Potential use of ITS2 and *matK* as a Two-Locus DNA Barcode for Identification of *Vicia* Species

Sebastin Raveendar, Jung-Ro Lee, Jae-Wan Park, Gi-An Lee, Young-Ah Jeon, Yun Jeong Lee, Gyu-Taek Cho, Kyung-Ho Ma, Sok-Young Lee, Jong-Wook Chung*

National Agrobiodiversity Centre, National Academy of Agricultural Science, Rural Development Administration, Jeonju 560-500, Republic of Korea

ABSTRACT We investigated the species discriminatory efficiency of the proposed plant barcoding loci ITS2 and *matK* in *Vicia* species. In 2011, China Plant BOL Group proposed the addition of nuclear ITS2 to *matK* be accepted as a 2-locus DNA barcode to classify plant species. The *matK* region was chosen as a DNA barcode because of its effective species discriminating power, high quality sequence recovery, and easy experimental analysis. Integration of *matK* sequences into *Vicia* phylogeny could improve phylogenetic reconstruction of this species. To assess the ability of barcoding loci to resolve *Vicia* species, we sampled 36 of the taxonomically best known groups in the genus. Topologies of the phylogenetic trees based on ITS2 and *matK* analyses were similar but a few accessions were placed into distant phylogenetic groups. Neither ITS2 nor *matK* analyses alone could discriminate some closely related *Vicia* species. Thus, we have proposed a concatenated data approach to increase the resolving power of ITS2 and used *matK* as an additional tool for phylogenetic analysis in *Vicia* because characterization of the nucleotide sequences of the *matK* region was easier to recover and more cost-effective than those of the ITS region.

Keywords *Vicia*, Barcode DNA, Phylogenetic analysis, Species differentiation

INTRODUCTION

Legumes (Fabaceae) are the third largest flowering family and are second only to cereals in their importance for human nutrition. Archaeological evidence suggests that the Mediterranean region is the principal center of origin for legumes (Perrino 1988). Legumes are inexpensive, nutrient-dense, and high-quality sources of protein that can be substituted for dietary animal protein (Anderson *et al.* 1999). Legumes also produce a high diversity of secondary metabolites which serve as medicinal, nutraceuticals, and eco-friendly by-products such as tannins, gums, insecticides, resins, varnish, paints, dyes, and biodiesel. Moreover, there is renewed interest in grain legumes for their use in animal feed. Equally important is the role of legumes to sustainable agriculture, because of their ability to fix soil nitrogen via symbiotic nitrogen-fixation. They also play an important

role as a rotation crop with cereal grain and vegetable crops (Arianoutsou and Thanos 1996; Velazquez *et al.* 2010)

The genus *Vicia* L. is a member of the legume tribe Viciae of the subfamily Papilionoideae (Frediani *et al.* 2004). The temperate and herbaceous genus *Vicia* L. comprises 210 annual or perennial species that are widely distributed in temperate regions of Europe, Asia, and the Americas (Maxted 1993; Jaaska 2005). The identification of *Vicia* species using a method based on their morphological characteristics has proved tricky and is rather difficult to account for the entire genetic variation existing in *Vicia* species (Haider *et al.* 2000). Hosseinzadeh *et al.* (2008) reported that there are several species with intermediate morphological characters which makes species diagnosis a difficult task for non-specialists. Large scale structural changes were observed in *Vicia* chromosomes; thus, cytological and karyological studies have been performed

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*Corresponding author Jong-Wook Chung, jwchung73@korea.kr, Tel: +82-63-238-4872, Fax: +82-63-238-4829

to discriminate their taxonomy (Maxted *et al.* 1991; Navratilova *et al.* 2003); however, earlier morphological and cytogenetic studies had limited ability to discriminate the species. Thus, a robust and reliable method is crucial to discriminate plant species to secure their diversity. Recently, DNA barcoding has become a hotspot of biodiversity research (Gregory 2005).

