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ITS1 LOCUS: A MAJOR DETERMINANT OF GENETIC DIVERSITY OF *PLANTAGO* SPP. (PLANTAGINACEAE)

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Abstract

By this study, ITS (Internal Transcribed Spacer) regions in the nuclear DNA of 10 *Plantago* samples collected from Mongolia (5 samples) and Viet Nam (5 samples) were sequenced and constructed Maximum Parsimony (MP) and Neighbor-Joining (NJ) phylogenetic trees for establishing the genetic relationship. The results showed that 10 samples belonged to 2 species (7 *Plantago major* and 3 *Plantago depressa*). The length of sequences ranged from 632 to 644 bp (ITS1 ranged from 210-222 bp, 5.8S was 162 bp, and ITS2 259 - 261 bp). The ITS1 region was highly variable among the sequences whereas ITS2 and 5.8S regions were more conservative. The MP and NJ trees apparently separated *P. major* and *P. depressa* into 2 different groups, supported with high bootstrap values. *P. depressa* was first time reported in Mongolia. The results highlighted that ITS sequences could distinguish *P. major* and *P. depressa*, which is certainly important for pharmacist to use crude drugs derived from *Plantago*.

Key words: Plantago, Plantaginaceae, ITS (internal transcribed spacer), Phylogeny, Genetic variation.

Introduction

Among popular medicinal plants, plantain (*Plantago* sp.) belonging to Plantaginaceae family is widely distributed and considered as a potential herb. Recently, this plant has attracted much attention and became economically important (Ho, 2003). The plantain plant contains biologically active substances such as iridoid glycosides, polysaccharides, flavonoids, lipids, caffeic acid derivatives, terpenoids and some organic acids that are involved in the wound healing activity, anti-inflammatory, analgesic, antioxidant, weak antibiotic, and antihypertensive activity effects. Indeed, it was mentioned as an old medicinal plant that has been used more than a millennium ago for wound healing remedy and in the treatment of a number of diseases related to the skin, respiratory organs, digestive organs, the circulation, against cancer, pain relief and against infections, etc. all over the world (Kolak *et al.*, 2011; Suh *et al.*, 1991). Besides being recorded as a traditional medicine, nowadays, this plant is appreciated in many aspects. It has not only been used as a vegetable but also a functional food in Vietnam, Laos, Thailand and Cambodia. In Mongolia, plantain is also used for medicinal purposes like treating stomachache, bloody urine, cough, improving eyesight, diuretic, even also valued as forage for livestock (Maria, 2000; WHO Regional Office for the Western Pacific, 2013). Study on plantain through conventional and modern methods was successfully achieved for several other species, but positive results regarding Mongolian and Vietnamese plantains based on application of molecular techniques which analyze the genetic diversity of this plant have not been reported.

Conventionally many studies have investigated genetic diversity among plants based on morphological and marker assisted analysis (Mumtaz *et al.*, 2010; Mumtaz *et al.*, 2011; Turi *et al.*, 2012; Sultan *et al.*, 2013; Shinwari *et al.*, 2014; Hussain *et al.*, 2016). Of the various molecular techniques available, according to many authors, nuclear DNA data provide valuable information in phylogenetic study of plants, and the internal transcribed spacer (ITS) regions of the nrDNA have been shown as a valuable source of evidence to resolve phylogenetic relationships at different taxonomic levels (Wendel *et al.*, 1995, Baldwin *et al.*, 1995, Sang *et al.*, 1995, Becerra and Venable, 1999). To date, sequence data of nuclear DNA have not been used in phylogenetic study of *Plantago* spp., especially in Mongolia and Vietnam.

In this study, we analyzed the nucleotide sequences of ITS region of the nuclear ribosomal DNA from 10 taxa of *Plantago* spp. and 2 outgroups to 1) address the circumscription of the genus and species within the genus; 2) reconstruct the phylogeny within the genus. This information should contribute to develop a reasonable classification system and to a better understanding of the evolution of *Plantago* spp. in Mongolia and Vietnam.

Materials and Methods

Sample collection: *Plantago* spp. were collected from 10 different regions in Mongolia and Vietnam (Fig. 1 and Table 1). A whole plant was taken for species determination. Voucher specimens of all the samples, morphologically authenticated by Professor Le Van Hoa, were deposited at College of Agriculture and Applied Biology, Can Tho University, Vietnam.

