USING THE CHLOROPLAST rbcL GENE TO CLARIFY THE RELATIONSHIP BETWEEN SPECIES OF THE GENUS Stephania (Menispermaceae) FROM VIETNAM

Vu Tien Chinh¹²*, Tran Thi Lieu¹, Duong Van Tang¹

¹Vietnam National Museum of Nature, VAST, Vietnam
²Graduate University of Science and Technology, VAST, Vietnam

Received 23 April 2020, accepted 15 June 2020

ABSTRACT

The rbcL gene of the chloroplast genome is widely used as an additional data for the study of species origin, molecular evolution and phylogeny. In this study, we used the rbcL gene to separate three species of genus Stephania: S. japonica, S. polygona, S. rotunda and one subspecies S. japonica var. discolor from Vietnam. Molecular analysis was performed on 523 bp segment of the rbcL genes with 4 examined samples of the genus Stephania and 18 other genbank sequences of five genera Pachygone, Antizoma, Cissampelos, Cyclea and Syntriandrium. The dataset consists of 22 sequences used to reconstruct the evolutionary tree using two methods: Bayesian Infer (BI) and Maximum Likelihood (MP). The results showed that S. rotunda was able to distinguish from S. japonica or S. polygona, while S. japonica, S. japonica var. discolor and S. polygona could not distinguished each another. The phylogenetic tree split three examined species into two groups, representing the two main groups of morphology in genus Stephania: a group with tuberous rootstock and another group with main root.

Keywords: Chloroplast genome, gene rbcL, phylogeny, Stephania.

Citation: Vu Tien Chinh, Tran Thi Lieu, Duong Van Tang, 2020. Using the chloroplast rbcL gene to clarify the relationship between species of the genus Stephania (Menispermaceae) from Vietnam. Academia Journal of Biology, 42(2): 109–115. https://doi.org/10.15625/2615-9023/v42n2.15006.

*Corresponding author email: tienchinhvu@gmail.com

©2020 Vietnam Academy of Science and Technology (VAST)
INTRODUCTION

*Stephania* Lour. is a large genus of family Menispermaceae with about 60 species, most of which are distributed in tropical and subtropical regions of Asia and Africa, some species are also found in Oceania. Recently, 37 species were recorded in China, 15 species in Thailand (Lo, 1978; Hu, 2008; Chinh, 2016; Phuc, 2019). In Vietnam, this genus comprises ca. 20 species with the similar dioecious flower (Hang, 2014). In Vietnam, *Stephania* species have long been used in the traditional medicine to treat various diseases, such as asthma, tuberculosis, dysentery, hyperglycemia, malaria and cancer (Hang, 2014; Xie, 2015; Chinh 2019).

Presently, DNA data are widely and regularly used to provide additional evidence at the molecular level for plant taxonomic studies. The trend of combination morphological characteristics and chemical and genetic fingerprints into a dataset for species identification, becomes very important for systematic studies. Molecular analysis have been used as a tool to determine the evolutionary relationships among taxa at level the genus or family or order. DNA considered to be suitable for phylogenetic tree because nucleotide difference were accumulated over time in different groups of organisms, associated with the process of splitting species into new species. So, normally, close species will have a small genetic distance and vice versa, distant species will have a more large genetic distance. Molecular data is not only evidence for identifying species or supporting evidence to new species, it is also used to study the evolution process. The contribution of studies at the molecular level helps to rearrange the classification system more accurately and easily.

DNA data has been proposed as a tool to identify species through the comparison of short DNA sequences from an unknown sample to a library of DNA sequences of known species (Chase, 2005; Kress, 2005; Cowan, 2006). Although controversial, DNA data is still an effective tool to review taxon of plant (Fazekas, 2008; Lahaye, 2008). One of the difficulties in studying DNA data in plants is the poor ability to distinguish close species (sister species). Previous studies has shown that only 17% to 41.50% of examined species have different rbcL gene sequence (Bafeel, 2012, Kang, 2017) and this is low level of variability. However, the differences on DNA sequences between genera are obvious and large enough to identify different genera (Bafeel, 2012). The chloroplast genes *rbcL*, *matK*, *trnH-psbA*, and the nuclear ITS gene regions are considered to be DNA Barcode for species identification. According to the suggestion of The Consortium for the Barcode of Life, DNA Barcode of plant should be a combination marker as *matK* and *rbcL* genes (Kress, 2007). In this study, we tested the ability to distinguish species in the genus *Stephania* of chloroplast *rbcL* gene sequences. The purpose of the study is to assess the ability to identify species names base on this marker and review quickly taxonomic status of *Stephania*, from which orientation for further research.