The barcoding method has been used to identify a specific region of the plant genome that can be sequenced routinely in diverse sample sets, resulting in easily comparable data which enable species to be distinguished from one another (Chen *et al.* 2010). Many recent papers have been reviewed about DNA barcoding in plants (Vijayan and Tsou 2010; Hollingsworth *et al.* 2011). There has been considerable debate regarding locus choice for DNA barcoding and a number of single locus and combined loci have been proposed as barcode sequences (Chase *et al.* 2007; Kress and Erickson 2007), but no consensus has emerged on the use of a standard region. To facilitate the selection of a plant barcode, the Consortium for the Barcode of Life (CBOL) was formed from the systematics research community. Subsequently a number of loci have been tested for their suitability as plant bar-codes and CBOL research groups have proposed combinations of *rbcL* and *matK* loci as their preferred plant barcodes (Group *et al.* 2009). Several researchers have already demonstrated the potential for using ITS2 for taxonomic classification and phylogenetic reconstruction at both the genus and species levels for eukaryotes, including animals, plants, and fungi (Schultz *et al.* 2005; Yao *et al.* 2010; Bellemain *et al.* 2010). The ITS2 region of nuclear DNA provides a powerful tool because of sufficient variation in primary sequences and secondary structures. To achieve maximum species discrimination, the China Plant BOL (Group Li *et al.* 2011) proposed the addition of nuclear ITS2 to the *matK* + *rbcL* combination as a plant barcode. We therefore evaluated the use of a nuclear region ITS2 along with a chloroplast region *matK* for discrimination of *Vicia* L. species and to compare phylogenetic tree topologies of *matK* and ITS2 analyses.

MATERIALS AND METHODS

Plant Material and DNA extraction

Seeds of genebank accessions (a total of 105 accessions) belonging to 36 species of *Vicia* were provided by the Genetic Resource Center of the National Academy of Agricultural Science, Rural Development Administration, Republic of Korea (Table S1). To determine which of a subset of the proposed barcoding loci are necessary to resolve *Vicia* species, we sampled a total of 105 accessions which contains one to five individual accessions. Seeds were germinated and leaf tissue was harvested from 3 weeks old seedling in the greenhouse. Total DNA was extracted using the DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Fresh leaf tissue from each accession was used for each extraction and was ground well in liquid nitrogen. DNA was resuspended in 100 µL water, and dilutions were made to 10 ng/µL followed by storage at either -20°C or -80°C. Genomic DNA was quantified using a Nanodrop/UVS-99 instrument (ACTGene, USA), and the A260/A280 nm ratio was determined. DNA quality was confirmed on a 0.8% agarose gel.

PCR amplification and Sequencing

The internal transcribed spacer ITS2 nuclear region and the *matK* chloroplast region of 105 individuals, belonging to 36 species of *Vicia* were tested for their ability to resolve congeneric species and to infer their applicability and efficacy as DNA barcodes. The sequences of the universal primers for ITS2 and *matK* regions and general PCR reaction conditions were obtained from previous studies (Chen *et al.* 2010). Amplification reactions were carried out in a total volume of 20 µL containing 1× PCR buffer, 0.1 mM primers, 0.2 mM each dNTP, 1 U Taq DNA polymerase, and 200 ng template DNA.

Approximately 1 µg DNA was used to generate a DNA library to use with the Genome Sequencer GSFLX Titanium System (Roche, 454 Life Science, Branford, CT, USA). The DNA fragment ends were polished (blunted), and two short adapters were ligated to both ends according to standard procedures (Moe *et al.* 2011). Finally, the quality

of this single stranded template DNA library was assessed using a 2100 BioAnalyzer (Agilent, Waldbronn, Germany). The library was quantified to determine the optimal amount needed as input for emulsion-based clonal amplification. Multiplex identifiers were used to specifically tag unique samples in a GS FLX Titanium sequencing run, which were recognized by the GS data analysis software after the sequencing run and provided high confidence for assigning individual sequencing reads to the correct sample.

Phylogenetic analysis

Sequences of ITS2 and *matK* regions were manually edited with MEGA6 (Tamura *et al.* 2013) and aligned using the “ClustalW” option. Manual adjustment in the alignment of the nucleotide sequences for the ITS2 region was made to improve alignment. Aligned ITS2 and *matK* sequences were evaluated with bootstrap analysis (1000 replicates) (Felsenstein 1985) and Kimura 2-parameter distances were calculated to construct a neighbor-joining (NJ) dendrogram using the MEGA6 with the program (Kimura 1980). Aligned ITS and *matK* sequences were also used for the maximum parsimony (MP) method. MP trees were obtained using the tree-bisection-regrafting (TBR) algorithm (Nei and Kumar 2000) with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). MP trees were tested with bootstrap analysis (1000 replicates). All positions containing gaps in the ITS2 and *matK* sequences were eliminated. MP analyses were conducted using the program MEGA6 (Tamura *et al.* 2013).

