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DNA Barcoding of Fagopyrum Sp. (Buckwheat) of the Himalayan Regions of Sikkim and Nepal

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Abstract

Buckwheat (*Fagopyrum* spp.) is a pseudo cereal and economically important nutraceutical crop. The two most cultivated species grown in the Himalayas are *Fagopyrum esculentum* (Common Buckwheat) and *Fagopyrum tartaricum* (Tartary Buckwheat). *Fagopyrum tartaricum* is a highly suitable crop that grows in extremely in cold conditions. It contains rutin (antioxidant polyphenol), quercetin, vitexin and D-chiro inositol (soluble carb). In tartary buckwheat seed proteome, a total of 3363 Proteins were found, and their biological significance was explored and annotated. While the growing season significantly affected the flavonoid content of the hulls, location was the primary source of variation for the flavonoid and rutin concentrations of the seed. The rutin rich buckwheat seeds needs to be verified and encouraged to be cultivated. DNA barcoding using two sections of the chloroplast genes like *rbcl* and *matK* along with the combinations of *ITS2* and *psbA-trnH* yields a better identification result. ITS2 is more suited for species recognition because to its short length and excellent PCR amplification efficiency. The cultivar NGRC03777 (*Fagopyrum esculentum)* had 73.75731 mg/100g rutin and NGRC03731 *(Fagopyrum tataricum* (L.) Gaertn) had 433 mg/100g of the Nepal Himalayas, and E 16-KUMREK had 67 mg/100g of rutin of from Sikkim Himalayas were used for DNA barcoding. The alignment using BLASTn with NCBI, showed 100% and some 98% identity with collected specimens. The future for identifying these rutin specific verities could help the public greatly.

Keywords: *Fagopyrum* spp., *rbcl* and *matK,* rutin, BLASTN,

Introduction

The *Fagopyrum esculentum* (Common Buckwheat) and *Fagopyrum tartaricum* (Tartary Buckwheat) are pseudo cereal and economically important nutraceutical crop that are cultivated species in Himalayas. It contains rutin (antioxidant polyphenol), quercetin, vitexin and D-chiro inositol (soluble carb). In tartary buckwheat seed proteome, a total of 3363 proteins were recognised, and their biological processes were explored and annotated. Thus, identifying their nutrient value will make us to promote the use of those nutraceutical important cultivars. The cultivar NGRC03777 (*Fagopyrum esculentum)* had 73.75731 mg/100g rutin and NGRC03731 *(Fagopyrum tataricum* (L.) Gaertn) had 433 mg/100g in of the Nepal Himalayas, and E 16-KUMREK had 67 mg/100g of rutin of from Sikkim Himalayas.

Rutin (quercetin3-rutinoside) is a glycoside of flavonoids found in nearly all the parts of the plant including, leaves, stems, inflorescence, cotyledons, and seeds. It possesses a variety of pharmacologically advantageous qualities, including actions that are anti-inflammatory, anti-thrombotic, cytoprotective, and vasoprotective in humans (Nidhi Gupta et al., 2011**).** In tartary buckwheat seed proteome, a total of 3363 proteins were noted, and their biological roles were explored and described. The rutin rich cultivars of the Himalayas needs to be identified and promoted as to get the best use of these cultivars.

As the cornerstone of all biology, the identification, naming, and classifying living things at the species level has emerged as one of the essential standards in biodiversity analysis and management, preservation, and breeding (Vu and Le., 2019). Using sequence divergence based on nucleotide alignment, a DNA barcode is a set of one or more short gene sequences (often 200–900 base pairs) selected from a standardised region of the genome to help identify and find new species (Emerson et al., 2011). This genetic tool's primary goal is to efficiently and successfully identify any biological sample's species independent of the sample's visual categorisation by comparing barcode patterns to refer to databases.

Verification of plant items covering medicinal plants to food products has used barcoding technologies (Chen et al.2010) to kitchen spices (De Mattia, et al. 2010), Berries (Jaakola et al. 2010), olive oil (Kumar et al. 2011) and tea (Stoeckle et al. 2011). Numerous real-world scenarios and classification tests had proven the value of the barcoding technology (Vu and Le 2019). The selected loci and algorithms in a barcoding technique based on sequences directly influence the identification outcomes. Phylogenetic trees have been constructed using genetic sequences obtained by DNA barcoding for application in phylogenetic community ecology.

