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DNA BARCODING OF *BERGENIA STRACHEYI* (HOOK. & THOMS.) ENGL. (SAXIFRAGACEAE) FROM MUZAFFARABAD, AZAD JAMMU AND KASHMIR, PAKISTAN

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Abstract

DNA barcoding is impending towards the generation of universal standards for species discrimination with a standard gene region that can be sequenced accurately and within short span of time. The genus *Bergenia* is a perennial herb that is evergreen and a member of the Saxifragaceae family. The majority of people in Kashmir harvest and dry the rhizomes of *Bergenia,* which are stored for long term use. In Pakistan, two distinct species of *Bergenia*, specifically *Bergenia ciliata* and *Bergenia stracheyi,* have been documented. This plant is highly medicinal and comprises a lot of bioactive compounds, but an essential chemical component of the species is bergenin. The extraction of genomic DNA that can be amplified by PCR has become a crucial prerequisite. As a result of the known existence of polyphenols and polysaccharides in medicinal plants, the isolation of high-quality DNA presents a significant challenge. To achieve this, high-quality DNA was extracted from Bergenia species and subjected to PCR amplification using both non-protein-coding (ITS) and protein-coding (rbcL, MatK) primers. These DNA samples were obtained from specimens gathered from three different locations having sub alpine zones within AJ&K, Pakistan with the leaves serving as the primary source of genetic material.

The consensus length of the ITS gene in *B. stracheyi* included 513 base pairs. Out of 513 sites, 487 (94.9%) were conserved and 26 (5.06%) were variable characters, with 18 parsimonies informative and 8 singleton variables having the best fit model: JC 1195.470 BIC and 1239.676 BIC values, respectively. To establish the phylogenetic relationships of these *Bergenia* species, phylogenetic trees were constructed, revealing a remarkable 99% to 100% sequence similarity with their corresponding species in publically available databases. Both the BOLD and GenBank databases were utilized to validate the query sequences. More specifically, *B. ciliata* exhibited an average genetic divergence of 0.008%, accompanied by a mean nucleotide divergence within the species of 0.003%. The intraspecific divergence of *B. stracheyi* samples varied from 0.000 to 0.081, suggesting a comparatively high degree of inter-specific distance. When it comes to the success rates of amplification for different molecular markers, the overall success rates were 99% and 85% for rbcL and ITS, respectively. However, matK did not yield any amplification, resulting in a zero success rate. Consequently, based on these findings, rbcL is recommended as the most reliable plant barcode among potential molecular markers for *Bergenia* species. It would be a useful study to understand the evolutionary relationship, sketched in emigration and mislabeling, that could be a probable assessment for its biodiversity.

Key words: *Bergenia*; DNA barcoding; Phylogenetic; ITS; rbcL; matK; BOLD

Introduction

The historical relationship between humans and plants may be traced back to the earliest stages of human civilization (Goudie *et al.,* 2018). *Human impact on the natural environment: Past, present and future*. John Wiley & Sons. Nature has always been a rich source of novel and diverse chemical substances (Rates *et al.,* 2001). In Pakistan, two distinct species of *Bergenia*, specifically *Bergenia ciliata* and *Bergenia stracheyi*, have been documented (Amin *et al.,* 2025). The *B. stracheyi* has been reported from Afganistan to Uttarakhand (India) between 3300 to 4800 m in Alpine slopes (Bhat *et al.,* 2018). The presence of broad petiolar sheath, hood and bulblike structures distinguishes *B. stracheyi* from other species of this plant. The *Bergenia stracheyi* is an important Himalayan medicinal herb belongs to genus *Bergenia* and family Saxifragaceae. *Bergenia stracheyi,* commonly known as 'Pashanbheda' or Zakhm-e-Hayat, is a perennial herb that has been recognized for its diverse medicinal properties (Khan *et al.,* 2017).

The Azad Jammu and Kashmir is situated in the northwest Himalaya region, boasts a complex and varied terrain, encompassing a wide range of ecosystems such as meadows, steep mountain slopes, lakes, rivers, streams, springs, and wastelands. Remarkably, the Kashmir Himalayas alone are home to nearly 2,000 plant species, this is responsible for nearly 20% of the total plant species, although being confined to a mere 2.15% of the overall geographical area (Dar *et al.,* 2018).

Introduction of the *Bergenia stracheyi*: The *Bergenia stracheyi* is an important Himalayan medicinal herb belongs to genus *Bergenia* and family Saxifragaceae. *Bergenia stracheyi*, commonly known as 'Pashanbheda' or Zakhm-e-Hayat, is a perennial herb that has been recognized for its diverse medicinal properties (Tiwari *et al.,* 2017). In Pakistan, two distinct species of *Bergenia*, specifically *Bergenia ciliata* and Bergenia stracheyi, have been documented. The *B. stracheyi* has been reported from Afganistan to Uttarakhand (India) between 3300 to 4800 m in Alpine slopes (Tiwari *et al.,* 2017; Bhojak *et al.,* 2025). The presence of broad petiolar sheath, hood and bulblike structures distinguish *B. stracheyi* from other related species of the genus (Chowdhary & Verma, 2010).

