

DNA Barcoding of *Myrica esculanta* (Kafal), An Indigenous, Multipurpose and Medicinal Plant Species from Nepal, by rbcL and matK Gene

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Received: February 24, 2020

Published: March 09, 2020

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Abstract

Myrica esculanta (Kafal), is one of the most important native medicinal plant of Nepal which is domesticated for economic growth for the rural people. To authenticate and develop molecular database we collect samples all over Nepal. DNA barcodes can be used as reliable tools to facilitate the identification of medicinal plants for the safe use, important for metal absorption and value added products. In this study, investigation of *Myrica esculanta* for its molecular identification by DNA barcoding was done through sequence analysis with three barcode loci rbcL, matK and ITS genes, and found only first two are the best for its authentication based on database search on NCBI. The nucleotide sequence of internal transcribed spacers (ITS2) and chloroplast maturase kinase gene (matK) and rbcL have determined close relationship among 12 other species of *Myrica*; to construct the phylogenetic tree. The phylogenetic relationships of accessions based on the matK and rbcL region showed that all accessions in this study were related to three geographical origins. Based on sequence alignment and phylogenetic analyses and comparative sequence analysis based on sequence based taxonomic parameter on iBOLD database, we concluded that the rbcL and matK sequences can distinguish *M. esculanta* accessions from iBOLD database.

Keywords: DNA Barcoding; Internal Transcribed Spacer; Maturase K; Phylogenetic; iBOLD

Introduction

The genus *Myrica* consists about 97 species of small tree and aromatic shrubs belonging to family *Myricaceae*. These are reported to be globally distributed in both temperate and sub-tropical regions of the world [1]. Kafal trees are found on hills of Nepal and Northern India, between the altitudes of one and two thousand meters above sea level. Kafal changes to reddish purple color ellipsoid-shape fruit at its maturity. In scientific journals, Kafal is mostly called *Myrica esculenta*, but also referred as *Myrica integrifolia* and *Myrica nagi*. In ancient Sanskrit language, Kafal is often called Kaiphala or Katphala and believed to have a medicinal property in its bark [1]. The bark is yellow colored and contains the chemical substances myricetin, myricitrin and glycosides [2,3]. *M. esculenta*

is a small tree or large shrub native to Hills of Nepal and northern India. Its common names include Box myrtle, Bayberry, Kafal (local name) [2]. It is a well-known medicinal plant in Ayurveda or Himalayan Wild Fruit – Kathfal. Nepali name Kaaphal or Kafal Common name - Bay-Berry, Box myrtle. Kaaphal is one of many extremely delicious wild fruits found throughout mid-Himalayan region. The fruit looks somewhat like deep-red colored raspberries [4]. They barely have any pulp, have a big round seed in the center. Since they are very refreshing to eat, they are well liked by many Nepalese. The fresh fruits have a reputation for being a little acidic even when they are ripe, but more sour when unripe [5]. They have a limited harvesting period and available for a short period of time only. When they were in season, local villagers picked and gathered

the berries carefully from the wild growing areas and transported to Kathmandu in a wicker baskets (doko-daalo). It is not a good timber as it warps and splits badly, but is used occasionally for low-grade construction and, agricultural implements. In spite of being a multipurpose tree, the cultivation of the plant is very limited and most of the traditional and commercial uses of *M. esculenta* plant depend exclusively on the collections from the wild sources by indigenous people. Thus, the species is under imminent danger of extinction from wild sources due to increase in urbanization, over harvesting, negligence of sustainable utilization and over exploitation of forests and waste lands for commercial uses [6,7]. Poor regeneration in natural habitat due to high anthropogenic activity is another important factor, which affect the natural population of this plant species [8]. Screening of various Himalayan species of medicinal plants have been already done in Nepal [9], but lack the *M. esculenta* species with molecular based analysis is yet to perform.