DNA amplification and sequencing: DNA extraction: Total nuclear DNA was extracted according to the CTAB method described by Kyndt *et al*. (2009). The DNA was dissolved in 200 µL 0.1X TE Buffer. DNA samples were stored at -20°C for further analysis.

PCR targeting ITS region and sequencing: PCR amplification of the ITS region, including the 5.8 S rDNA region, was conducted using the primers ITS1 and ITS4 (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'; ITS4: 5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990), using the C1000 Touch™ thermal cycler (Bio-Rad, USA). The thermal cycle was initially 94°C for 90 s, and then continued with 30 cycles of 95°C for 50 s, 55°C for 70 s and 72°C for 90 s; and a final step at 72°C for 10 min. A final hold at 10°C was set up if the reaction was not taken out after the program finished. Each PCR tube contained 50 ng DNA, 0.5 µM of each primer, 0.2 mM dNTPs, 0.5 U Taq DNA polymerase (Promega), PCR buffer (containing 2.5 mM $MgCl_2$). PCR products were purified by PureLink™ PCR Purification kit (Invitrogen) before sequencing using the ABI prism BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) on an automated sequencer (ABI prism 3130, Applied Biosystems).

Sequence analyses: The sequences were checked for failures or multiple peaks using the Sequensing Analysis software v5.3.1 (Applied Biosystems, USA) before conducting the sequence alignments, and constructed phylogenetic tree with 2 out group accessions (FJ024618 and AY101874) of *P. australis* by MEGA 6 program (Tamura *et al.*, 2013). The length and GC content of DNA sequences were calculated using the Endmemo software (http://www. endmemo.com/bio/gc.php).