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In honour of German physician and botanist Karl August von Bergen, Conrad Moench came up with the name Bergenia in 1794. Genus Bergenia is an evergreen perennial herb that harbours highly valued ornamental, rhizomatous, and temperate medicinal species (Koul *et al*., 2020). Since ancient times, in folk medicine the plant has been used to dissolve kidney stones and gallbladder (Singh & Pandey, 2019). *B. ciliata* is a 50cm tall herb, mostly found in temperate Himalayan regions (Pandey *et al*., 2019). The native population in the Sikkim Himalayas utilizes the Bergenia rhizome for the treatment of several ailments, including bone fractures, new lacerations, wounds, diarrhea, respiratory infections, vomiting, fever, cough, and boils (Idrisi *et al.,* 2010).

Hierarchy of Bergenia

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Saxifragales
Family: Saxifragaceae
Genus: *Bergenia*
Species: *stracheyi*

The plant under investigation possesses secondary metabolites, namely bergenine, gallic acid, and catechin, which exhibit therapeutic properties and have been historically employed in traditional medicine. These compounds are mostly accountable for the advantageous attributes associated with plant (Singh *et al.,* 2017). Furthermore, *Bergenia* has been utilized in traditional medicinal practices for the treatment of many ailments including haemorrhoids, gastrointestinal disorders, ocular impairments, and cardiovascular issues. It is also acknowledged for its antimalarial, analgesic, antiviral, and anti-inflammatory properties. In traditional medicine, *B. stracheyi* has been utilized as a restorative tonic for its therapeutic potential in treating stomach ulcers (Ahmed *et al.,*2013; Akhtar *et al.,* 2013).

Global biodiversity is experiencing a serious and unprecedented decline, with about one million species on the verge of extinction, a trend expected to accelerate in the future decades (Palombo, 2021; Turnhout & Purvis, 2020; Borthakur & Singh, 2024;). Among the many influencing factors, climate change amplified by human activities stands as the leading and intensifying cause of ecological disruption (Ripple *et al.,* 2017; Arneth *et al.,* 2020; Verrall & Pickering, 2020). The destabilizing effects pose a serious threat to ecosystem resilience and balance (Nazar *et al.,* 2020). However, the IUCN Red categorise of Threatened Species does not officially categorise *Bergenia stracheyi* as threatened plant (Krishna & Francis, 2012; Borthakur & Singh, 2024). Plant has lovely flowers, due to its limited range, ornamental value, and medicinal overuse, the plant is rare and requires both in situ and ex situ conservation (Wan *et al*., 2016; Zegeye, 2017; Wani *et al.,* 2024).

Similarly, morphometric changes caused by long-term isolation and interbreeding might have an impact on population diversity. Limited population sizes can lead to an increase in genetic drift and inbreeding. Genetic drift refers to the stochastic alteration in the frequency of alleles across successive generations due to the transmission of a limited number of alleles. The impact of genetic drift is more conspicuous in populations with smaller sizes, and groups that consistently have small effective sizes are particularly vulnerable to the depletion and recombination of genetic diversity due to drift (Ouborg *et al*., 2006).

Moreover, populations experiencing sporadic fluctuations in size, such as bottlenecks or founder/ colonization events, may also experience a random loss of genetic diversity. Allelic variation tends to decrease as population size reduces dramatically, although heterozygosity might remain relatively stable as population size increases rapidly. The genetic effects of bottlenecks and founder events influence population structure and composition (Barrett, 1992).

A fundamental aspect of molecular evolution research is understanding the relationships among genes, phylogeny, and diverse fields, ranging from theoretical biology to applied biotechnology. Molecular phylogeny reconstructs the evolutionary link between species using DNA and protein sequences. By conducting a comprehensive analysis involving a substantial number of DNA and protein sequences, as well as their respective lengths, researchers have been able to identify which sequences display variations due to natural selection and highlight the regions that have evolved gradually over time. These investigations use phylogenetic tree-based comparative approaches to discover the evolutionary links between various sequences (Lynch & Walsh, 2007).