Phylogeny reconstruction within closely related species may be difficult because of incomplete lineage sorting, introgression,

short evolutionary scale, and lack of molecular markers in poorly studied taxa [10]. In this circumstance, reduced-representation genome sequencing methods allow us [11] to sequence the regions flanking restriction sites with deep coverage, then to align orthologous sequences across multiple samples to discover thousands of genetic markers for systematics, population genomics and adaptive evolution studies [12,13]. Sequence based analysis play important role in the study of species identification [14]. The phylogenetic trees constructed to show the relatedness and distance of the analyzed plants in the history of evolution by the analysis of richness of clades. The construction of DNA barcode library of medicinal plants is an introductory research arena in Himalayan region of in helping the routine identification of plants [15], and developing guidelines for detection of adulterants in herbal medicines as well as protection of biodiversity conservation [16] and economic growth by value added product, production from plants fruits.

DNA Barcoding of *M. esculenta* in Nepal has not been yet reported with any barcode loci ITS (non coding region), *rbcl* nor even *matK* [17-19].

Figure 1: Leaf, Tree and Fruits of *M. esculanta*.

Materials and Methods

Study area

Collection of *M. esculanta* from three different location of Nepal was done in the month of May 2017 from Gulmi, Palpa and Kathmandu, three different geographical location as shown in figure 2.

Sample preparation

The DNA was isolated using ZR Genomic DNATM-Tissue Mini-Prep (Zymo Research, USA). According to the manufacturer's

manual [2], Folmer region was amplified using MyTaq™ Red DNA Polymerase (BioLine, UK). The primers used for the amplification of the *rbcl* gene were *rbcl*La -F (forward ATGTCACCACAAA-CAGAGACTAAAGC and *rbcl*La -R (reverse GTAAAATCAAGTCCAC-CRCG) and for *matK*-xF (forward) TAATTTACGATCAATTCATTC and *matK*-MALPR1 (reverse ACAAGAAAGTCTGAAGTAT) table 1. The thermal program consisted of initial step of 2 minutes at 54°C followed by 35 cycles of 40 seconds at 94°C, 40 seconds at 55°C and 1 minute at 72°C followed in turn by final extension of 10 minutes at

72°C. The PCR products were visualized on 1.2% agarose gels, purified and sequencing. Amplicons were purified using DNA Clean and Concentrator TM-25. Products were labelled using the BigDye Terminator V.3.1 Cycle sequencing kit (Applied Biosystems, Inc.) and sequenced bidirectionally using ABI 3730 Automated Sanger

sequencer (Macrogen, Inc.). Sequence was evaluated, assembled, and aligned using Geneious V8.1 software and submitted to GenBank database. Refined sequence was used to identify the species using DNA related database (BOLD and BLASTn) Sequence Editing, Aligning, Assembly and Data Analysis

Figure 2: Sample collection area (Kathmandu, Gulmi and Palpa).

Barcode genes	PCR Primers	Primer sequence 5' -3'	Annealing Temp for (PCR)	Sequencing Primers
rbcl	rbclLa -F (forward)	ATGTCACCACAAACAGAGACTAAAGC	55°C	rbclLa -F* rbclLa -R*
rbcl	rbclLa -R (reverse)	GTAAAATCAAGTCCACCRCG		
matK	matK-xF (forward) TAATTTACGATCAATTCATTC		56°C	matK-xF matK-MALPR1 (choice I)* matK-1RKIM-f matK-MALPR1 (choice II)*
matK	matK-MALPR1 (reverse) ACAAGAAAGTCGAAGTAT			
matK	ACCCAGTCCATCTGGAAATCTTGTTTC			

Table 1: List of PCR primers and sequencing primers used in the DNA barcoding.