RESULTS

We obtained 105 ITS2 and 105 *matK* sequences from 36

Vicia species, resulting in 210 new sequences. With regard to universality of primers and success of sequence amplification, the proportion at each of the regions was 100% for both the chloroplast *matK* and nuclear ITS2 region (data not shown). The ClustalW aligned sequence length of ITS2 was 301 bp and the sequence length of *matK* was 665 bp; the resulting concatenated sequence length of ITS2 and *matK* was 966 bp. All positions containing gaps and missing data were eliminated. There were a total of 957 positions in the final dataset.

A total of 210 sequences were analyzed from 36 *Vicia* species in this study. The sequence analysis revealed the average nucleotide frequencies as A = $34.5 \pm 0.20\%$, T = $28.9 \pm 0.30\%$, G = $19.7 \pm 0.30\%$, C = $16.9 \pm 0.20\%$. The phylogenetic utility of ITS2 and *matK* regions is shown in Table 1. The number of segregating sites (S) = 314 and nucleotide diversity was (Pi) = 0.0556. Tajma’s statistics was (D) = -1.0021 and it was not significantly ($p < 0.01$) different among sequences. Kimura 2 Parameter (K2P) genetic distance in *Vicia* species is given in Table S2. The overall K2P distance between the 36 species was 0.060.

The NJ tree developed using Kimura (1980) distances based on nucleotide sequences of the ITS2 region had a similar topology as the MP tree, shown in Figure 1. The phylogenetic tree based on the NJ analysis of Kimura (1980) distances of the nucleotide sequences of the *matK* region was topologically similar to the MP tree in Figure 2. However, trees generated by NJ and MP analyses differed in their branch-length values (data not presented).

Phylogenetic analyses based on the nucleotide sequences of the ITS2 region generally discriminated the *Vicia* species (Fig. 1). Although the ITS2 region separated most of the *Vicia* species from each other, some closely related species could not be discriminated (Fig. 1). For example, *V.*

Table 1. Phylogenetic utility of ITS2 and *matK* regions.

	ITS2	<i>matK</i>	ITS2 + <i>matK</i>
Individuals (n)	105	105	210
Aligned length (bp)	301	665	966
Nucleotide diversity (Pi)	0.1133	0.0296	0.05563
Variable sites	187	127	314
Informative sites	181	101	282
Tajima’D	-1.0293	-0.9271	-1.0021

aintabensis, *V. Americana*, *V. amoena*, *V. anatolica*, and *V. articulata* accessions were located in the same clade without any differences. Phylogenetic analysis of the ITS2 region also demonstrated that, for several species, accessions were located in distant clades, suggesting that there might be mixtures or interspecies variation in the accession. For example, an accession of *V. villosa* subsp. *villosa* was

located in a clade with the accessions of *V. sativa* (Fig. 1).

Similarly, the phylogenetic analyses based on the nucleotide sequences of *matK* region generally discriminated the *Vicia* species (Fig. 2). Although most of the species were also discriminated by *matK*, some closely related species could not be distinguished (Fig. 2). For example, accessions of *V. anatolica*, *V. amoena*, *V. articulata*, *V. benghalensis*, and

Fig. 1. Phylogenetic analysis of 36 *Vicia* species based on the nucleotide sequences of the ITS2 region. The NJ tree was developed using Kimura 2-parameter method on nucleotide sequences of the ITS2 region. Numbers next to the branches are the bootstrap test values.

V. villosa were placed in the same clade with no clear differences. *matK* analysis also confirmed mixtures. For instance, different accessions of *V. hyrcanica* and *V. sativa* were placed in distant clades.

Topologies of the phylogenetic trees based on concatenated ITS2 and *matK* regions were similar, but there were some

Vicia species that were placed in different clades after ITS2 or *matK* analysis. For example, ITS2 analysis placed *V. aintabensis* and *V. Americana* accessions into the same clade of *V. amoena*, *V. anatolica*, and *V. articulata* (Fig. 1). However, *matK* analysis placed *V. aintabensis* and *V. Americana* accessions into different clades (Fig. 2).

Fig. 2. Phylogenetic analysis of 36 *Vicia* species based on the nucleotide sequences of the *matK* region. The NJ tree was developed using the Kimura 2-parameter method on nucleotide sequences of the *matK* region. Numbers next to the branches are the bootstrap test values.