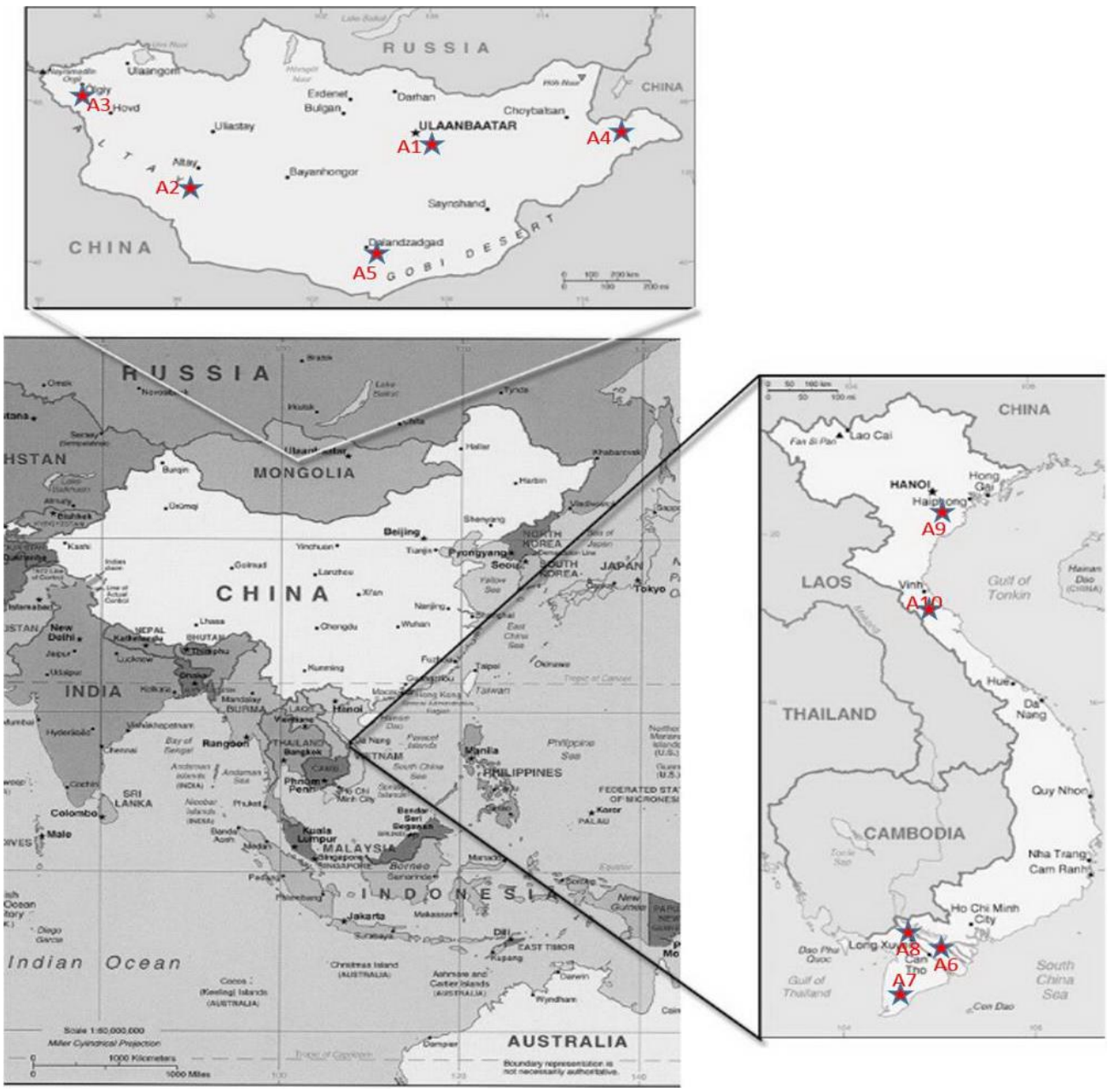

Fig. 1. Geographical locations of *Plantago* spp. collected from Mongolia and Vietnam. Star marks show collection sites.
From A1 to A10: 10 collected ecotypes belong to plant geographical regions which are Middle Khalkh dry steppe, Dzuungariin Gobi Desert, Mongolian Altai Mountain steppe, East Mongolia steppe and Alashaa Gobi Desert (Mongolia); Can Tho, Ca Mau, Kien Giang, Nam Dinh, Thanh Hoa (Vietnam), respectively.

Table 1. Geographical locations of *Plantago* spp. collected from different locations in Mongolia and Viet Nam.

Plant geographical region name	Country	Code	Location (Latitude, Longitude)		Date of collection
Middle Khalkh dry steppe		A1	47.8864° N	106.9057° E	July, 2015
Dzuungariin Gobi Desert		A2	45.4511° N	95.8506° E	July, 2015
Mongolian Altai Mountain steppe	Mongolia	A3	48.3983° N	89.6626° E	July, 2015
East Mongolia steppe		A4	47.4658° N	115.3927° E	July, 2015
Alashaa Gobi Desert		A5	43.5000° N	104.2861° E	July, 2015
Can Tho		A6	10.0452° N	105.7469° E	August, 2015
Ca Mau		A7	9.1527° N	105.1961° E	August, 2015
Kien Giang	Vietnam	A8	9.8250° N	105.1259° E	August, 2015
Nam Dinh		A9	20.4388° N	106.1621° E	August, 2015
Thanh Hoa		A10	20.1291° N	105.3131° E	August, 2015

Table 2. Identification of ITS sequences in *Plantago* species based on BLAST search.

No.	Sample	Sampling location site		Identified species	Ref. GenBank Accession No.
1.	A1	Middle Khalkh dry steppe		*Plantago depressa*	AB281168.1
2.	A2	Dzuungariin Gobi Desert		*P. depressa*	AB281168.1
3.	A3	Mongolian Altai Mountain steppe	Mongolia	*Plantago major*	AY101861.1
4.	A4	East Mongolia steppe		*P. depressa*	AB281168.1
5.	A5	Alashaa Gobi Desert		*Plantago major*	AY101861.1
6.	A6	Can Tho		*P. major*	AY101861.1
7.	A7	Ca Mau		*P. major*	AY101861.1
8.	A8	Kien Giang	Vietnam	*P. major*	AY101861.1
9.	A9	Nam Dinh		*P. major*	AY101861.1
10.	A10	Thanh Hoa		*P. major*	AY101861.1

Table 3. Length and GC content of ribosomal DNA sequences of *Plantago* spp.

