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# Phylogenetic Analysis of Native *Vigna sinensis* in Korea Using DNA Sequence of Internal Transcribed spacer (ITS) Region

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Cowpea (*Vigna unguiculata* (L.) Walp.) is recognized as a potential source of protein and other nutrients. The genus *Vigna* includes 100 wild species of plants. Especially, *Vigna unguiculata* includes annual cowpeas (ssp. *unguiculata*) and ten wild perennial subspecies. DNA sequence of internal transcribed spacer (ITS) region was determined for *Vigna sinensis,* one of native plant, which was found in recent but thought to have gone extinct in Korea. The seeds of *Vigna sinensis* used in this study were donated from Dong-Young Jo. The DNA sequence of ITS-5.8S-ITS2 for *Vigna sinensis* obtained from this study was deposited as *Vigna sinensis* AY195581 on GenBank of NCBI (National Center for Biotechnology Information). We investigated the sequence-based phylogenetic relationships of plants related and clarified its taxonomical position. DNA similarities among subspecies including *Vigna unguiculata* showed the range 98 to 100% in sequence-based phylogenetic analysis using total 507 base pairs of ITS1, 5.8S and ITS2. *Vigna unguiculata* and subspecies were grouped independently as one cluster from other *Vigna* species used in the phylogenetic analysis. In this study, based on the phylogenetic analysis using the ITS1-5.8S-ITS2 sequence of *Vigna sinensis*, it may be concluded to be classified to one of *Vigna unguiculata* substrains.

**Key words** : Cowpea, ITS, phylogeny, *Vigna sinensis*, *Vignia unguiculata*

## Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is recognized as a potential source of protein and other nutrients. It is cultivated for its immature pods and mature seeds and is consumed by people all around the world, especially in the developing nations [5, 10]. Cowpea [*Vigna unguiculata* (L.) Walp.] is an important food legume in less-developed countries of the tropics and subtropics, especially in sub-Saharan Africa, Asia, and Central and South America [8] and this region in West and Central Africa represents over 66% of the 12.5 million ha grown worldwide. It is a staple food for millions of poor people living in the dry areas of the world where it is difficult to grow any other crop. The worldwide production of dry cowpea for 2002 was estimated at 7.4 billion pounds from 20 million acres and the major producing countries are of Africa, Asia and Latin America.

The genus *Vigna* includes 100 wild species of plants [6]. Especially, *V. unguiculata* includes annual cowpeas (ssp. *unguiculata*) and ten wild perennial subspecies. Subspecies *unguiculata* includes all the domesticated (var. *unguiculata*), as well as wild and weedy, forms [var. *spontanea* (Schweinf.) Pasquet] [4]. Six species of them are staple crops of cultivation and it is presumed that five of the six species be originated from Asia. Previously, according to some researchers, *Vigna unguiculata* (L.) Walp. has been mixed with *V. sinensis*, *V. catjang*, *V. repens*, *Dolichos unguiculata*, *D. sinensis*, or *D. datjang* and so on, but it was mainly called as *V. sinensis.* Usual name has been also called as cowpea, black-eye pea, catjang, catiang, asparagus bean, yard-long bean or others. Even though plant taxonomy for the genus *Vigna* still has been much argued, the species including the genus *Vigna* are classified into five subspecies including two wild type species (subsp. *dekindtiana* growing naturally in regions of Ethiopia and Saharan Africa and subsp. Mensensis) and three cultivation type species (subsp. *unguiculata,* subsp. *cylindrical* and subsp. *sesquipedalis*) [11]. Maréchal *et al.* [3] clas-

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sified three cultivation type subspecies as variants and called them bound with cv-gr. *Textilis* Subsp. *unguiculata*, and classified four variants, subsp. *stenophylla*, subsp. *stenophylla* and subsp. *tenuis* into subsp. *dekindtiana*.

At present, most of researchers classify cultivation type into three subspecies, but others have been still classifying the subspecies to distinct species. *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* is called as cowpea or common cowpea and it as single subspecies occupies the biggest cultivation area in the world.

In this study, DNA sequence of internal transcribed spacer (ITS) region was determined for *Vigna sinensis,* one of native plant, which was found in recent but was thought to have gone extinct in Korea. We investigated the Sequence-based phylogenetic relationships of plants related and clarified its taxonomical position.

## Materials and Methods

### The seed of Vigna sinensis

The seed of *Vigna sinensis* used in this study was donated from Dong-Young Jo.