*Bidirectional Sequencing

Reagents	rbcl (µl)	matK (µl)	Volume for unit reaction (1X) (µl)
10X Buffer	20	20	2.0
50 mM MgCl ₂	3	3	0.3
10µM Primer Forward	2	2	0.2
10 µM Primer Reverse	2	2	0.2
10 mM dNTPs	2	2	0.2
DNA Polymerse (5U/µl)	1	1	1
DNA Template (20 - 40 ng/µl)	10	10	10
ddH ₂ O	60	60	60
Total Reaction volume	100	100	100

Table 2: Combinations and concentrations of reagents used for PCR reactions of *rbcl*, *matK* and ITS2 for analysis of *M. esculanta*

Tempera- ture	Time	Cycle	Function	Annealing Temp for (PCR)
98°C	45 sec			55°C <i>rbcl</i>
98°C	10 sec	35	Denaturation	
59°C	30 sec		Annealing	59°C ITS
72°C	40 sec		Extension	
72°C	10 min		Final extension	56°C <i>matK</i>

Table 3: PCR Program for DNA amplification for all the 3 gene loci.

PCR and sequencing

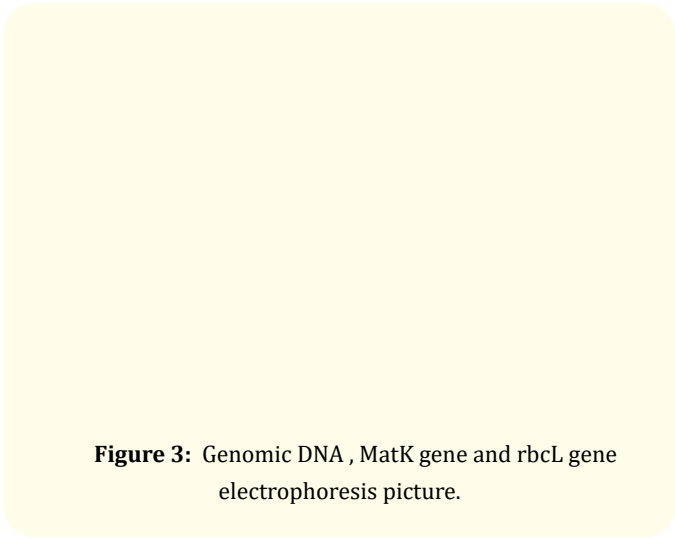


Figure 3: Genomic DNA , MatK gene and *rbcl* gene electrophoresis picture.

Sanger seq *matK* data

>*Kafal_data*

CCTTCGCTACCGGGTGAAAGATGCCTCCTCTTTGCATTATT-

GCGGTTCTTTCTTCATGAGTATTCTAATTGTAGCATTTCGTATTATTC-
CAAAAAAAAAACGAATCCATTTCTATTTTTTTAAAAAGTAATCCAA-
GATTATTGTTATTTTTATATAATTCTCATATATGTGAATACGAATC-
CGTCTTCTTTTTTATCCGTAACCAATCTTCTCATTTACGATTAA-
CATCTTCTGGAGTCCTTTTTGAGCGAATCTATTTACATAGAAAAATG-
GAACATCTTGTCAAAGTCTTTGCTAATAATTTTCGGGGCATCCTAT-
GCTTCCCGAAGGATCCTTTCATTCAATTATGTTAGATATCAAGGAAAAT-
CAATTCTGGTTTCAAAAGATACACCTCTTCTGATAAATAAATG-
GAAATATTACCTTGTCAATTTATGGCAATGTCATTTTTCT-
GTGTGGTCTCGCCTGGGAAGGATCTATATAAACCAATTATC-
CAAGCATTCCCTCGACTTTTTGGGTATTTTTCAAGTGTGCGACTA-
AATCTACAATGGTGCGTAGTCAAATGCTAGAAAATTCATTTATAAT-
CAAAAATGCTCCCAAGAAGCTCGATACAATAGTTCCAATTATTCCTCT-
GATTGGATCATTGGCTAAAGCGAAATTTTGTAACGCATTAGGG-
TATCCATTAGTAAGCTGACTCGGGCCGATTTATCGGATTTTGATAT-
TATCAATCGATTTGTGCGTATATGCAGAAATCTTCTCATTTATTA-
CAGCGGATCTTCAAAAAAAAAAGAGTATGTATCGAACAAA

Figure 4: NCBI Blast output result show 100% similarity with *M. rubra* sub species of *M. esculanta* with *matK* gene.

