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Some Barcoding DNA Sequence Analysis of *Sphagnum nepalense* H. Suzuki, a Bryophyte Species Endemic to East Nepal

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Abstract

Sphagnum nepalense is a bryophyte endemic to Nepal. The objective of the present study is to analyze DNA barcoding markers useful for delineating the *Sphagnum* species. Here, a specimen of *Sphagnum nepalense* collected from the bank of Maipokhari lake, Ilam (2107 m asl) was used. Three chloroplast loci from the sample viz. *rbcL*, *psbA-trnH* and *trnF-trnL*, the latter two being intergenic spacers, were amplified and sequenced. Four accessions of plastome sequences of *S. junghuhnianum*, *S. multifibrosum*, *S. palustre* and *S. subsecundum* were retrieved from the National Center for Biotechnology Information (NCBI). Evolutionary analysis was performed following the Maximum Likelihood approach using MEGA X. The result showed that the evolutionary tree generated with single locus *trnF-trnL* and combined sequences of *trnF-trnL* and *psbA-trnH* was better compared to that generated with the sequence of other single locus and even the combined sequence of *rbcL*, *psbA-trnH* and *trnF-trnL*. The sequence data generated in this study for *Sphagnum nepalense* are novel to the scientific community.

Keywords: Bootstrapping support, Evolutionary tree, GenBank accession, Molecular markers, Plastome

Introduction

Bryophytes rank second position among land plants after angiosperms in terms of species diversity (Goffinet & Shaw, 2008). There are 11 species of *Sphagnum* recorded from Nepal (Pradhan & Shrestha, 2022). *Sphagnum nepalense* H. Suzuki is an endemic bryophyte reported first from east Nepal (Hara, 1966). Correct identification of species is a prerequisite for species conservation and management. DNA barcoding, a process that involves sequencing of specific regions of DNA as a molecular tool for species identification, could be the best option for precise and rapid identification [Consortium for the Barcode of Life's (CBOL) Plant Working Group, 2009].

Existing literature show the use of diverse markers for different taxa of plants. For example, CBOL Plant Working Group 2009 recommends *rbcL* and *MatK* for land plants. Similarly, regarding the mosses, various studies have recommended different markers for identification (Heck et al., 2021; Hofbauer et al., 2016; Liu et al., 2011). In some cases individual markers have worked well, for instance, *ITS2* worked well in *Schistidium* (Hofbauer et al., 2016), *psbA-trnH* in the moss genera of Grimmiaceae (Liu et al., 2011) and *BRK1* for the genus *Sphagnum* (Heck et al., 2021). Whereas in other works, markers have proved efficient when they were combined. For example, for the genus *Dicranum*, species were distinguishable with combined sequence data of *ITS1*, *trnF-trnL*, *rps4-trnT*, *psbA-trnH*, *rps19-rpl2* and *rpoB* (Lang et al., 2014).

In this paper three commonly used molecular markers have been used to illustrate the molecular identity and relationship of *Sphagnum nepalense* with its congeners. This is a first step towards building a DNA barcode database of Nepal's flora. We believe it is prudent to initiate the DNA barcoding work from the endemic plants and then proceed to other categories that have had doubts or contestations. Further, DNA barcoding the endemic plants of Nepal will: (a) validate the taxa through molecular method (b) contribute to proper identification and classification of the taxa and (c) build knowledge base for floristic studies of Nepal and the wider Himalayas.

Materials and Methods

Plant material and DNA extraction

During exploration in February 2021 we encountered *Sphagnum nepalense* at the bank of Maipokhari lake (Altitude 2107m, latitude 27.00723ºN and longitude 87.93075ºE) (Figure 1), which formed a dense mat. DNA material of *Sphagnum nepalense* was collected and preserved in silica gel with all the necessary field notes about the specimen. The sample code was assigned as BT-2. Voucher specimens were collected and deposited at KATH (specimen no. B1_9/2/2021). Total genomic DNA was isolated from silica-dried samples using CTAB method (Keb-Llanes et al., 2002).

