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PHYLOGENETIC STUDY OF LIVING FOSSIL *PSILOTUM NUDUM* L. FROM HIMALAYAN RANGE OF PAKISTAN USING DNA BARCODES

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Abstract

The genus *Psilotum* belongs to *Psilotaceae* family, with about 133 to 189 species having outstanding social, economic and medically important relict plants. However, being living fossil plant, many hurdles come in way while trying to discern its phylogenetics reconstruction. The partial conserve nucleotide sequences of *rbcLa, trnA, trnV, matK, ITS, ycF3* and *rpoB* genes were used to select the best suitable barcodes in *Psilotum nudum* L. The amplified fragments were subjected to Sanger sequencing and then Maximum likelihood, Maximum Parsimony and Neighbor Joining trees were generated using bioinformatics software. i.e., MUSCLE, BioEdit and Mega-7. Current population showed the best match between *P. complanatum* (KY099851.1), population reported from New Zealand and *P. nudum* (F384430.1) with identity value 100 and 99% having E-value 0.0 and 0.0 by *rbcLa* and *trnA* barcode region, respectively. The findings showed that *P. nudum* L. sequences are ever first reported from the Himalaya regions of Pakistan and could be utilized for further comparison among different populations of this relict plant species across the globe.

Introduction

The floristic studies still fall behind in Pakistan. Although comprehensive data are available for angiosperms but grievously only limited work has been done on pteridophytes, predecessors to modern flora that still require vast level exploration. First ever preliminary checklist of 133 species of 39 dwells in District Mansehra, KPK (Stewart 1972). Later on, the Japanese team in 1992 and 1993 contributed a lot in adding information in pteridophytes study (Sundas *et al.* 2012). and furthermore, Frazer-Jenkins discovered that 189 species of pteridophytes are endemic to Western Himalaya of Pakistan (Gul *et al*. 2016). So far, no record has been found from any area of Pakistan neither in wild nor in cultivated form.

Nearly 400 million years ago, *Psilophytes*, first ever fossil records were found from Scotland in the same way as *Psilotum* is studied today. It is the most pre-historic primitive vascular plant, abundantly available in tropical and sub-tropical regions of the globe (Zhang 2013, Gul *et al.* 2016). A Greek world "*Psilotum"* as well as Latin word *"Nudum"* both have same meaning i.e., "naked" (Yamazaki *et al*. 2001, Nazarian *et al*. 2010). Recently its imprints were reported for the first time from Elum mountains of Buner and Hazara, KP in Pakistan and was named as *Psilotum hazaricum* (Rahman *et al*. 2015). *Psilotum* carries ancestral twiggy structural qualities having diminutive flowering broom, fruits and seeds with prehistoric Y-shaped branches; reproductive structures occupy with little pillboxes like spores along the side of the stem that's seems to be due

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to unfavorable environmental conditions. Its origin and dispersal are yet to be identified (Miriam 1951, Ray *et al*. 1983). A single population was recorded reflecting its rarity in the particular area. It also contains certain medicinal attributes (Gul *et al.* 2016). Hence, the best *in situ* as well as *ex situ* conservation strategies are needed to be implemented for such remnant plants in Pakistan (Fazli *et al*. 2015).

Recently, DNA based techniques have totally changed the dimensions of the molecular genetics. These molecular approaches have been successfully used to study the extent and distribution of variation in plant species gene-pool, identification, baseline conservation strategies and to address the typical taxonomic and evolutionary questions of such exotic plants (Karp *et al*. 1997). The evolutionary studies are the mirror image of the natural history to understand the biodiversity in their ecosystems. The previous studies were based on morphological traits along with some plasid and nuclear genes analysis but there is still lack of phylogenomic studies for their proper delimitation and conservation. Hence the correct species delimitation that is a very crucial step for conservation and biodiversity preservation (Hebert *et al*. 2003, CBOL Plant working group 2009). DNA sequences have become the major source of new information for genetic relationship and evolutionary studies because of the progress in sequencing and computational technologies (Hajibabaei *et al*. 2007, Dick and Kress 2009). Plastid as well as the nuclear genome of plant thus suggested the best optimization and yielding of some appropriate sequences for DNA fingerprinting, i.e., that will be variable enough to discriminate among species and at the same time have less intra than infra-specific inconsistency (Chase *et al.* 2005, Kress *et al*. 2005). It basically involves the evolutionary aspects depending upon character based (Maximum Parsimony and Maximum Likelihood) and distance-based method (Neighbor-Joining). Being sensitive Maximum Parsimony method is generally less effective for phylogenies but the Maximum Likelihood is the comparatively good method. Although these two methods are robust to determine the heterogeneity while the Neighbor-Joining method is not much enough in this respect (Felsenstein 1985).