The necessity for precise identification of species to both protect and use plants underpins the significance of plant DNA barcoding (de Vere et al., 2015). Unfortunately, none of their markers have been found to fully satisfy each and every need for DNA barcodes. For instance,

the rbcL fragment is easy to amplify, sequence, and align while having limited discriminatory strength. However, it is challenging to multiply the matK barcode, which might be the closest plant analogue to COI in animals because to the absence of appropriate universality primers (Braukmann et al., 2017; de Vere et al. 2015; Fang et al. 2019; Li et al. 2015).

In this study, we need to use particular plant DNA barcoding markers. Candidates for plant barcoding include the nuclear-encoded ribosomal internal transcribed spacer (ITS) region and the chloroplast intergenic spacer trnH-psbA. These candidates are followed by the coding sequences from the two plant loci that are now most frequently utilised, rbcL and matK, from plastid genes (Kress et.al.,2007, Loera-Sanchez et al., 2020; Yao et al., 2010), and generate specific gene sequences are unique to each cultivar which identifies and differentiates from the rest of cultivars in Nepal and Sikkim Himalayas.

Materials and Methods

Collection of Seeds

The buckwheat seeds with high rutin content of *Fagopyrum esculentam* and Fagopyrum tartaricum were collected from Nepal Agriculture Genetic Resources Centre (NAGRC) Khumaltar*, Lalitpur, Kathmandu, Nepal* and from the farmers of Sikkim Himalayas. The T 5 (NGRC03731) which had rutin of 433 mg/100g, and E 10 (NGRC03777)-74 mg/100g are from NAGRC and E 16-KUMREK-67 mg/100g from Sikkim were chosen for DNA barcoding purpose.

DNA Extraction

The DNA was extracted from buckwheat seeds using modified 2 % CTAB method. (Porebski L. et al., 1997). The seeds of buckwheat, 100 mg was measured and ground using mortar and thistle with adding gradually up to 1 ml of 2% CTAB solution (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 1% beta-mercaptoethanol, 2% CTAB). The extract was transferred to 2ml Eppendorf tubes and kept at water bathe for one hour at 60° C, with inverting gently every 15 minutes. After cooling it for fifteen minutes, 10 minutes of centrifuging at 10,000 rpm (rotation per minute) 700 µL of the supernatant was added into a new 2 ml Eppendorf tube. To this, equivalent amount of phenol Chloroform: Isoamyl Alcohol was added in a 25:24:1 ratio. centrifuged for 10 minutes at 10,000 rpm after being gently mixed 5 to 6 times by inversion. After being gently mixed five to six times by inversion, the mixture was centrifuged for 10 minutes at 10,000 rpm. To this, freshly prepared 50 µL of 5M Potassium Acetate was added

and an equal volume of ice-cold ethanol was added and allowed the DNA to precipitate for 20 minutes at 4° C. when the pellets appeared, tube was centrifuged at 10,000 rpm for 10 minutes. After discarding the supernatant, the pellet was washed twice with 500 µL of 80% freshly prepared ethanol at 10,000 rpm for 3 minutes and allowed to air dry for 20 minutes. The pellet was dissolved in 50 L of Milli-Q water devoid of nucleases and kept at -20° C for later use.

Agarose gel electrophoresis

The clean gel casting tray with comb was poured with agarose gel (0.8% w/v). The agarose gel was prepared by adding 0.8 gm of agarose (SRL, Mumbai) in 100 mL of 1X TAE (Tris-acetate-EDTA) and boiled. To this, after cooling it to room temperature, 1 µL of ethidium bromide (EtBr) was added (10mg/ml) was added. The gel was allowed to solidify for about 30 minutes. After placing it in the gel tank with freshly prepared 1X TAE (Tris-acetate-EDTA) from 10X stock solution, the genomic DNA was mixed with the gel loading dye and was loaded on to the wells and the DC current at 70 Volts. After 45 minutes, the gel was placed on a Gel Doc and images were captured using the Mediccare System.

Sequencing and PCR amplification of DNA

50 ng of total genomic DNA was utilised as the template for the PCR, which amplified DNA barcode markers using the standard primers. (Table-1 for matK, rbcL, ITS2 and psbA-tmH (Kress et al. 2007, Fazekas et al, 2008). 1X buffer containing 1.5 mM MgCl, 200 mM dNTPs, 5 pmol primers, and 1 unit of Taq DNA polymerase were included in the PCR reaction mixture (30 ml). PCR was done in a thermal cycler (Applied Biosystems, CA, USA) utilising the following protocol: initial denaturation at 95 °C for 5 minutes, 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minutes, final extension at 72 °C for 5 minutes, and hold at 16 °C (Nithaniel et al, 2014). Using a Silica Spin Column PCR Purification Kit, the PCR products were purified after being confirmed using agarose gel electrophoresis (Livegen Biotechnologies, Bangalore). Using the same PCR primers, the purified PCR products were sequenced in SeqStudio (Applied Biosystems, CA, USA) from both ends. With the aid of Gene Mapper v. 7.0, the sequences were manually modified (Applied Biosystems, CA, USA) and full-length sequences were put together.