MATERIALS AND METHODS

**Sampling**

In this study, four leaf samples of three species *S. japonica*, *S. polygona*, *S. rotunda* and one subspecies *S. japonica var. discolor* were collected in Ha Giang, Hoa Binh, Lam Dong Provinces and Ha Noi city. Plants were identified basing on leaf morphological characteristics and then stored in silicagel (Table 1).

**DNA extraction, amplification and sequencing tag segment**

Total DNA was extracted from leaf samples using DNeasy Plant Kits (Qiagen, Germany), then checked by electrophoresis on agarose gel 0.8% contained dye Florosafe DNA Stain and observed under UV light. The concentration and purity of total DNA were assessed by index OD\textsubscript{260nm}/OD\textsubscript{280nm}. Amplification of *rbcL* gene was performed by PCR reaction with primers *rbcL1F*: 5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3', *rbcL724R*: 5'-TCG CAT GTA CCT GCA CAA ACA GAG ACT AAA GC-3', *rbcL724R*: 5'-TCG CAT GTA CCT GCA CAA ACA GAG ACT AAA GC-3'.
Using the chloroplast rbcL gene to clarify

GTA GC-3’ (Fay, 1997). Amplification reaction were conducted with a volume of 25 µl, including the components: 1X PCR Buffer, 2.5 mM MgCl₂, 2 mM dNTPs, 0.5 mM for per primers, 0.5 unit Taq polymerase and 50 ng of total DNA. Amplification was performed on PCR systems 9700 in the following cycle: 1) 94°C: 5 minutes; 2) 94°C: 1 minute; 3) 55°C: 1 minute; 4) 72°C: 1 minute and repeat 35 cycles from step (2) to (4); and finish reaction at 72°C: 10 minutes. PCR products were carried out electrophoresis on agarose gel 0.8%, and then purified using the QIquick Gel Extraction Kit (Qiagen, Germany). Purified PCR products were used as template DNA for sequencing reactions with same PCR primers. Sequencing was performed on the ABI PRISM® 3100 Avant Genetic Analyzer (Applied Biosystems) at The National Key Laboratory of Gene Technology, Institute of Biotechnology, VAST.

Table 1. List of sequences used in the study

| No | Accession Genbank/Voucher | Species | Origin |
|----|---------------------------|---------|--------|
| 1  | S1                        | Stephania japonica var. discolor | This study |
| 2  | S2                        | Stephania polygona | This study |
| 3  | S3                        | Stephania japonica | This study |
| 4  | S4                        | Stephania rotunda | This study |
| 5  | KF496796                  | Stephania japonica | Genbank |
| 6  | JN051689                  | Stephania abyssinica | Genbank |
| 7  | JN051692                  | Stephania elegans | Genbank |
| 8  | FJ626601                  | Stephania longa | Genbank |
| 9  | JN051691                  | Stephania cephalantha | Genbank |
| 10 | JN051690                  | Stephania brachyandra | Genbank |
| 11 | FJ026509                  | Stephania rotunda | Genbank |
| 12 | EU526996                  | Stephania venosa | Genbank |
| 13 | DQ099437                  | Antizoma angustifolia | Genbank |
| 14 | JQ025032                  | Cissampelos capensis | Genbank |
| 15 | GQ436370                  | Stephania tetrandra | Genbank |
| 16 | JX944483                  | Stephania tetrandra | Genbank |
| 18 | KF181462                  | Cyclea polypetala | Genbank |
| 18 | FJ026482                  | Cyclea hypoglauca | Genbank |
| 19 | FJ026481                  | Cyclea burgmani | Genbank |
| 20 | FJ026508                  | Stephania laetificata | Genbank |
| 21 | JN051685                  | Pachygone loyaltiensis | Genbank |
| 22 | HQ260804                  | Syntriandrium preussii | Genbank |

Phylogenetic analysis

The dataset consists of 22 sequences, of which 18 sequences from genbank were used for analysis. The most fittest substitution model was found by Partitionfinder 2.1 for 3 sub-datasets which correspond to the 1st, 2nd, and 3rd positions of codon. Phylogenetic tree was generated by 2 methods: Bayesian Infer using Mr. Bayes and Maximum Likelihood using Treefinder.

RESULTS

Extraction, amplification and sequencing of target gene segments

We successfully isolated, amplified and sequenced the rbcL gene for 4 Steniphia samples from Vietnam. The total DNA has an ratio OD₂₆₀/₆₅₀/OD₂₈₀/₆₅₀ of 1.89, which shows good DNA quality because this ratio ranges from 1.8-2.0. The concentration of total DNA
was estimated at 560 ng/µl. The PCR of target segment obtained a single specific band (Fig. 1). The size of the PCR products was about 600 bp in length as expected. The sequencing reactions were successfully performed in both the forward and reverse directions and all sequences with clear fluorescence peaks, strong intensity, clarity and corresponding to each nucleotide. The sequences were checked for the target gene by Blast Nucleotide on NCBI (National Center for Biotechnology Information), and resultly, all of them were chloroplast rbcL gene.