Indeed, the strange *Psilotum nudum* L., i.e., 'Living Relics' requires a vast level critical comprehensive molecular study for their survival. Pakistan is blessed to have these evolutionary dead-end plants available for exploring their phylogenetic issues. So, it is hoped that upcoming researchers will be successful to solve the riddle of practically relics pteridophytes through advance molecular fingerprinting techniques.

The purpose of the present study was to obtain best DNA barcodes for species in the genus *Psilotum*, in order to determine their existence of the phylogenetic relationships in the respected genus. The present research work was conducted to evaluate the phylogenetic diversity of *Psilotum* and its condition under these objectives: (1). To characterize the collected samples *P. nudum* L. on the basis of standard DNA barcode markers. (2). To perform the phylogenetic analysis of *P. nudum* L. (3). To propose the suitable barcodes for the species identification.

Materials and Methods

Plant material *Psilotum nudum* L. was collected from 1,038 m (3,406 ft) high mountains of District Battagram, Khyber Pakhtunkhwa, Pakistan. The collected sample was correctly identified and kept at Herbarium of Hazara University Mansehra. Description of the research material is given in Table 1.

The total nucleic acid was extracted by using plant genomic DNA purification kit Tiangen [cat. no. DP309] from the desired tissues of selected plant species. For quantification and clarification of DNA 0.1% agarose gel electrophoresis was done. The DNA barcode regions were amplified by using universal DNA barcoding markers from CBOL Plant Working Group (2009).

Primarily, seven plastid and nuclear plant DNA barcode regions (*rbcLa, TrnA, TrnV, matK, ITs, ycF3 and rpoB*) because of their suitability, universality, robustness, good resolving power and reliable sequence consistency were selected to test the effectiveness in *P. nudum* L. (Chase *et al*. 2005).

Table 1. Descriptive information of research material.

Plant sample	Research material	Location of collection	GPS co-ordination	Altitude
Psilotum nudum L.	Leaves/stem	Battagram Mansehra, Pakistan	34.672654, 73.024309	1,038 m (3,406 ft)

For confirmation of the present studied plant species, its sequences were BLAST at NCBI or species. Clipping and low-quality sequences and primer areas were cleaned by CodonCode Aligner 3.7.1 (Codon Code, Co. Centreville, MA. USA). Alignment of DNA sequences and phylogenetic analysis of current specie *P. nudum* L. was carried by using ClustalX version 1.81 (Thompson 1997), MUSCLE, BioEdit and cladistics analysis was carried out by Mega 7 (Kumar *et al*. 2012). The bootstrap consensus inferred from 500 replicates along with pairwise deletion of missing sites for the evolutionary study of taxa (Felsenstein 1985). The genetic distance was calculated on the basis of Kimura-2 parameter model (Kimura 1980).

Results and Discussion

A 25 µl PCR reaction mixture was amplified according to the procedure described by Zhang *et al*. (2014) by using the conditions mentioned in Table 2.

Table 2. Various DNA barcode regions used in current study.

Sl. No	Primer name	Sequence 5´ to 3´
1	*rbcLa*-F	5'ATGTCACCACAAACAGAGACTAAAGC3'
	R	5'GTAAAATCAAGTCCACCCRCG3'
2	*trnA*-F	5'GGTTCAAGTCCCTCTATCCC3'
	R	5'ATTTGAACTGGTGACACGAG3'
3	*trnV*–F	5'GTAGAGCACCTCGTTTACAC3'
	R	5'CTCGAACCGTAGACCTTCTC3'
4	*ITs*-F	5'GCCGTTAAGACCAGGGAT3'
	R	5'TGATTCACGGGATTCTGC3'
5	*rboB*-F	5'ATGCAACGTCAAGCAGTTCC3'
	R	5'GATCCCAGCATCACAATTCC3'
6	*Mat-K*-F	5'CCCTATTCTATTCAYCCNGA3'
	R	5'CGTATCGTGCTTTTRTGYTT3'
7	*ycF3*-F	5'AGAACCGTACTTGAGAGTTTCC3'
	R	5'CTGTCATTACGTGCGRCTATCT3'

PCR products were purified via vender of gene elution kit [Cat. GF-GP-100] and sequencing was performed by Macrogen, (Seoul, Korea and Amsterdam, The Netherlands). Nearly all the DNA barcode regions showed good amplification while sequencing of *matK, ITs* and *rpoB* did not give good results.