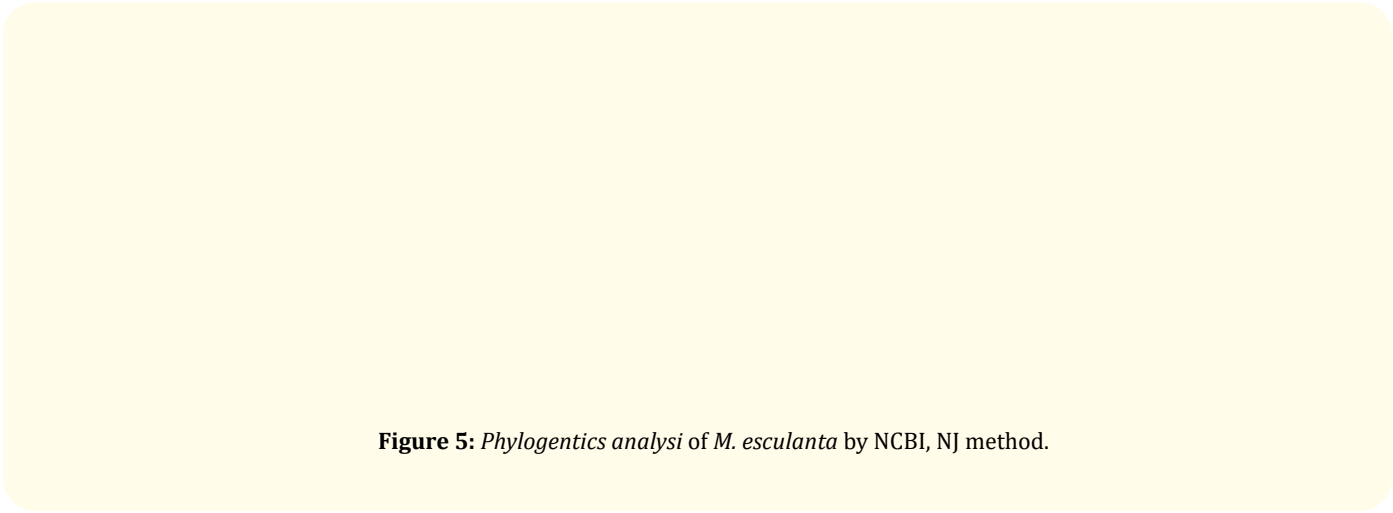


Figure 5: Phylogenetics analysi of *M. esculanta* by NCBI, NJ method.

Identification and comparison of barcodes sequence with ref-
erence to BOLD database:

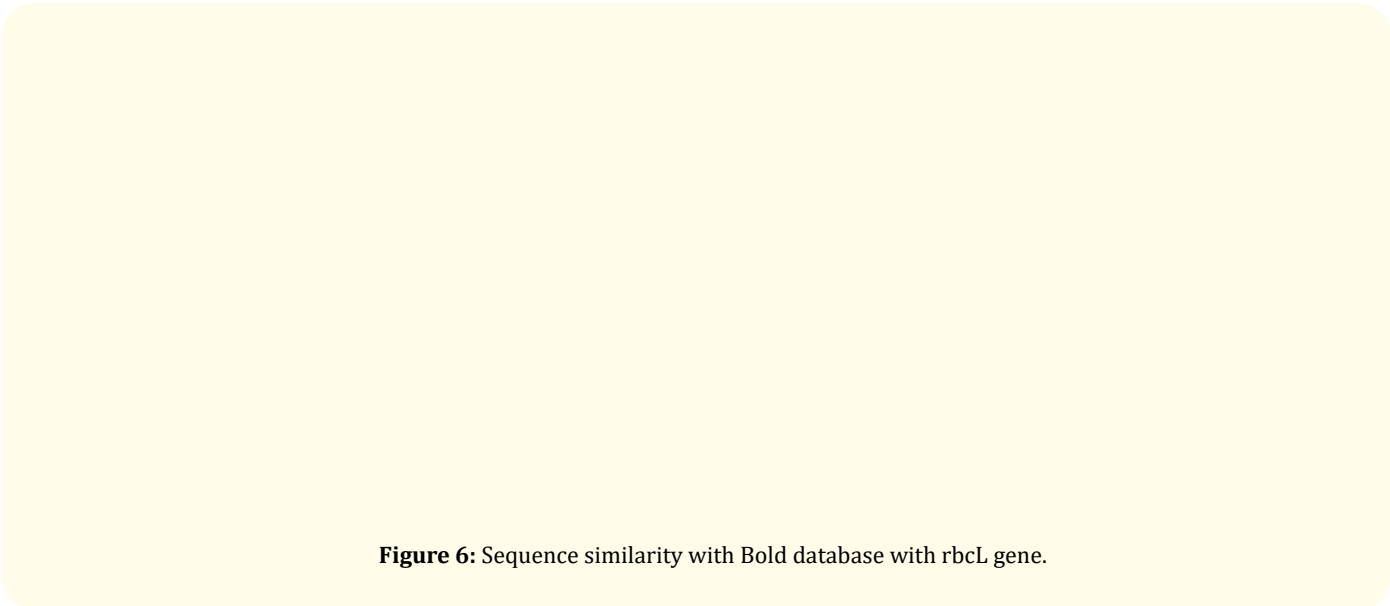


Figure 6: Sequence similarity with Bold database with *rbcl* gene.

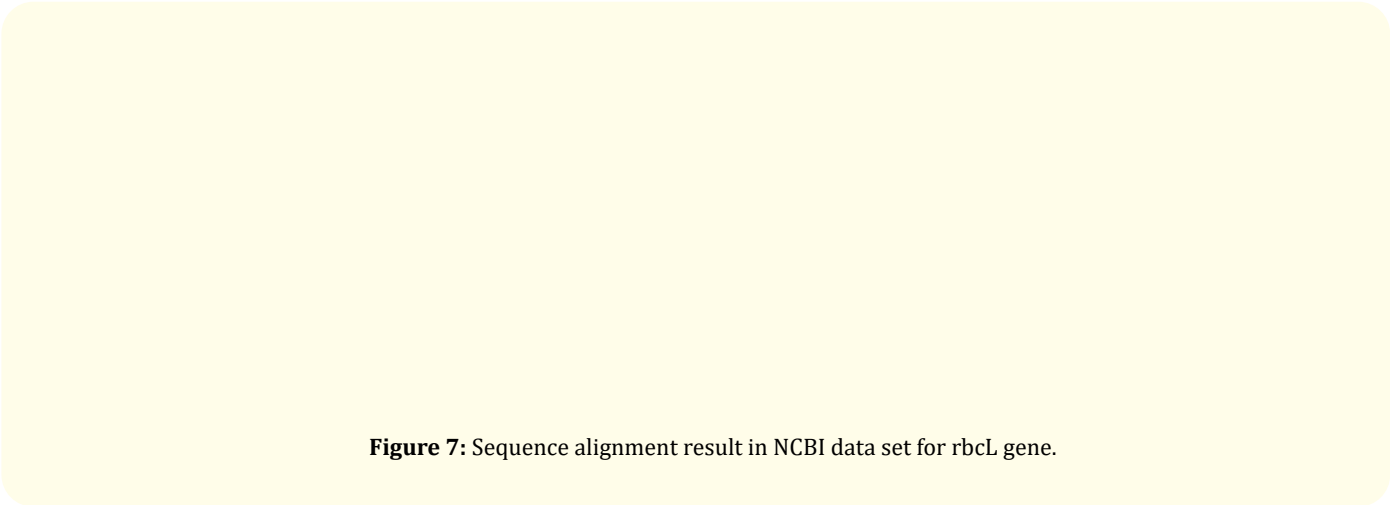


Figure 7: Sequence alignment result in NCBI data set for *rbcl* gene.