Sample	Species	Entire region		ITS1		5.8S		ITS2	
		% GC	Length	% GC	Length	% GC	Length	% GC	Length
A1	*Plantago depressa*	53.9	636	51.9	213	56.2	162	53.4	261
A2	*P. depressa*	54.0	635	52.1	212	56.2	162	53.6	261
A3	*Plantago major*	55.2	632	53.0	210	56.2	162	55.2	260
A4	*P. depressa*	53.9	635	52.1	213	56.2	162	53.3	261
A5	*P. major*	55.0	633	52.8	211	56.2	162	55.2	260
A6	*P. major*	54.8	637	53.7	215	56.2	162	54.0	260
A7	*P. major*	54.5	637	53.4	216	55.6	162	53.9	259
A8	*P. major*	54.8	639	53.4	216	55.6	162	54.2	261
A9	*P. major*	55.1	644	54.4	222	56.2	162	54.0	260
A10	*P. major*	54.6	634	52.8	212	55.6	162	54.4	260

Results and Discussion

Divergence of ITS sequences in *Plantago* spp.: ITS sequences of *Plantago* samples were searched for similarity on the NCBI database, and the BLAST results are shown in Table 2. The most of the samples were *Plantago major* (7 sp.), subspecies of *P. major* and the others were identified as *Plantago depressa* (3 sp.) which have geographical origin in Mongolia, subspecies of *P. depressa*. All samples collected in Vietnam were *P. major*. *Plantago* encompasses about 200 species. They are found worldwide, including America, Asia, Australia, New Zealand, Africa and Europe. They can also be found in alpine and semi-alpine or coastal areas. According to Flora of China editorial committee (2011) and Ho (2003), *P. major* can grow in many areas, at mountain slopes, ravines, riverbanks, fields, roadsides, wastelands, lawns; near sea level to 3800 m. It is distributed in China, Pakistan, Bangladesh, Bhutan, India, Vietnam, Indonesia, Japan, Korea, Malaysia, Mongolia, Nepal, and Sri Lanka. Whereas *P. depressa* is just distributed in seaside sandy areas; near sea level to 4500 m (China, Afghanistan, Bhutan, India, Kashmir, Kazakhstan, Korea, Kyrgyzstan, Mongolia, Pakistan, and Russia). In this present study, however, the molecular evidence supported that *P. depressa* is also distributed in Mongolia while *P. major* presents in Mongolia and Vietnam.

The amplified ITS sequences of *Plantago* included the 5.8S rDNA gene and two ITS regions (ITS1 and ITS2). The length of sequences ranged from 632 to 644 bp (ITS1 ranged from 210-222 bp, 5.8S was 162 bp, and ITS2 from 259 to 261 bp) (Table 3). The sizes of ITS sequences were in the range of angiosperm ITS lengths which are from 500 to 700 bp (Alvarez & Wendel, 2003; Baldwin *et al.*, 1995). The lengths of ITS1 and 5.8S were similar to the sequences of *Plantago* genus analyzed by Ronsted *et al.* (2002).

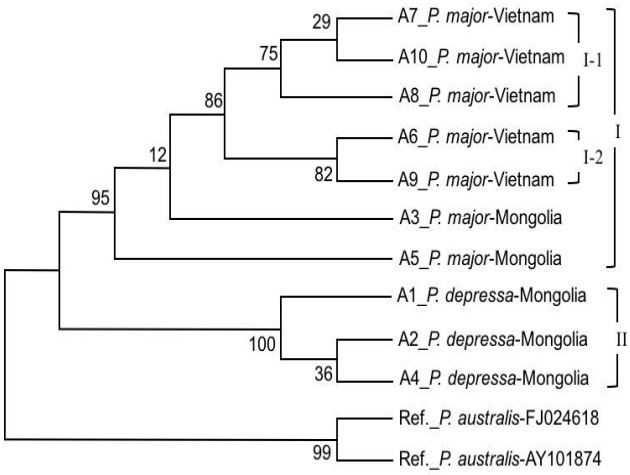

Fig. 2. Maximum Parsimony phylogenetic tree of 12 *Plantago* taxa (2 references obtained from NCBI database included FJ024618 and AY101874).