Target	Nam of the Primer	Direction	Sequence (5' → 3')

matK	matK_xf	Forward	TAATTTACGATCAATTCATTC
matK	matK_MALPR1	Reverse	ACAAGAAAGTCGAAGTAT
rbcL	rbcLa_f	Forward	ATGTCACCACAAACAGAGACTAAAGC
rbcL	rbcL_724_rev	Reverse	GTAAAATCAAGTCCAOCRCG
ITS2	ITS-F3	Forward	CCGTGAACCATCGAGTCTTT
ITS2	ITS-R2	Reverse	CTCGCCGTTACTAGGGGAAT
psbA-trnH	psbA3_f	Forward	GTTATGCATGAACGTAATGCT
psbA-trnH	trnHf_05	Reverse	CGCGCATGGTGGATTCACAATCC

Table:1 Showing the details of the common plant DNA Barcoding markers along with their name and the details of the primer sequences

Basic Local Alignment Search Tool (BLASTn)

The Basic Local Alignment Search Tool (BLAST) identifies areas where sequences are locally similar. It may assess the statistical significance of matches between nucleotide or protein sequences and sequence databases. The rbcL, ITS2, psbA-trnH, matK, and fully modified sequences with original trace files were retrieved and BLAST, a basic local alignment search tool, were used to identify species in databases against the NCBI's non-redundant nucleotide database (www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and discussion

Banding Paterns of *rbcL,* ITS2, psbA-trnH **and *matK* markers and BLAST analysis of DNA sequence data**

The banding pattern of the PCR products clearly corresponds to their respective base pair sizes (Fig 1). The 100 kbs ladder is used as the reference. The lanes 1 to 3 are of the sample T5 (NGRC03731), E 10 (NGRC03777) and E 16-KUMREK respectively. The marker used is ***rbcL.*** The 4th & 5th lane is of ITS2 marker of E 10 and E16 cultivars. The 5th lane has no band present. The 6th and 7th lanes are of psbA-trnH of E10 and E16 samples. The 8th lane is of ***matK*** marker of E-16 cultivar.