Figure 1. Agarose gel electrophoresis (0.8%) showing PCR products (600 bp) from rbcL gene. Lanes: lane M, 1Kb DNA ladder; lane S1: S. japonica var. discolor; lane S2: S. polygona; lane S3: S. japonica; lane S4: S. Rotunda

The level of genetic variation between the three examined species of Stephania ranged from 0% (between S. japonica and S. polygona) - to 1.0% (between S. rotunda and S. japonica or S. polygona) (Table 2). The average level of diversity on the studied gene segment was 0.48%. This was a low level of diversity and often found in chloroplast genomes among closely related species (Kress, 2007). After cutting of primer sequences, the obtained rbcL gene segment with length of 523 bp contains: 27.72% A, 28.59% T, 22.23% G and 21.46% C, respectively and coding for 173 amino acids includes: Ala, Cys, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Tyr (20 types). The average rate of transversion (A ↔ T and G ↔ C)/transition (A, T ↔ G, C) mutation was \(R = 4.036\). The data set of 22 sequences contained 494 conservative sites, 27 variable sites with 16 parsimony information sites and 10 single mutation sites.

Reconstruct phylogenetic tree

Phylogenetic trees was obtained with the same topology for both methods: Bayesian Inference (BI) and Maximum Likelihood (MP). Based on rbcL 523 bp data, the phylogenetic tree has separated 3 studied species into 2 different groups. The first group includes S. japonica, S. polygona and sequences from Genbank such as S. abyssinica, S. longa, S. terrandra, S. elegans; the second group includes S. rotunda and other sequences from Genbank such as S. brachyandra, S. cephalantha and S. venosa (Figure 2). Both two groups were supported highly by bootstrap values (PP = 1, BS = 97 and PP = 0.96, BS = 87, corresponding to BI and MP methods. These two clades correspond to species groups with the tuberous rootstock and species groups with the main roots. The root structure is a key feature of species identification to genus Stephania (Chinh, 2015).

Genetic distance between S. japonica or S. polygona and S. rotunda was 1.0%, while between S. japonica and S. polygona was 0%. Thus, based on the results of the analysis here, we found that this rbcL gene segment could distinguish S. rotunda from S. Japonica or S. rotunda with S. polygona but it could not distinguish S. japonica with S. polygona. Phylogenetic analysis showed genus Stephania are not monophyly, instead of the genera Antizoma, Cissampelos, Cyclea nested in the genus Stephania. This
result was similar to previous molecular studies, confirming that genera *Stephania* are polyphyletic (Jacques et al., 2008; Wang et al., 2007), which have been grouped together but do not share an immediate common ancestor. The inconsistency between the molecular system and the traditional classification system has been pointed out in the genera of Menispermaceae (Jacques, 2008; Xie, 2015). Therefore, a combination of morphological and molecular characteristics is needed to rearrange the classification system of *Stephania* genus in the future.

Table 2. Genetic distance between 4 studied samples and 18 Genbank datas based on *rbcL* segment (calculated by MEGA v6.0 and the value have not yet multiplied by 100%)

| Sample | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|--------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| S1     | 0.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| S2     | 0.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| S3     | 0.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| S4     | 0.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| KF496796 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JN051689 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JN051692 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JN051691 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JN051690 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JX944483 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JN051689 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JX944483 | 0.01 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

Figure 2. Phylogenetic tree based on the sequence of 523bp gene *rbcL* (examined samples in bold letters)

**Differences between S. rotunda, S. japonica and S. Polygona**

Base on the 523 bp *rbcL* sequences in this study, *S. rotunda* has 5 different nucleotide compared with sequences of *S. japonica* or *S. polygona*. The 523 bp *rbcL* segment was aligned with reference full chloroplast DNA (contained *rbcL* sequence) (GenBank accession: KU204903) to locate sites of
mutation nucleotides. The alignment result showed that the mutations occurred at sites: 127 (A ↔ T), 147 (G ↔ A), 183 (C ↔ T), 189 (A ↔ G), 284 (G ↔ A). The characterized nucleotides of *S. rotunda* are: 127A, 147G, 183C, 189A, 284G, while the these of *S. japonica* or *S. polygona* are: 127T, 147A, 183T, 189G, 284A. We recommend the use these different nucleotide sites for distinguishing the species groups of the genus *Stephania*. Genetic distance between *S. rotunda* and *S. japonica* or *S. polygona* is 1.0% on the analyzed segment.