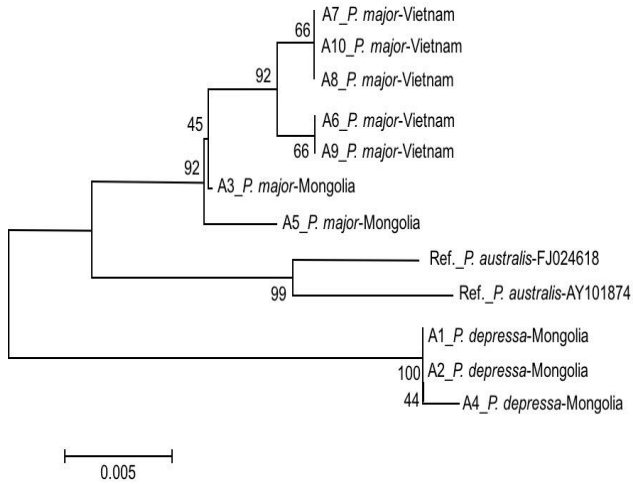

Fig. 3. Evolutionary relationships of 12 *Plantago* taxa (2 references obtained from NCBI database included FJ024618 and AY101874) based on the NJ analysis.

The highest length divergence of ITS sequence was observed from the ITS1 region which had 12 bp indel (insertion/deletion), whereas this figure was 0 bp in the 5.8S rDNA gene (Table 3). The ITS2 region had a slight difference in sequence length divergence with only 3 bp. This means the ITS1 sequence had more informative in term of genetic alternative than ITS2 and 5.8S regions in the case of *Plantago* spp. The ITS regions are supported to have higher genetic changes than other sequences because they are not functioned in encoding rRNA subunits.

The GC contents of entire sequences differed in 1.3% with the lowest percentages found in A1 (Middle Khalkh dry steppe, Mongolia) and A4 (East Mongolia steppe, Mongolia) sequences (Table 3). The highest variation in CG content (2.5%) was recorded in the ITS1 region, and the least divergence was 5.8S sequence (only 0.6%). Interestingly, the A7, A8, and A10 sequences had a slight difference of GC content compared to the others. This variation caused by some nucleotide substitutions because the sequence lengths were equal.

Phylogenetic analysis: The maximum parsimony (MP) tree (Fig. 2) and Neighbor-Joining (NJ) tree (Fig. 3) were constructed and compared. Both MP and NJ trees resulted in 2 major clusters according to their classification (*P. major* and *P. depressa*), but slight differences were observed in the clades between the NJ tree and the MP tree. The consistency index (CI) and retention index of the MP tree was 1.00 which indicated that the tree was strongly supported.

Group I (*P. major*) consisted of 5 taxa distributed in Vietnam and 2 species in Mongolia (A3 and A5). However, the location of the sample A5 in the group was strongly supported by 95 and 92% bootstrap values in both MP and NJ trees, respectively, whereas the sample A3 arrangement was not confirmed due to the low bootstrap value.

This group was also separated in 2 sub-group including I-1 (with A7, A8, and A 10) and I-2 (with A6 and A9). Among taxa collected in Vietnam, A6, A7, and A8 originated from nearby regions. However, the A6 taxon had a small distance from the others which is supported by 86 and 92% bootstrap values in MP and NJ phylogenetic trees, respectively. The taxa A9 and A10 were not in the same sub-group despite collected in similar geographical origins (Nam Dinh and Thanh Hoa, Vietnam).

Group II (*P. depressa*), which included the sequences A1, A2, and A4 (originated from the same geographic location, Mongolia), was supported with 100% bootstrap value. Discriminating of *P. major* from *P. depressa* using ITS sequences is not only really important for plant taxonomist, but this result is also necessary for pharmacist to identify the herbal species in *Plantago* genus due to phenotypic characteristics depending on environmental variation (Sahin *et al.*, 2007). In addition, not all *Plantago* species have been used as traditional herbs. Among them, *P. major* is listed in Japanese and Chinese Pharmacopoeia (Sahin *et al.*, 2007).

The NJ tree showed the genetic distance of the taxa. With 0.06 total length of branches, the genetic variability among taxa was not high. The figure indicated that although the taxon A4 distributed in the same group with other *P. depressa*, it had a small genetic difference. The taxa within the sub-group I-1 and I-2 had no genetic variability.

Conclusions

The findings confirmed the genetic evolution and relationship of *P. major* and *P. depressa* distributed in Mongolia and Vietnam based on ITS sequences. The most genetic variation was found in ITS1 region among 10 taxa. The MP and MJ phylogenetic trees suggested the strong biogeographical relationship of *P. major* collected in Mongolia and Vietnam.

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