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

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ABSTRACT

The *rbc*L 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 *rbc*L 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 *rbc*L 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 splited 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 *rbc*L, phylogeny, *Stephania*.

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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 taxons 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 *rbc*L 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 *rbc*L, *mat*K, *trn*H-*psb*A, 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 *mat*K and *rbc*L genes (Kress, 2007). In this study, we tested the ability to distinguish species in the genus *Stephania* of chloroplast *rbc*L 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 $OD_{260nm/280nm}$. Amplification of *rbc*L gene was performed by PCR reaction with primers *rbc*L1F: 5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3'/, *rbc*L724R: 5'-TCG CAT GTA CCT GCA

GTA GC-3' (Fay, 1997). Amplification reaction were conducted with a volume of 25 µl, including the components: 1X PCR Buffer, 2.5 mM $MgCl_2$, 2 mM dNTPs, 0.5 pM 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 burmanii*	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 *rbc*L gene for 4 Steniphia samples from Vietnam. The total DNA has an ratio $OD_{260/nm}/OD_{280nm}$ 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 choroplast *rbc*L gene.

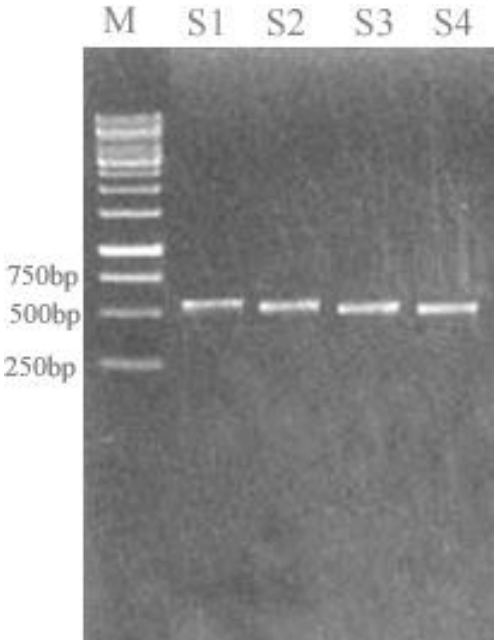

Figure 1. Agarose gel electrophoresis (0.8%) showing PCR products (600 bp) from *rbc*L gene. Lanes: lane M, 1Kb DNA ladder; lane S1: *S. japonica* var. *discolor*; lane S2: *S. polygona*; lane S3: *S. japonica*; lane S4: *S. Rotunda*

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 *rbc*L 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 tranversion (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 *rbc*L 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. tetrandra*, *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 boostrap 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 *rbc*L 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 gneus *Stephania* are polyphyly (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 *rbc*L segment (calculated by MEGA v6.0 and the value have not yet multiplied by 100%)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. S1 S. japonica var. discolor																					
2. S2 Stephania polygona	0.00																				
3. S3 Stephania japonica	0.00	0.00																			
4. S4 Stephania rotunda	0.01	0.01	0.01																		
5. KF496796 Stephania japonica	0.00	0.00	0.00	0.01																	
6. JN051689 Stephania abyssinica	0.00	0.00	0.00	0.01	0.00																
7. FJ626601 Stephania longa	0.00	0.00	0.00	0.01	0.00	0.00															
8. JN051692 Stephania elegans	0.00	0.00	0.00	0.01	0.00	0.00	0.00														
9. JN051691 Stephania cephalantha	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01													
10. JN051690 Stephania brachyandra	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00												
11. FJ026509 Stephania rotunda	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00	0.00											
12. EU526996 Stephania venosa	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00										
13. FJ026508 Stephania laetificata	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01									
14. GQ436370 Stephania tetrandra	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01								
15. JX944483 Stephania tetrandra	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00							
16. DQ099437 Antizoma angustifolia	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01						
17. JQ025032 Cissampelos capensis	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00					
18. KF181462 Cyclea polypetala	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00				
19. FJ026482 Cyclea hypoglauca	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00			
20. FJ026481 Cyclea burmanii	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.00	0.00	0.00		
21. JN051685 Pachygone loyaltiensis	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	
22. HQ260804 Syntriandrium preussii	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02

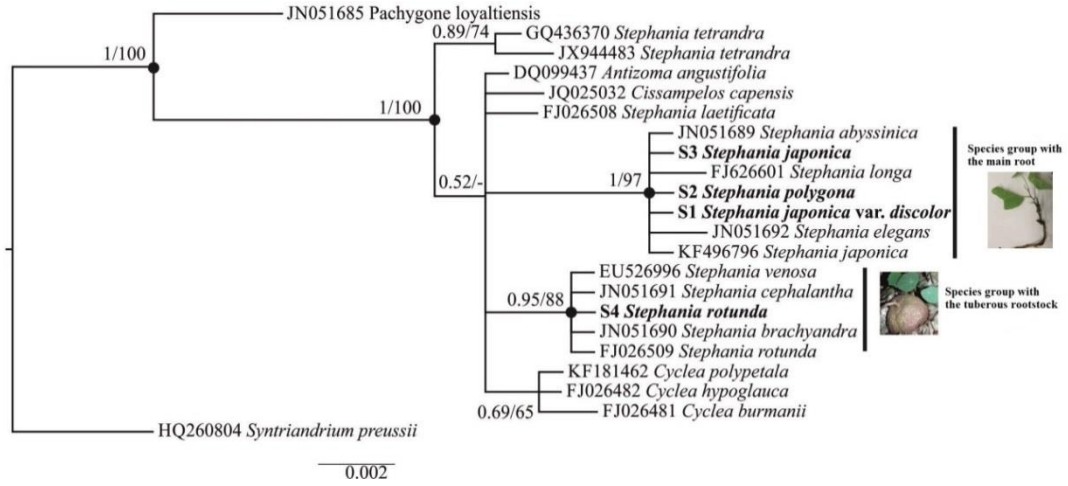

Figure 2. Phylogenetic tree based on the sequence of 523bp gene *rbc*L (examined samples in bold letters)

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

Base on the 523 bp *rbc*L sequences in this study, *S. rotunda* has 5 different nucleotide compared with sequences of *S. japonica* or *S. polygona*. The 523 bp *rbc*L segment was aligned with reference full chloroplast DNA (contained *rbc*L 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 *rbc*L 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 *rbc*L gene sequence (Kang, 2017) because of the 100% similarity. In this study, with 4 samples of 3 species and 1 subspecies were used, the *rbc*L 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 furthely other locus such as nuclear ITS, chloroplast *trn*H - *psb*A space or study a combination of multiple gene locus.

CONCLUSION

Using data of the *rbc*L 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 *rbc*L 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.

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