Figure 1: *Sphagnum nepalense* plant

PCR amplification and sequencing

Three plastid markers *rbcL* (Ribulose-1,5-bisphosphate carboxylase), *psbA-trnH* (the intergenic spacer between the gene coding protein D1, a polypeptide of the photosystem II reaction center (psbA) and gene coding histidine accepting tRNA (trnH)) and *trnF-trnL* (the intergenic spacer between two genes coding for transfer RNA) were amplified (Figure 2) and sequenced using primers listed in Table 1. The PCR conditions for all the three markers were 35 cycles of denaturation at 94°C for 30 sec., annealing at 54°C for 30 sec. and extension at 72°C for 1 min.

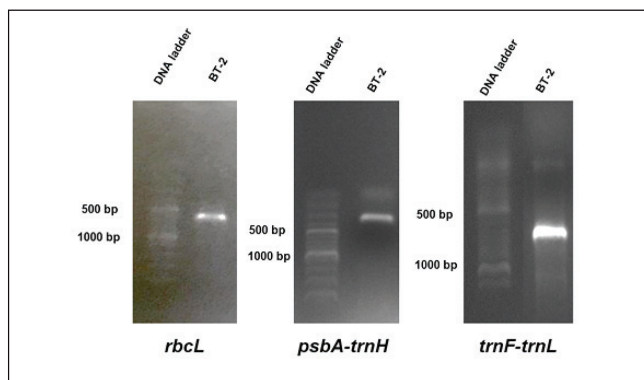

Figure 2: PCR amplification of *rbcL*, *psbA-trnH* and *trnF-trnL* from BT-2

The sequencing was carried out in ABI310 Genetic Analyzer. The raw sequences were quality trimmed, and the sequences with both forward and reverse reads were aligned into a consensus sequence. We also compared the DNA sequence data with chromatogram in SnapGene Viewer tool and edited the sequence manually whenever required. The newly generated sequences were registered at the NCBI; the assigned NCBI accessions are presented in Table 2.

Table 2: GenBank accessions generated in the study

Species	Locus	GenBank Accession
Sphagnum nepalense H. Suzuki	*psbA-trnH*	OP918673
	rbcL	OP985339
	trnF-trnL	OP985340

Sequence downloads and data analysis

Four accessions of plastome sequences were retrieved from the NCBI, representing four *Sphagnum* species viz. *S. junghuhnianum*, *S. multifibrosum*, *S. palustre* and *S. subsecundum*. Similarly, one accession of plastome of a bryophyte species *Andreaea rupestris* was also retrieved (Table 3). Respective aligned

Table 1: Primers used in the study

Locus	Primer name	Sequence (5'⟶ 3')	Remarks
rbcL	rbcL-F	ATGTCACCACAAACAGAGACTAAAG	Modified from Kress et al., 2009
	rbcL-R	GTAAAATCAAGTCCACCACG	
psbA-trnH	psbA	GTTATGCATGAACGTAATGCTC	Modified from Sang et al., 1997
	trnH	CGCGCATGGTGGATTCACAATC	Modified from Tate et al., 2003
trnF-trnL	trnF	ATTTGAAGTGGTGACACGAG	Taberlet et al. 1991
	trnL	CGAAATCGGTAGACGCTACG	

sequences of *rbcL*, *psbA-trnH* and *trnF-trnL* were extracted from each accession manually using SnapGene viewer tool.

Table 3: Plastome sequences retrieved from NCBI

S.N.	Species	GenBank Accession
1.	*Andreaea rupestris*	MW561627.180840-81296
2.	*Sphagnum junghuhnianum*	NC_060704.162998-63198
3.	*Sphagnum multifibrosum*	NC_060705.164202-64400
4.	*Sphagnum palustre*	MW822172.163056-63255
5.	*Sphagnum subsecundum*	NC_060384.163996-64195

The DNA sequences were aligned by MUSCLE. Phylogenetic analysis was performed following the Maximum Likelihood approach and Kimura 2 Parameter (K2P) model with 1000 bootstrapping replications using Molecular Evolutionary Genetics Analysis (MEGA X) tool. The sequence of *Andreaea rupestris* was used as an out-group to root the tree.