After PCR amplification and sequencing, the sequences were queried via BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the online nucleotide database. BLAST analysis was used to evaluate the identification power of tested markers at species level. Aligned sequences were searched in National Centre for Biotechnology Information (NCBI) database through BLAST (Altschul 1990). Top matching hit having the highest (> 98%) maximal per cent identity score was the criteria for successful conspecific/congeneric delimitation. The comprehensive report comprised of the query cover, E-value, indent value, sequence length, identity value and gaps along their accession numbers were mentioned in Table 3.

Table 3. Summary of BLAST results.

Sl. no.	Species	Accession nos.	Query cover %	E-value	Indent	Sequence length	Identity	Gaps (%)
rbcLa								
1	*Psilotum complanatum*	KY099851.1	100	0.0	100	1309	(553/553)	00
2	*Psilotum complanatum*	MF349812.1	100	0.0	100	553	(553/553)	00
3	*Psilotum nudum*	L11059.1	100	0.0	99	1905	(546/553)	00
4	*Psilotum nudum*	KR816696.1	100	0.0	98	1328	(540/553)	00
5	*Psilotum nudum*	KY099852.1	96	0.0	98	1292	(526/536)	00
6	*Psilotum nudum*	EF469944.1	99	0.0	94	1258	(514/521)	00
7	*Psilotum nudum*	KJ710862.1	94	0.0	98	1248	(511/552)	00
8	*Psilotum nudum*	AB626657.1	91	0.0	99	1197	(500/507)	00
9	*Psilotum nudum*	AB574682.1	91	0.0	98	1205	(500/507)	00
10	*Psilotum nudum*	U30835.1	91	0.0	98	1206	(499/507)	00
11	*Psilotum nudum* L.	LC371057	-	-	-	553	-	-
trnA								
1	*Psilotum nudum*	FJ384430.1	96	0.0	99	796	406/409	3/409
2	*Psilotum nudum*	AY241586.1	96	0.0	99	862	406/409	3/409
3	*Psilotum nudum*	AY327836.1	90	0.0	99	828	378/381	3/381
4	*Psilotum nudum.* L	LC374378	-	-	-	419	-	-

Fig. 1. *P. nudum.* L. showing morphological features.

Fig. 2. Representative map of the study area (Hazara division, KP Pakistan).

There are no records of *P. Nudum* L. sequences of *trnV* and *ycf3* at the species level in the DNA databases. The sequences of the present work were successfully submitted at DNA Data Bank of Japan (DDJB).

Total 11 sequences were used, 1 from the present study while others were retrieved from NCBI for the investigation of phylogenetic reconstruction. The length of tree was -4542.51 with 553 nucleotides having 2 conserved regions, 505 variables, 440 parsimony informative and 65 singleton sites. Two major clades were constructed comprising of eight and three species. *P. nudum* L. of the present study showed close similarity with *P. complanatum* (MF349812.1) with the bootstrap value 51 (Fig. 3a). While in neighbor joining tree, *P. nudum* L. showed close similarity with *P. complanatum* (KY099851.1) with bootstrap value 36. On the other hand, the tree based on Maximum Parsimony Method showed similarity of *P. nudum* L. with *P. complanatum* (MF34912.1) at bootstrap value of 36 and with the tree length of 1179. (Fig. 3c). The branches less than 50% are collapsed. Tajima's relative rate was conducted. The identical sites in all these three sites were 507, while the divergent site was 00. Unique differences in both *P. nudum* L. (present studied) and *P. nudum* (EF469944.1) were 00 and in *P. nudum* (MF349812.1), the unique difference was 0 (Table 4).

Table 4. Description of different sites of *rbcLa* and *trnA*.