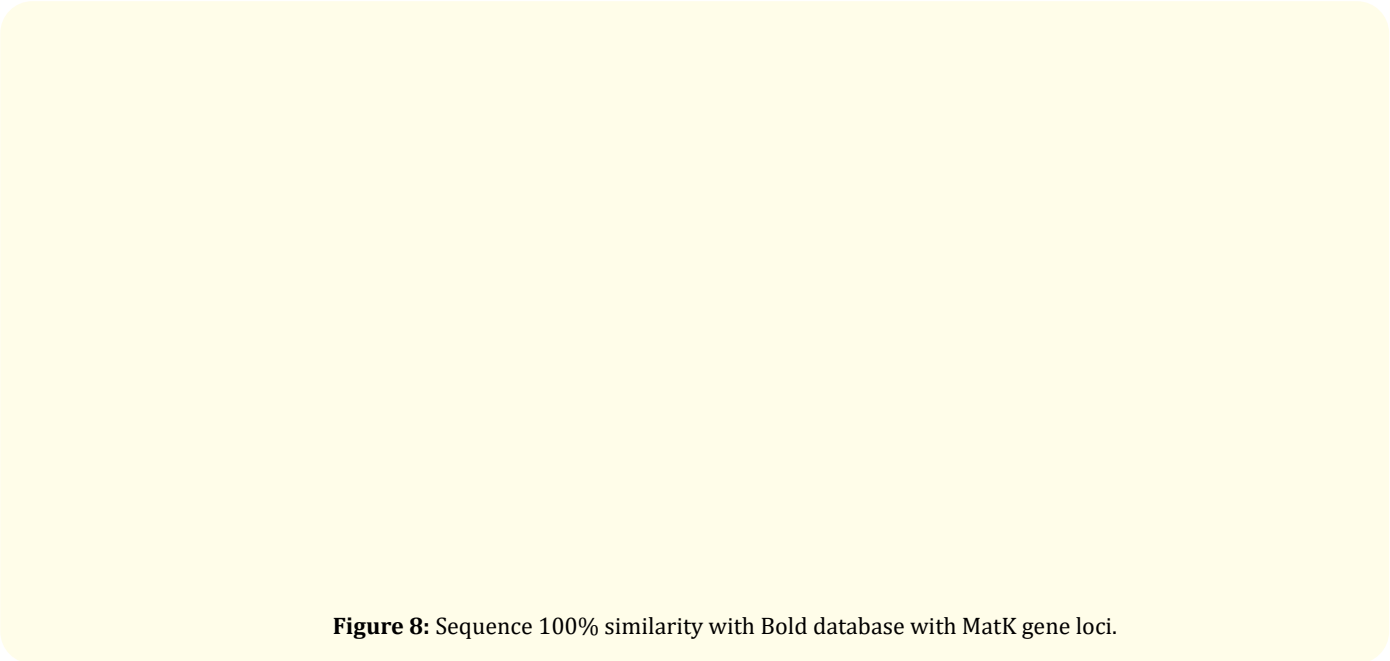


Figure 8: Sequence 100% similarity with Bold database with *MatK* gene loci.

Figure 9: Identification of *M. esculanta* by matK gene in Barcode database.**Sequence Submitted to NCBI Database for Gi Number.**

LOCUS Kafal_ 804 bp DNA linear 11-FEB-2020

DEFINITION data_MatK.

ACCESSION Kafal_

VERSION

KEYWORDS.

SOURCE chloroplast *Myrica esculenta*ORGANISM *Myrica esculenta*

Unclassified.

REFERENCE 1 (bases 1 to 804)

AUTHORS Sharma,D. and Lamichhane,J.

TITLE DNA barcoding of *Myrica esculanta* (Kafal), An Indigenous, Multipurpose and Medicinal plant species from Nepal, by rbcL and matK gene

JOURNAL unpublished

REFERENCE 2 (bases 1 to 804)

AUTHORS Sharma,D. and Lamichhane,J.