Results and Discussion

rbcL and psbA-trnH are weaker marker for Sphagnum

The phylogenetic analysis using *rbcL* and *psbA-trnH* sequences showed rather poor species discrimination. Though different species formed separate clades, bootstrapping support values were very weak, less than 50 in the majority of clades. Also, the phylogenetic position of individual species was not consistent in two trees (Figure 3 and 4). Liu et al. (2010) suggested *rbcL*, *rpoC1*, *rps4*, *psbA-trnH* and *trnL-trnF* as suitable barcode loci for moss, out of which the best performing single loci are *rbcL* and *rpoC1*. Consistent with our finding, *psbA-trnH* exhibited poor performance as a barcoding marker for delineating closely related bryophyte taxa of selected moss (Hassel et al., 2013)

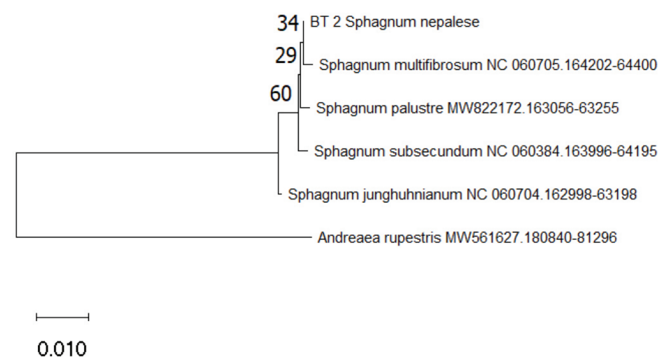

Figure 3: Maximum Likelihood tree generated using *rbcL* sequences based on the K2P model. The number on the branches represents bootstrapping support after 1000 bootstrap replications test. Scientific names are followed by respective GenBank accession numbers. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 530 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

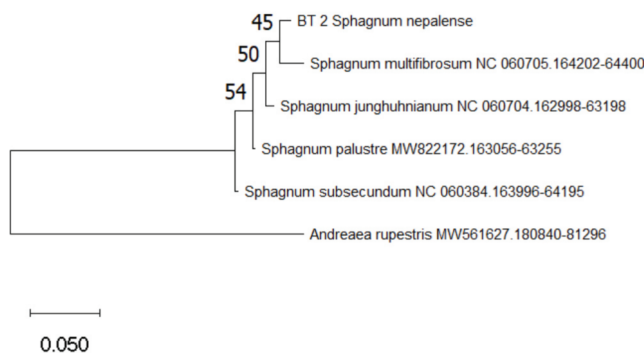

Figure 4: Maximum Likelihood tree generated using *psbA-trnH* sequences based on the K2P model. The number on the branches represents bootstrapping support after 1000 bootstrap replications test. Scientific names are followed by respective GenBank accession numbers. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 208 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Tree generated with trnF-trnL and combined psbA-trnH and trnF-trnL is better

Interestingly, the tree generated with *trnF-trnL* sequence is better compared to that generated with *rbcL* and *psbA-trnH* sequences. Here, each species formed distinct clades supported with significantly higher bootstrap values (Figure 5), suggesting that the *trnF-trnL* could be the single locus marker for species delineation of *Sphagnum*. Lang et al. (2014) also found *trnF-trnL* as one of the most promising single locus markers for *Dicranum*.

The tree generated with combined sequences of *psbA-trnH* and *trnF-trnL* was also better than that generated with single locus *rbcL* and *psbA-trnH* respectively (Figure 3, 4 and 5). The tree is comparable to that generated with *trnF-trnL* sequence. Specifically in *S. nepalense*, the bootstrapping support value was found significantly increased from 68 to 84. Furthermore, phylogenetic positions of all the species are consistent in both the trees (Figure 5 and 6).