Barcode regions	*rbcLa*	*trnA*
Conserved sites	2/553	13/419
Variable sites	505/553	406/419
Parsimony informative	440/553	63/419
Singleton sites	65/553	343/419

On the basis of Maximum Likelihood, Neighbor Joining and Maximum Parsimony, the tree length was -2186.09, 721 and 710, respectively. The present studied species showed close resemblance with *P. nudum* (AY327836.1) and *P. nudum* (AY241586.1) with bootstrap values 33 and 39 with Maximum likelihood and Neighbor-Joining Method. *P. nudum* L. had 419 base pairs out of which 25 of them were conserved, 474 were variables, 63 were parsimony informative and 411 were singleton sites. (Fig. 4). Tajima's relative rate showed that identical sites in all these

three sites were 32, divergent sites were 148, and unique difference in *P. nudum*, *P. nudum* (FJ384430.1) and *P. nudum* (AY241586.1) were 84, 81 and 74, respectively. Sequence of current research showed reasonable similarity between *P. nudum* L. and *P. nudum*. (FJ384430.1) (Table 5).

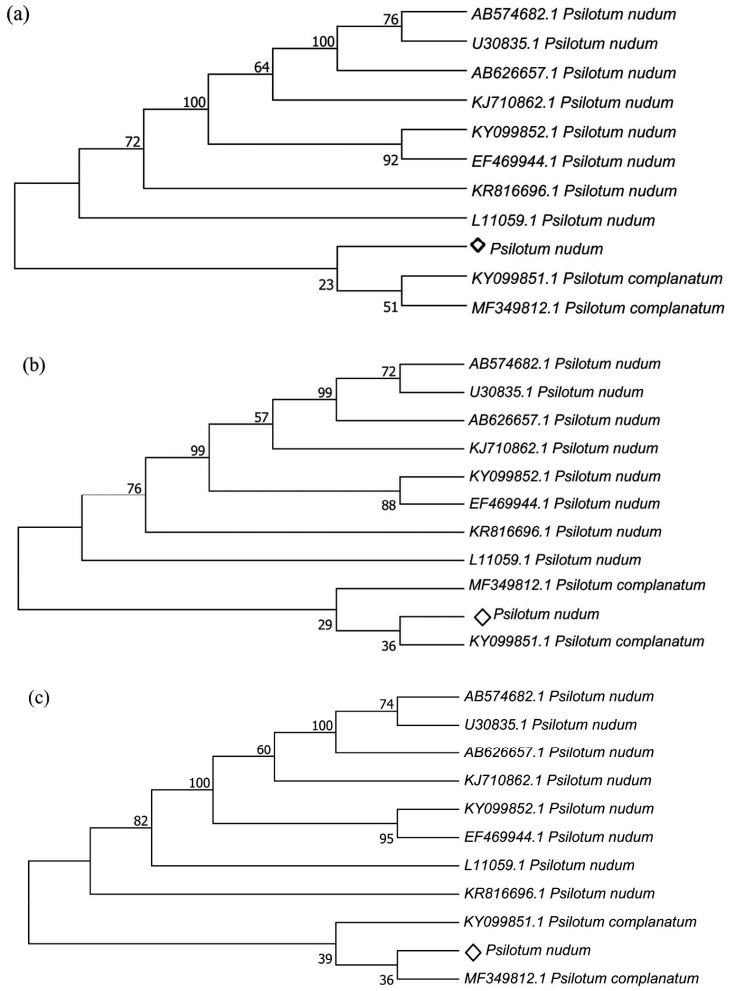

Fig. 3. (a, b and c) Tree of Maximum parsimony, Maximum likelihood, Neighbor Joining of *rbcLa* with the bootstrap values at the branches.

Model based phylogenetic gave the reliable authentication of success rate of DNA barcode regions by illustrating different substitution pattern from simpler to complex criterion. Basic evolutionary analyses were tested to check out the best model for the calculation of phylogenetic aspects. The best DNA model for estimating evolutionary distances is believed to be the nucleotide substitution model in which evolutionary distance between a pair of sequences was computed as the number of nucleotide substitutions occurring between them in a nucleotide-by-nucleotide comparison (Nei and Kumar 2000). It was used to infer the nucleotide frequencies, substitutions and more phylogenetic analyses. For each model carrying the lowest Bayesian