TITLE Direct Submission

JOURNAL Submitted (11-FEB-2020) Biotechnology, Kathmandu University,

Dhulikhel, Kavre, Nepal, Dhulikhel, 3 Bagmati 44600, Nepal

COMMENT

Bankit Comment: ALT EMAIL:deepakshrm59@gmail.com

Bankit Comment: TOTAL # OF SEQS:1

Bankit Comment: TAX: Yes, new species/combinations; SEE ATTACHMENT

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..804

/organism="Myrica esculenta"

/organelle="plastid:chloroplast"

/mol_type="genomic DNA"

/tissue_type="Plant Leaf"

/country="Nepal"

/altitude="1350 m."

/collected_by="Deepak Sharma"

/identified_by="Tirtha Maiya Shrestha"

BASE COUNT 242 a 147 c 126 g 289 t

ORIGIN

1 ccttcgctac cgggtgaaag atgcctcttc ttgcattha ttgcggttct ttctcatga

61 gtattctaata ttagcattc gtattattcc aaaaaaaac gaatccattt ctatttttt

121 aaaaagtaat ccaagattat tgtatttttt atataattct catatatgtg aatacgaatc

181 cgtcttcttt ttatccgta accaatcttc tcatttacga ttaacatctt ctggagtcct

241 ttttgagcga atctatttac atagaaaaat ggaacatctt gtcaaagtct ttgcta-

ataa

301 ttttcggggc atcctatgct tcccgaagga tcctttcatt cattatgta gatatcaagg
 361 aaaatcaatt ctggtttcaa aagatacacc tctctgata aataaatgga aatat-
 tacct
 421 tgtcaattta tggcaatgct atttttctgt gtggtctgc ctgggaagga tctatataaa
 481 ccaattatcc aagcattccc tcgactttt gggtttttt tcaagtgtgc gactaaatcc
 541 tacaatggg cgtagcaaa tgctagaaaa ttcat tata atcaaaaatg
 ctccaagaa
 601 gctcgataca atagtcca ttattcctct gattggatca ttggctaaag cgaaattttg
 661 taacgcatta gggatccca ttagtaagct gactcggcc gatttatcgg attt-
 gat
 721 tatcaatcga ttgtgcga tatgcagaaa tctttctcat tattacgcg gatcttcaaa
 781 aaaaaagagt atgtatcgaa caaa

Results and Discussion

M. esculenta is a multipurpose potent income generating species of Nepalese Himalaya. The species is in the verge of extinction from wild due to overexploitation by the indigenous people as well as poor regeneration of the species in their natural habitat. Immediate scientific intervention and some preliminary necessary action should be taken for the multiplication and establishment of the species in their natural habitat.

Phylogeny Study

The comparative analysis of molecular sequence data is essentially important for reconstructing evolutionary history of plants. The discriminatory influence of three barcode genes [20]. ITS, rbcL and matK was considered in pairwise distance matrix to construct phylogenetic tree using in MEGA X software [21]. The evolutionary history was inferred using pairwise distances in Maximum Composite Likelihood (MCL) [22]. The phylogenetic relatedness and evolutionary history drawn by means of neighbor-Join (NJ) method [23] using nucleotide sequence alignment of rbcLa [24] and ITS2 [25] and matK [26] (Figure 2 and Figure 3). The evolutionary distance analysis conducted using Kimura 2-parameter [27] method of base substitution per site and mentioned by units. The pairwise distance matrix value indicates their closeness during evolution and ultimately helps to draw their phylogenetic tree and the history of evolution. The clades formed in the trees were mostly mixture of several plant species and the big and small branches showed their relatedness and distances clearly among the plant species [5].

Acknowledgements

The research part of this project has been supported by UGC-Nepal Faculty Grant 2017 and publication support by NAST PhD Grant 2014.

Conflicts of Interest

The authors declare no conflict of interest.

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