**DISCUSSION**

The *rbcL* gene was used as DNA barcode to identify species in flowering plant, but its limitations have also been shown by previous studies. Previous studies showed that 58.5% of sister species were not identifiable by the *rbcL* gene sequence (Kang, 2017) because of the 100% similarity. In this study, with 4 samples of 3 species and 1 subspecies were used, the *rbcL* gene of 523 bp segment was able to distinguish *S. rotunda* with *S. japonica* or *S. polygona* but could not distinguish *S. japonica* with *S. polygona*. However, this is a taxonomically significant result which helps botanist to separate species group by molecular analysis, thereby guiding to find for morphological differences among groups. Molecular data is a good tool to support for morphology in species identification and rearrangement of classification system (Xie, 2015).

In an analysis that included genbank sequences, we found that the tag gene segment used in this study could not distinguish between species in the same group; group (1) *S. abyssinica*; *S. longa*; *S. japonica*; *S. elegans* and group (2) *S. venosa*; *S. cephalantha*; *S. brachyandra*; *S. rotunda*. However, it distinguishes very well between two species belonging to two different groups. These are two groups with different morphological characteristics of root, a major feature used in key to species identification of *Stephania*. About DNA barcode for genus *Stephania*, we suggest that should examine furthey other locus such as nuclear ITS, chloroplast *trnH* - *psbA* space or study a combination of multiple gene locus.

**CONCLUSION**

Using data of the *rbcL* gene can distinguish several species *Stephania* from each other, but is restricted to close species because the nucleotide sequence difference between species is quite small. Phylogenetic tree based on partial *rbcL* gene have divided 3 examined species into two groups, corresponding to the morphological characteristics of the tuberous rootstock and the main roots. The study has added molecular data for 3 species and 1 subspecies of *Stephania* which were collected in Vietnam.

**Acknowledgements:** The authors would like to thank the Vietnam Museum of Nature and the Graduate University of Science and Technology for their supports and facilitation to complete this study.

**REFERENCES**

Bafeel S. O., Arif I. A., Bakir M. A., Al Homaidan A. A., Al Farhan A. H., and Khan H. A. (2012). DNA barcoding of arid wild plants using *rbcL* gene sequences. *Genetics and Molecular Research*, 11 (3): 1934–1941.

Chase M. W., Salamin N., Wilkinson M., 2005. Land plants and DNA barcodes: short-term and long-term goals. *Phil Trans Roy Soc B*, 360: 1889–1895.

Cowan R. S., Chase M. W., Kress W. J, Savolainen V., 2006. Three hundred thousand species to identify: problems, progress, and prospects in DNA barcoding of land plants. *Taxon*, 55: 611–616.

Fazeekas A. J., Burgess K. S., Kesnakurti P. R., 2008. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PloS ONE*, 3: 2802.

Hu C. M., Luo H. S., Chen T., Gilbert M. G., 2008. Flora of China. Vol 7: 1–166.

Jacques F. M. B., Bertolino P., 2008. Molecular and morphological phylogeny
Using the chloroplast rbcL gene to clarify

of tribe Menispermaceae (Menispermaceae) inferred from chloroplast and nuclear sequences. Perspectives in Plant Ecology, Evolution and Systematics, 8: 141–154.

Vu Tien Chinh, Bui Hong Quang, Ritesh Kumar Choudhary, Nian He Xia, Jongku Lee, 2016. Stephania subpeltata H.S.Lo (Menispermaceae): A New Recorded Species from Vietnam. Journal Korean Journal Plant Taxon, 46 (3): 288–294.

Vu Tien Chinh, Bui Hong Quang, Tran Thi Phuong Anh, 2015. Morphological Characteristics and Key to Genera of Family Menispermaceae in Vietnam, Proceedings of the 6th National Scientific Conference on Ecology and Biological Resources Hanoi, Agricultural Publishing House: 27–32.

Vu Tien Chinh, Duy Nong Van, Van Tien Tran, Nian he Xia, 2019. Stephania polygona (Menispermaceae): A new Species from Southern Vietnam. Phyto taxa, 400 (3): 211–214.

Vu Tien Chinh, Trinh Thi Phuc, Tong Y Hoa, Xia Nian He, 2019. Stephania brevipes Crail., (Menispermaceae): A new record for the Flora of Vietnam. Journal of Tropical and Subtropical Botany, 27(3): 323–326.

Xie D. A., He J. A., Huang J. A., Xie H. A., Wang Y. A., Kang Y. A. C., Jabbour F. B., Guo J. A., 2015. Molecular phylogeny of Chinese Stephania (Menispermaceae) and reassessment of the subgeneric and sectional classifications. Australian Systematic Botany, 28: 246–255.