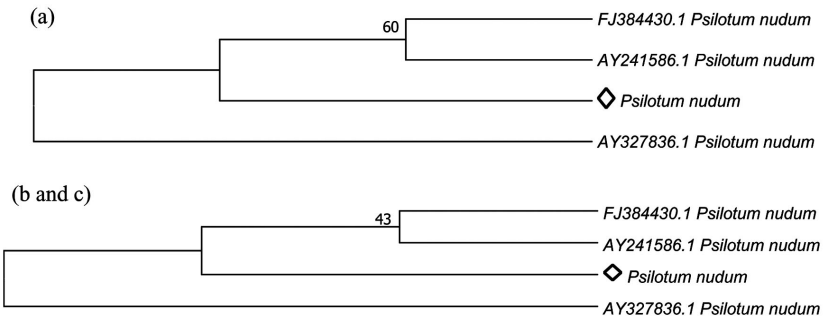

Fig. 4. Tree of Maximum Likelihood, Neighbor Joining and Maximum Parsimony of *trnA* with the bootstrap values at the branches.

Table 5. Tajima's rate relative test of DNA barcode regions *rbcLa* and *trnA*.

Configuration	Counts	
	rbcLa	*trnA*
Identical sites in all three sequences	507	323
Divergent sites	0	148
Unique differences in sequences (*Psilotum nudum* L)	0	84
Unique differences in sequences (*B*)	0	81
Unique differences in sequences (*C*)	0	74
Identical sites in all three sequences	507	323

B = KY099851.1 *(rbcLa*) and FJ384430.1 (*trnA*) and C= MF349812.1 *(rbcLa),* AY241586.1 *(trnA*).

Information Criterion [BICS], AICc-value (Akaike Information Criterion, corrected) is the minimum theoretical model describing good fit criteria. The results showed that the kimura2-model (K2) was the best followed by the Jukes-Cantor (JC) and Hasegawa Kishino-Yano (HKY) method with the parameter values 21, 22 and 22 for *rbcLa* and 5, 6 and 9 for *trnA* and BICS values were 8936.9, 8940.8 and 8929.1 for *rbcLa* and 4630.0, 4630.05 and 4438.9 for *trnA*, respectively. More and less similar pattern of evolution was observed in the rest of tested evolutionary set of parameters (Table 6). The log likelihood value [inL], with the help of discrete Gamma distribution [+G] non-uniform sites are modeled along certain invariable evolutionary sites [+I]. "R" was the estimated transition/ transversion bias for each model "r" and "f" were the relative rates and frequencies of substitution in the nucleotide base pairs whose sum is equal to (1) for every model. Maximum likelihood was determined by the complete deletion of gaps as well as missing data. Phylogenetic information was inferred on the basis of relative rates of evolutionary sites which are considered to be lower than 1, hence they were highly conserved as compared to the average conservation of sites in the alignment (Tamura *et al.* 2013).

The Consortium for the Barcode of Life (CBOL), officially confirmed that *rbcLa+ matK* are more efficient while others need to be explored more. *rbcLa* indicates the highest distinctive power, while *matK* is not good enough core DNA barcode region (Ebihara and Mitta 2010, de Groot *et al.* 2011, Hollingsworth *et al.* 2011. Similarly, the study showed close association among *P. nudum*. L, *P. complanatum* (KY099851.1), *P. complanatum* (MF349812.1) and *P. nudum* (AY241586.1) inhabitant of temperate creepy regions of New Zealand giving efficient resolution with *rbcLa* and *trnA* barcode regions, respectively.

Table 6. Five different substitution models based on Maximum likelihood.