Similarly, an accession of *V. hyrcanica* (68) was placed into the same clade as *V. hyrcanica* accessions, which was misplaced by ITS2 (Fig. 2). Phylogenetic trees based on concatenated ITS2 and *matK* regions have improved the topologies of the phylogenetic tree (Fig. 3). Although this barcoding region successfully discriminated all 36 *Vicia* species, the closely related species were not discriminated. For example, concatenated ITS2 and *matK* analysis failed to discriminate the closely related species *V. amoena*, *V. anatolica*, and *V. articulata* (Fig. 3).

DISCUSSION

Taxonomy of the genus *Vicia* L. has been problematic as the taxonomic history of the genus is extensive and contentious, having 20 major classification of the genus (Maxted 1993). The high economic importance of the genus *Vicia* has led to a large body of studies on the molecular characterization and investigation of phylogenetic relationships among species that belong to this genus. Various studies have been reported for investigation of

Fig. 3. Phylogenetic analysis of 36 *Vicia* species based on the nucleotide sequences of the ITS2 and *matK* region. The NJ tree was developed using the Kimura 2-parameter method on nucleotide sequences of the ITS2 and *matK* region. Numbers next to the branches are the bootstrap test values.

phylogenetic relationships among species based on rDNA (Raina and Ogihara 1995), in situ hybridization with repetitive sequences (Navratilova *et al.* 2003), RAPD analysis (Haider *et al.* 2000; Sakowicz and Cieslikowski 2006), repetitive DNA sequences as probes (Frediani *et al.* 2004), capillary electrophoresis (Piergiorganni and Taranto 2005), and SDS-PAGE on seed storage proteins (Mirali *et al.* 2007). However, none of these could resolve the taxonomic problems.

Previously, the ITS region was suggested for phylogenetic analysis in plants (Chen *et al.* 2010; Gao *et al.* 2010). Recently, nucleotide sequences of some DNA regions in the chloroplast genome (*matK*, *rpoC1* *rpoB*, *trnH-Psba* *rbcL*, *atpF-atpH*, and *psbK-psbI*) and their combinations were tested as potentials for barcoding plant species (Starr *et al.* 2009). Among these regions, *matK* and *rbcL* were accepted as a 2-locus DNA barcode by the CBOL Plant Working Group (Group *et al.* 2009). In this study, the potential use of ITS2 nucleotide sequences combined with *matK* was tested as a two-locus DNA barcode for discrimination of the *Vicia* species. According to our results, ITS2 and *matK* regions were both easy to amplify with PCR. Species resolution of both DNA regions was similar because the topologies of trees of ITS2 and *matK* were similar. Both DNA regions generally separated sections in *Vicia*. However, several species could not be discriminated from each other by either ITS2 or *matK* regions alone. It was reported that the ITS region requires cloning before sequencing because of the allelic polymorphisms, pseudogenes, and paralogous copies of the ITS region in a plant species (Bailey *et al.* 2003; King and Roalson 2008). However, there was no allelic polymorphism or insertion/deletion in the *matK* region within the chloroplast genome of *Vicia* species. Therefore, we were able to efficiently amplify and sequence-characterize the *matK* region without cloning.

Phylogenetic relationships among *Vicia* species based on both ITS2 analyses were in agreement with the previous studies using the ITS region (Shiran *et al.* 2014). Genetic relationships among 49 *Vicia* species were recently analyzed using the polymorphisms within the region of nrDNA which comprises both internal transcribed spacers, ITS1 and ITS2. The sequence data obtained for ITS2 and

matK were used to discriminate the species used. However, the dendrogram did not indicate any clear division among the *Vicia* accessions. Although it did not perform well in the strict sense, it can still be used to discriminate both *V. faba*, *V. sativa*, *V. ervilia*, and *V. benghalensis*. Chung *et al.* (2013) reported a cluster analysis of *V. sativa* subsp. *sativa* in which no clear clustering pattern of geographically close accessions was observed, indicating that the association between genetic similarity and geographical distance was of low significance. The placement of the other species used in this study in the phylogenetic tree cannot be directly compared with placements made in other studies owing to the methods used to construct the tree. Thus our results suggest that ITS2 and *matK* could make a significant contribution to resolving phylogeny at multiple taxonomic levels from generic relationships within *Vicia* species.

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