When it comes to plant DNA extraction, maintaining both the quality and yield of DNA faces greater difficulties as compared to animal DNA extraction. This phenomenon is caused by the presence of stiff cell walls in plants, which are mostly made of cellulose and a number of other chemical components such as proteins, lipids, polysaccharides, and polyphenols. Medicinal plants, including *Bergenia* species, are known for their richness in polyphenols. These compounds further complicate the DNA extraction process. To address these challenges, a standard DNA extraction protocol called hexadecyltrimethyl-ammonium bromide (CTAB) extraction procedure has been developed and standardized. This protocol is effective in removing contaminants and ensuring the successful extraction of DNA (Heikrujam *et al.,* 2020).

Although, in DNA barcoding, approximately 20% of the raw medications were contaminated, and approximately 6% came from plants with completely different detrimental medicinal properties. Raw pharmaceuticals in the form of dried roots, powders, and complete plants were shown to be more susceptible to adulteration than rhizomes, fruits, and seeds. Co-occurrence, physical similarity, mislabeling, deceptive vernacular names, and fraudulent or illegal substitutions could all have had an impact on species adulteration in raw pharmaceuticals (Pan *et al.,* 2013).

Hence, the use of this technique on a regular basis can serve as a means of authentication for sold raw pharmaceuticals, thereby benefiting all relevant parties involved, including traders, customers, and regulatory bodies. The process of DNA barcoding involves the sequencing of short segments of DNA in order to enable

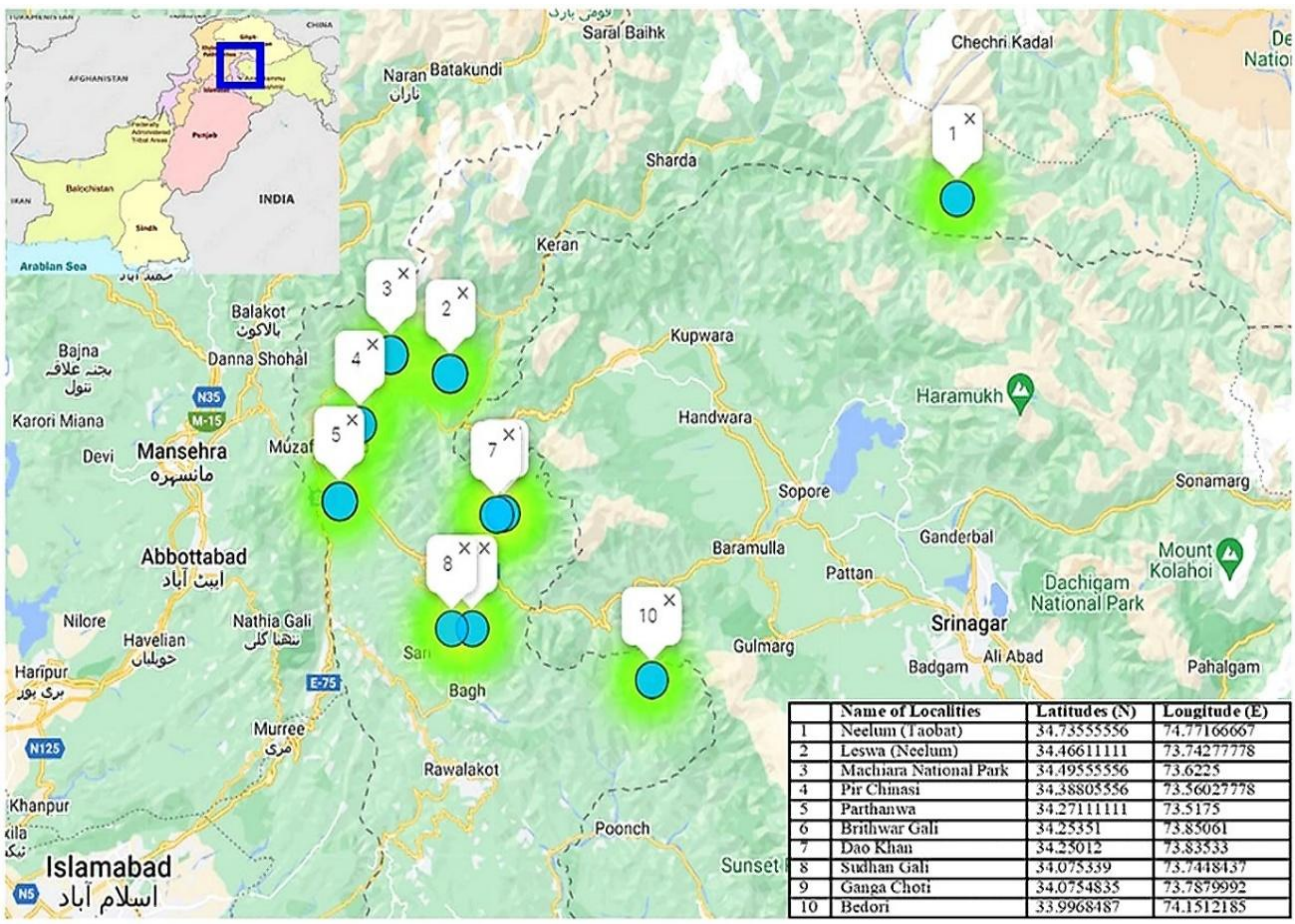
efficient and accurate identification of species, particularly those that possess a significant level of genetic resemblance. DNA barcoding has gained significant popularity as a method for the examination of invasive plant species, determination of the geographical range of plant roots, and discrimination between species that are visually indistinguishable at the macroscopic level. In addition, the genetic sequences employed for DNA barcoding exhibit a significant degree of conservation across species, hence facilitating the development of universal sequencing primers. The publication of Hebert's fundamental work drew significant attention to the field of barcoding research. They proposed the use of the mitochondrial gene CO1 as the generally approved barcode for animal species. Genetic markers derived from PCR have become increasingly valuable in several domains of animal, plant, and food science. These markers are employed for genotyping, as well as for sample identification and authenticity purposes (Pan *et al.,* 2013; Hebert *et al.,* 2003).