### DNA isolation

For DNA extraction, 100 mg seed sample from conservation treatment of frozen in liquid nitrogen) was finely ground in liquid nitrogen. The pulverized sample was incubated in buffer (2% cetyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris–HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1% polyvinylpyrrolidone [mass weight 10,000], 0.2% β-mercaptoethanol, and 0.1 mg $mL^{-1}$ proteinase K) at 55°C for 60 min [1]. After this step, the solution was extracted twice with chloroform:isoamyl-alcohol (24:1 v/v). DNA was precipitated by the addition of cold isopropanol to the solution followed by centrifugation; the resulting pellet was washed with 70% ethanol and allowed to air dry. The DNA pellet was resuspended in 50 µL TE buffer (10 mM Tris–HCl [pH 8.0] and 0.1 mM EDTA [pH 8.0]) containing ribonuclease A (10 µg $mL^{-1}$) and incubated at 37°C for 30 min [7].

### PCR amplification

To evaluate DNA suitability for PCR amplification, primers specific for the internal transcribed spacer (ITS) region of ITS1-5.8S-ITS2 ribosomal DNA [13] were used for amplification in 20-µL final reaction volumes containing 25 ng DNA, *Taq* polymerase buffer (50 mM KCl, 10 mM Tris–HCl [pH 8.8], and 0.8% Nonidet P40), 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, and 1 U *Taq* polymerase (Fermentas Life Sciences, Burlington, Canada). PCR amplification was performed as described by Spiridonov and Moens [9]. Two sets of primers were used in the PCR reactions: (1) TW81 (5-GTTTCCGTAGGTGAACCTGC-3) and AB28 (5-ATATGCTTAAGTTCAGCGGGT-3) or (2) 18S (5-TTGATTACGTCCCTGCCCTTT-3) and 26S (5-TTTCACTCGCCGTTACTAAGG-3) [2, 12], respectively. PCR products were purified using QIAquick PCR or Gel Purification Kit (Qiagen Ltd, Crawley, UK). Amplifications were conducted as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C, with a final extension at 72°C for 7 min. Amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained with SYBR Gold.

### DNA Sequencing

The refined PCR products were directly sequenced with the use of a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). The cycle sequencing reaction was performed with a PCR Thermal Cycler$^{MP}$ (Takara, TP3080, Japan). The sequencing reaction was performed for 5 min at 96℃ at first and consisted of 25 cycles of the following: 10 sec at 96℃, 10 sec at 52℃, and 2 min at 60℃. The cycle sequencing products were purified with use of CENTRI-SEP Spin Columns (Applied Biosystems, USA). DNA sequences were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA).

### Alignment and phylogenetic analysis

The nucleotide sequence of ITS1-5.8S-ITS2 was aligned with reference sequences obtained from plants related using BLAST Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.BlAST). After collecting DNA sequences, the phylogenetic tree was constructed on BLAST.

## Results and Discussion

The DNA sequence of ITS-5.8S-ITS2 for *Vigna sinensis* obtained from this study was deposited as *Vigna sinensis* AY 195581 on GenBank of NCBI (National Center for Biotechnology Information). DNA similarities among subspecies including *Vigna unguiculata* showed the range 98 to 100% in sequence-based phylogenetic analysis using total 507 base pairs of ITS1, 5.8S and ITS (data not shown). *Vigna un-*