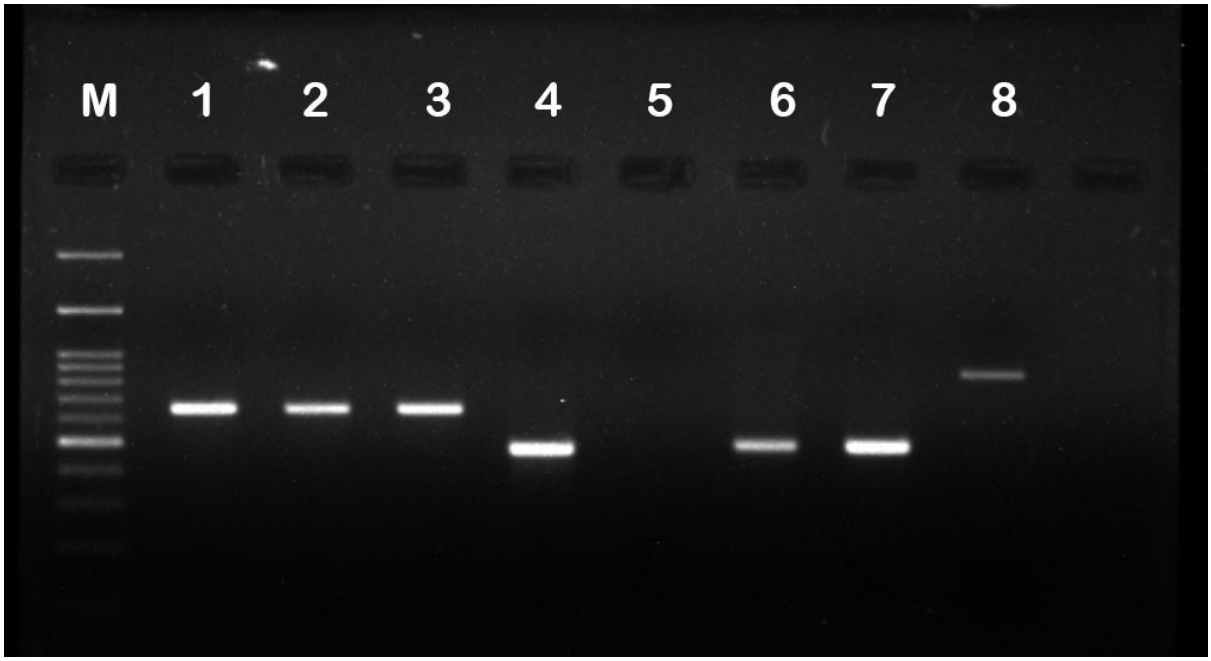

Fig 1: Agarose gel showing the PCR products of different markers: M-100 kbs Ladder. Lanes 1 to 3 **rbcL** Markers, Lane 4&5 are of **ITS2** Marker, Lanes 6 & 7 psbA-trnH marker and 8th Lane is of ***matK*** **markers**

rbcL **Marker**

The large subunit of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), which is encoded by the rbcL gene, is frequently utilised for molecular identification of different plant species. All the samples' coding regions were amplified, and amplicons of 580 bp were obtained in lanes 1-3. (Fig-1, lane 1,2&3).

These amplicons were further sequenced with both forward(rbcLa_f) and reverse (rbel724_r) primes. The strand of T5 sequenced with the forward primer was 535 bp in length(T5-rbcL-F) while the antisense strand sequenced with reverse primer was of 590 bp (T-5-rbcL-R) (Table :2).

Gene	Primer	Size	Sequence
rbcL	rbcLa_f	568	TGTTAAAGAATACAAATTGACTTATTATACTCCTGACTA TGAACCCCATGACCATGATATCTTGGCAGCATTTC GAGTAACTCCTCAACCTGGAGTTCCACCAGAAGAA GCAGGG

			GCCGCGGTAGCTGCCGAATCTTCTACTGGTACATG GAC AACTGTGTGGACCGATGGACTTACCAGCCTTGATC GTTACAAAGGACGATGCTACCACATCGAGCCTGTT CCTGGAG AAGAAAACCAATTTATTGCTTATGTAGCTTACCCAT TAGACCTTTTTGAAGAGGGTTCTGTTACTAACATGT TTACTTCCATTGTGGGGAATGTATTTGGGTTCAAAG CCCTGCGTGCTCTACGTTTGGAGGATTTACGAATCC CTCCTGCTTATACGAAAACTTTCCAAGGCCCGCCTC ATGGTATCCAAGTTGAGAGAGATAAATTGAACAAA TATGGACGTCCCCTATTGGGATGTACTATTAAACCT AAATTGGGGTTGTCTGCTAAGAACTACGGTCGAGC AGTTTATGAATGTCTTCGTGGCGGACTTGATTTTAC CAAAGATGATGAAAAC
rbcL	rbcL_724_re v	641	AATGGTTGGCAGTTCACGTTTTCATATCTTTGGTAA AATCAAGTCCGCCACGAAGACATTCATAAACTGCT CGACCGTAGTTCTTAGCAGACAACCCCAATTTAGG TTTAATAGTACATCCCAATAGGGGACGTCCATATTT GTTCAATTTATCTCTCTCAACTTGGATACCATGAGG CGGGCCTTGGAAAGTTTTCGTATAAGCAGGAGGGA TTCGTAAATCCTCCAAACGTAGAGCACGCAGGGCT TTGAACCCAAATACATTCCCCACAATGGAAGTAAA CATGTTAGTAACAGAACCCTCTTCAAAAAGGTCTA ATGGGTAAGCTACATAAGCAATAAATTGGTTTTCT TCTCCAGGAACAGGCTCGATGTGGTAGCATCGTCC TTTGTAACGATCAAGGCTGGTAAGTCCATCGGTCC ACACAGTTGTCCATGTACCAGTAGAAGATTCGGCA GCTACCGCGGCCCCTGCTTCTTCTGGTGGAACTCCA GGTTGAGGAGTTACTCGAAATGCTGCCAAGATATC ATGGTCATGGGGTTCATAGTCAGGAGTATAATAAG TCATTTGTATTCTTTACACAGCTTTGATCAACACTT

			GCTTAGTCCGTTTTGTGTGTGTGTGTGATAAAATAT AACCG

Table 2: *rbcl* gene sequence of *Fagopyrum esculentum* (E16) as obtained by forward and reverse primer

The strand of E10 sequenced with the forward primer was 575 bp in length(E10-rbcL-F) while the antisense strand sequenced with reverse primer was of 539 bp (E10-rbcL-R. The strand of E-16 sequenced with the forward primer was 561 bp in length (E-16 -rbcL-F) while the antisense strand sequenced with reverse primer was of 549 bp (E-16-rbcL-R.