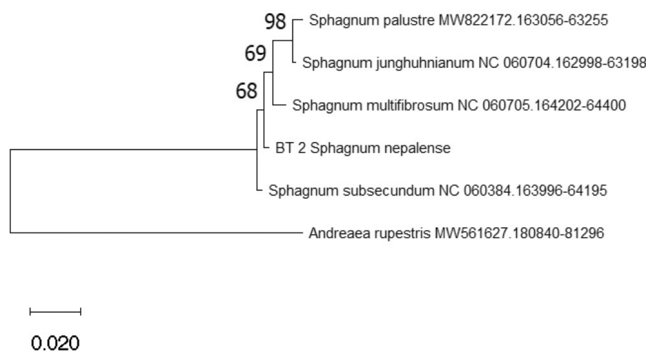

Figure 5: Maximum Likelihood tree generated using *trnF-trnL* sequences based on the K2P model. The number on the branches represents bootstrapping support after 1000 bootstrap replications test. Scientific names are followed by respective GenBank accession numbers. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 802 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

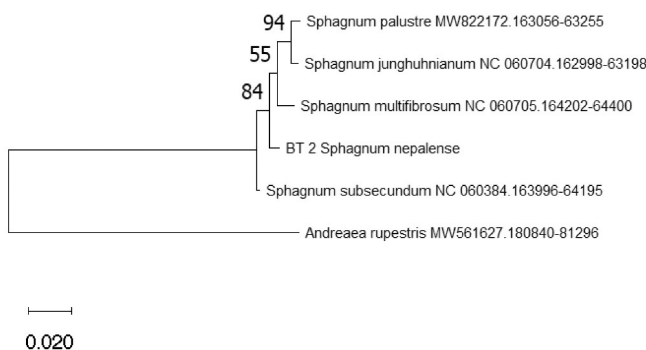

Figure 6: Maximum Likelihood tree generated using *psbA-trnH* + *trnF-trnL* sequences based on the K2P model. The number on the branches represents bootstrapping support after 1000 bootstrap replications test. Scientific name is followed by respective GenBank accession number. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1010 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Further, the sequences of *rbcL*, *psbA-trnH* and *trnF-trnL* were combined and the tree generated. Combination of sequence was done to get more robust tree. Contrastingly, the tree generated with three sequences combined is very poor (Figure 7). Similar results have also been reported in previous studies (Raskoti & Ale, 2021; Starr et al., 2009; Xiang et al., 2011; Xu et al., 2015), suggesting that combining the sequences need not always be a good strategy for phylogenetic analysis.

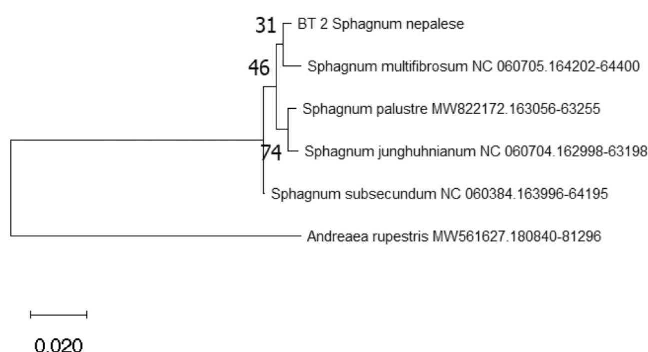

Figure 7: Maximum Likelihood tree generated using *rcbL* + *psbA-trnH* + *trnF-trnL* sequences based on the K2P model. The number on the branches represents bootstrapping support after 1000 bootstrap replications test. Scientific name is followed by respective Gene bank accession number. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1539 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Conclusion

From the present study, it was found that either single locus *trnF-trnL* or in combination with *psbA-trnH* could be the possible marker for species delineation of *Sphagnum*. More new accessions of *Sphagnum* and analysis of other barcoding markers such as *BRK1*, *MatK*, *ITS* etc. individual as well as in combination are necessary to get clearer picture of *Sphagnum nepalense,* particularly to assign its phylogenetic position. However, the study provided molecular evidence for *S. nepalense* as endemic species since the sequences are unique to other nucleotide sequences available in the public domain for *Sphagnum* species.

Author Contributions

MSTM designed the research. MSTM, SM, JP, DRK and GR performed experiments. MSTM analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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