected in Tuyen Quang and Cao Bang provinces are identical. There is no difference in any nucleotides and they are closely related to *P. notoginseng* (Fig. 2). This result confirmed new distribution areas for *P. notoginseng* in Tuyen Quang and Cao Bang provinces in Vietnam.

In plants, the nuclear genomic region (ITS-rDNA) is widely used for studying phylogenetic relationships, taxonomy, and species identification (CBOL Plant Working Group, 2009; China Plant BOL Group, 2011). Several molecular markers have been successfully used to identify *Panax* species such as nuclear gene region (ITS-rDNA*)* and chloroplast gene (*mat*K, *rbc*L, *rpo*B) (Wen *et al.*, 1996, Phan Ke Long *et al.*, 2014 a, b; Nguyen Thi Phuong Trang *et al*., 2016, 2017; Pham Quang Tuyen *et al*., 2018). The nucleotide sequence of the ITS-rDNA gene region of the studied samples after editing and removing all vacancies has a length of 588 bp. Nucleotide sequences obtained from these samples were checked for similarity with those available on Genbank using the BLAST tool. Because the results of BLAST gave inaccurate doubtful points, so we used the phylogenetic tree method to determine the scientific name for the ginseng samples in this study.

Research on building phylogenetic trees of species in the genus *Panax* so far has used the ITS gene region sequence as a reference standard as well as combined with other barcodes to get more comprehensive results (Lee & Wen, 2004; Zuo et *al*., 2011; Chen *et al*., 2013). The results of this study have shown that nuclear nucleotide sequence (ITS-rDNA) could fully identify *Panax* species without relying on the morphology of reproductive organs such as flowers and fruits.

CONCLUSION

The nuclear genome (ITS-rDNA) of the ginseng samples was collected in Tuyen Quang, Cao Bang, Yen Bai, and Ha Giang provinces sequenced with the size of 588 bp and successfully registered on GenBank with 24 codes (from OM190408 to OM190410, from OM213014 to OM213030, from OK376138 to OK376141). The ginseng samples collected in Tuyen Quang and Cao Bang provinces were determined as *Panax notoginseng* and the samples collected in Ha Giang and Yen Bai provinces were identified as *P. stipuleanatus*. This study also confirmed new distribution areas for *P. notoginseng* in Vietnam's Tuyen Quang and Cao Bang provinces.

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