rbcLa/trvA	Models	T92	HKY	JTR	K2	JC
rbcLa	Parameters	23	22	29	21	22
trnA		7	9	13	5	6
rbcLa	BICS	8950.3	8929.1	8937.1	8936.9	8940.8
trnA		4426.4	4438.9	4466.01	4630.0	4630.05
rbcLa	AICs	8776.9	8773.3	8756.4	8806.0	8803.6
trnA		4387.60	4387.60	4385.60	4388.60	4386.60
rbcLa	LnL	-4365.3	-4361.4	-4348.9	-4381.8	-4379.7
trnA		-2187.2	-2187.2	-2187.2	-2187.2	-2187.2
rbcLa	(+I)	n/a	n/a	n/a	n/a	n/a
trnA		n/a	n/a	n/a	n/a	n/a
rbcLa	(+G)	n/a	n/a	n/a	n/a	n/a
trnA		n/a	n/a	n/a	n/a	n/a
rbcLa	R	0.2433	0.2595	0.2684	0.5	0.21
trnA		0.30	0.31	0.56	1.67×10^{-7}	0.5
rbcLa	f(A)	0.2750	0.2662	0.2662	0.25	0.25
trnA		0.06	0.05	0.07	0.12	0.08
rbcLa	f(T)	0.27	0.28	0.28	0.25	0.25
trnA		0.34	0.33	0.33	0.25	0.25
rbcLa	F(G)	0.22	0.20	0.20	0.25	0.25
trnA		0.15	0.14	0.14	0.25	0.25
rbcLa	F(C)	0.22	0.22	0.22	0.22	0.22
trnA		0.15	0.16	0.16	0.25	0.25
rbcLa	r(AT)	0.11	0.11	0.18	0.08	0.1
`trnA		0.13	0.12	0.03	0.12	0.08
rbcLa	r(AC)	0.09	0.08	0.08	0.08	0.08
trnA		0.04	0.05	0.12	00	0.08
rbcLa	r(AG)	0.04	0.05	0.07	0.08	0.04
trnA		0.06	0.06	0.11	0.12	0.08
rbcLa	r(TA)	0.11	0.11	0.17	0.08	0.1
trnA		0.13	0.13	0.03	0.12	0.08
rbcLa	r(TC)	0.04	0.04	0.03	0.08	0.04
trnA		0.04	0.04	0	0	0.08
rbcLa	r(TG)	0.09	0.1	0.03	0.08	0.1
trnA		0.06	0.06	0.11	0.12	0.08
rbcLa	r(CA)	0.11	0.11	0.1	0.08	0.1
trnA		0.13	0.13	0.16	0.12	0.08
rbcLa	r(CT)	0.05	0.06	0.04	0.08	0.04
trnA		0.09	0.09	0	0	0.08
rbcLa	r(CG)	0.09	0.1	0.11	0.08	0.1
trnA		0.06	0.06	0	0.12	0.08
rbcLa	r(GA)	0.05	0.06	0.08	0.08	0.04
trnA		0.09	0.09	0.24	0	0.08
rbcLa	r(GT)	0.11	0.11	0.04	0.08	0.1
trnA		0.13	0.12	0.22	0.12	0.08
rbcLa	r(GC)	0.09	0.08	0.09	0.08	0.1
trnA		0.06	0.05	0	0.12	0.08

GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

The low performance of *trnA* in differentiating the species represents the solid indicator for the close phylogenetic association among *Psilotum* species. The results showed the poor sequencing performance for the DNA barcodes m*atK, ITs* and *rpoB.* Although *matK* most reliable coding regions, it is often difficult to be amplified in *P. nudum* L. The results suggested that *trn*A

could be a good alternative DNA barcode region in spite of *matK* and *ITs* across the ferns. The present study correlates with the previous reports about the evaluation of seven main candidate DNA barcode regions (*rbcLa, matK, rpoC1, rpoB, trnHpsbA, atpF-atpH,* and*psbK-psbI*) in land plants (Ching 1940). Any sequence of *ycF3* at tested species level in database was not found. Previous studies also showed that its expression was limited to just monocots (Li 2018). Further analyses revealed that *ITs, rpoB, matK* and *ycF3* barcode markers could not be used in this study because of low discriminatory efficacy. Based on the advance bioinformatics study, the Maximum Likelihood was the most favourite substitution method for inferring the phylogenetic reconstruction. Likewise, DNA substitution model based comparative analysis showed that K2-parameter model was the best evolutionary model (Nei and Kumar 2000, Yang 2007). Such findings surely open the new gateway for critical molecular systematics of fern and fern allies by the comparison of PCR amplification rate and sequence quality could be re-evaluated. The evolutionary data for such remnant plants could be of great significant value in building and strengthening phylogenetically inferred relationships among the extant groups by providing unique evidences on the sequences.

The prime barcode resource for the native endangered plant of scenic valley of Battagram, Mansehra were recorded. The barcode regions *rbcLa, trnA, trnV* and *ycF3* would be efficient and *matK*, *ITs* and *rpoB* need further research for future assessment of their evolutionary aspects, as they are still elusive in the present findings. The studied markers are new calculations for phylogenetic reconstruction and species identification which would be surely helpful to sort out the mysterious backbone of such extinct plants.

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