CULTIVARS	***rbcL***	ITS2	psbA-trnH	*matK*
T5	Yes (535-F) (590-R)	-	-	-
E10	Yes (575-F) (539-R)	Yes (292-F) (263-R)	Yes (426-F) (434-R)	-
E16	Yes (561-F) (549-R)	(313-F) (252-R)	Yes (436-F) (437-R)	Yes (607-F) (631-R)

Table 3: Showing presence of bands of markers of *rbcL,* ITS2, psbA-trnH and *matK* and their number of base pairs in each forward and reverse primers in T5, E10, and E16 Cultivars revealed in Sequencing.

ITS2 Marker

In E10 and E16 samples, the nuclear ribosomal internal transcribed Spacer (ITS2) region was amplified. Using agarose gel separation, a 313 bp amplicon was found (Fig 1 lane 4 &5). The strand of E10 sequenced with the forward primer was 292 bp in length (E10- ITS-F3-F) while the antisense strand sequenced with reverse primer was of 263 bp (E10- ITS-R2-R.)

The strand of E16 sequenced with the forward primer was 313 bp in length (E16- ITS-F3-F) while the antisense strand sequenced with reverse primer was of 252 bp (E16- ITS-R2-R).

Cultivars/Gene	**Description**	**Common name**	**Percentage of Identity**	**Accession Length**
T5-rbcL-F	Fagopyrum tataricum cultivar Miqiao 1 chloroplast, complete.	Tartarian bu.	98.86	159272
T5-rbcL-R	Fagopyrum tataricum cultivar Miqiao 1 chloroplast, complete...	Tartarian bu.	99.36	159272
E10-rbcL-F	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	99.01	159576
E10-rbcL-R	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	98.68	159576
E16-rbcL-F	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	100.0	159576
E16-rbcL-R	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	98.09	159576
E10-ITS2-F	Fagopyrum esculentum isolate YJ-19 small subunit ribosomal RNA	Common Buckwheat	93.80	736
E16-ITS2-F	Fagopyrum esculentum isolate YJ-17 small subunit ribosomal RNA	Common Buckwheat	94.10	736
E10-ITS2-R	Fagopyrum esculentum isolate DS-2 small subunit ribosomal RNA	Common Buckwheat	96.98	736

E16-ITS2-R	Fagopyrum esculentum isolate DS-2 small subunit ribosomal RNA.	Common Buckwheat	94.72	736
E10-trnH-F	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	99.56	159576
E16-trnH-F	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	99.78	159576
E10-trnH-R	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	99.12	159576
E16-trnH-R	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	99.12	159576
E16-*matK*-F	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	98.15	159576
E16- *matK* -R	Fagopyrum esculentum chloroplast, complete genome	Common Buckwheat	97.58	159576

Table 4: Sequences producing significant alignments in percentages in Nucleotide BLANTn

psbA-trnH Marker

An intergenic spacer called psbA-trnH exists in the chloroplast genome. The amplicon size in the E10 and E16 samples was 437 bp. The agarose gel separation revealed an amplicon size of 437bp (Fig 1 lane6 &7). The strand of E10 sequenced with the forward primer was 426 bp in length (E10- psbA3_f -F) while the antisense strand sequenced with reverse primer was of 434 bp (E10- trnHf_05-R.) The strand of E16 sequenced with the forward primer was 436 bp in length (E16- psbA3_f -F) while the antisense strand sequenced with reverse primer was of 437 bp (E16- trnHf_05-R.)

matK Markers

Additionally, the nucleotide sequence of the maturase enzyme-coding gene matK, which is encoded by the plastid, was examined. The DNA sequence length for Sample E16 after PCR amplification was 631 bp. The forward primer resulted in 607 bp in length (Fig 1: Lane 8) (E16- matK_xf -F). The reverse primer resulted in 631 bp in length (Fig1: Lane 8) E16-matK_MALPR1-R)

Thus, all the four markers showed high percentage of identity with the NCBI data confirming the identity of the cultivars. The E16 cultivar shows 100% similar identity with the NCBI database (Table:4). T5 cultivar shows 99.36 % identity while the nuclear marker ITS2 showed the lowest similarity which is 94.72%.

Conclusion

Among the buckwheat cultivars found in Sikkim and Nepal, these T5 (NGRC03731), E 10 (NGRC03777) and E 16-KUMREK are found rich in rutin, could be advocated for more cultivation, and use in the future. The 8 samples of Fagopyrum Sp. with prominent amplicons were selected for each barcode. They were subjected to sequencing which revealed a close and unique identity. These could be identified and promoted for wider consumption.

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