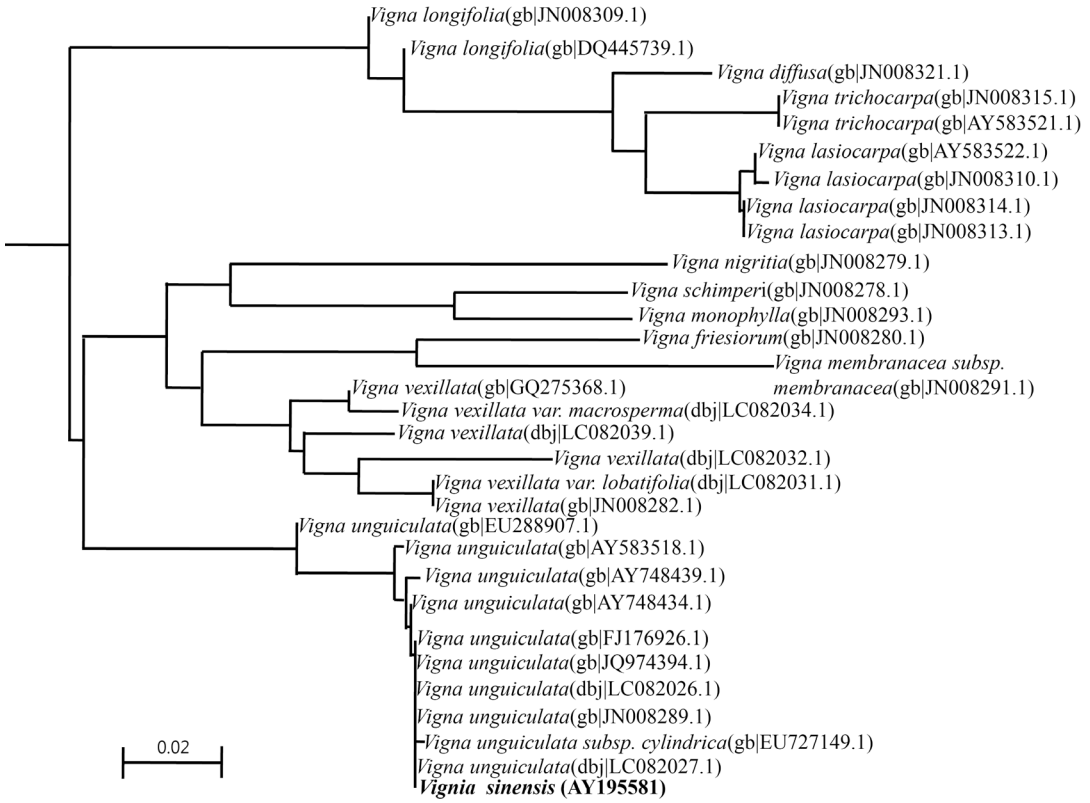


Fig. 1. Phylogenetic tree of ITS1-5.8S-ITS2 sequences for *Vigna* species using Neighbour-Joining method. A total of 507 bp aligned positions were used. *Vigna sinensis* of which the sequence was determined in this study is indicated in bold. Bar, 0.02 substitution per nucleotide position.

*guiculata* and subspecies were grouped independently as one cluster from other *Vigna* species used in the phylogenetic analysis (Fig. 1). It is presumed that the cluster of *Vigna unguiculata* subspecies be branched comparatively lately to other species of genus *Vigna*, and now occurred much on genetic divergence. In addition, it is showed that the cluster of *Vigna unguiculata* subspecies is genetically independent, compared to other species of genus *Vigna, Vigna longifolia, Vigna vexillata, Vigna membranacea, Vigna friesiorum, Vigna monophylla, Vigna schimperi, Vigna nigritia, Vigna lasiocarpa, Vigna trichocarpa* and *Vigna diffusa* (Fig. 1).

In this study, based on the phylogenetic analysis using the ITS1-5.8S-ITS2 sequence of *Vigna sinensis*, it may be concluded to be classified to one of *Vigna unguiculata* subspecies. The fact of this study is that the phylogenetic position of *Vigna sinensis,* one of native plant, found in recent but thought to have gone extinct in Korea, is clearly identified from plants related of *Vigna unguiculata* through sequence-based phylogenetic analysis.

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## 초록 : 토종 갓끈동부의 ITS1, 5.8S 및 ITS2의 염기서열을 이용한 계통 분석

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본 연구에서 밝혀진 갓끈동부의 ITS1, 5.8S 및 ITS2의 염기서열은 NCBI (National Center for Biotechnology Information)의 GenBank에 *Vigna sinensis* AY195581로 등록하였다. ITS1, 5.8S 및 ITS2의 총 염기서열 507 염기서열을 이용한 *Vigna sinensis* (AY195581)의 분자계통분석에서 *Vigna unguiculata* 및 그 아종들과 98~100% 범위의 염기서열 상동성을 보였다. *Vigna unguiculata*는 계통분석에 이용된 다른 종들로부터 독립된 하나의 cluster로 그룹핑(grouping)이 됨을 확인하였다. 본 계통분석은 *Vigna unguiculata*가 *Vigna* 속의 다른 종에 비해 비교적 최근에 분화되었으며, 현재 유전적인 변화가 많이 일어나고 있음 보이고 있다. 또한, *Vigna* 속, *Vigna longifolia, Vigna vexillata, Vigna membranacea, Vigna friesiorum, Vigna monophylla, Vigna schimperi, Vigna nigritia, Vigna lasiocarpa, Vigna trichocarpa, Vigna diffusa*의 다른 종들과 비교하여 유전적으로 독립적인 종임을 확인하였다. 본 연구의 *Vigna sinensis*의 ITS1, 5.8S 및 ITS2를 이용한 계통분석은 *Vigna sinensis*를 *Vigna unguiculata*로 분류하는 것이 타당한 것으로 보여진다. 본 종은 국내에서 멸종된 것으로 알려져 있었으나 최근 토착 식물로써 발견되었고 이 갓끈동부의 관련 식물 종들과의 분자계통학적 위치를 명확히 밝힘에 의의가 있다고 하겠다.