Similarly, overharvesting and local plant species extinction may result from growing demand for therapeutic items. Improper collection procedures may also result in the introduction of harmful or unsuitable species into the market, providing a health risk to end consumers. In order to identify an unknown sample, a barcode sequence is produced and compared to a reference library of barcodes from recognised museum specimens. This comparison is facilitated by the utilisation of an algorithm, which ultimately leads to the identification of the sample. DNA barcodes serve as molecular identifiers for each species, analogous to the machine-readable black-and-white barcodes employed in the retail sector for the purpose of identifying commercial products (Chase *et al.,* 2007; de Boer *et al.,* 2015).

Therefore, in light of the aforementioned features, the current work was designed in order to ensure accurate identification, and phylogenetic analysis of the *Bergenia stracheyi* in their native environment.

Material and Methods

Sites of collection of plant material: In Azad Jammu and Kashmir, plant samples of *B. stracheyi* were gathered from three different sub alpine locations i.e., Leswa (Neelum), Upper Brithwar and Bedori (Leepa Valley) (Figs. 1 and 2). Dr. Ashfaq, a taxonomist, identified the plant samples using the Flora of Pakistan database, which is available at http://www.tropicos.org/Project/Pakistan, as well as relevant literature. Plant names and taxonomic data, including genus, species, class, order, and family names, were documented and annotated. After labelling, the samples were securely packed inside polythene bags and returned to the University of Azad Jammu and Kashmir's Molecular Genetics Laboratory for further investigation. The samples were folded and wrapped in aluminium foil and kept in liquid nitrogen at -80°C following examination. Because Bergenia is lithotriptic plant found primarily on rocks and inhabit steep regions, their collection is quite challenging.



	Name of Localities	Latitudes (N)	Longitude (E)
1	Neelum (Taobat)	34.73555556	74.77166667
2	Leswa (Neelum)	34.46611111	73.74277778
3	Machiara National Park	34.49555556	73.6225
4	Pir Chinasi	34.38805556	73.56027778
5	Parthanwa	34.27111111	73.5175
6	Brithwar Gali	34.25351	73.85061
7	Dao Khan	34.25012	73.83533
8	Sudhan Gali	34.075339	73.7448437
9	Ganga Choti	34.0754835	73.7879992
10	Bedori	33.9968487	74.1512185

Fig. 1. The pic of studied areas from Azad Jammun and Kashmir.

Fig. 2. Inflorescence shown by *B. stracheyi* and *B. ciliata*.

DNA extraction, amplification and sequencing: On the molecular level, however, DNA barcoding is used to authenticate plant identity. DNA isolation is the first step in the process, which is followed by the polymerase chain reaction (PCR) and DNA sequencing of the amplified results. For the isolation of complete genomic DNA, Saghai-Maroof's approach was used with minor modifications (Saghai-Maroof *et al.,* 1984). A pestle and mortar was used to grind a sample that weighed around 100 mg. Then, for DNA extraction, 500 µL of CTAB buffer was added. This buffer contained 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, 2% CTAB, 1% beta-mercaptoethanol, and 2% polyvinylpyrrolidone (PVP). After homogenising the samples, they were placed in 1.5 ml centrifuge tubes and kept in water bath at 60°C for 30 minutes to 2 hours. Following an equal volume of chloroform extraction, the samples were centrifuged for 10 minutes at 10,000 rpm. After transferring the aqueous phase to 1.5 mL centrifuge tubes and precipitating it with an equivalent volume of ice-cold isopropanol, the samples were left to stand overnight. The samples underwent a 10-minutes centrifugation at 10,000 rpm in the next day. After two rounds of washing with 70% ethanol, the DNA pellet was allowed to air dry at room temperature and then dissolved in 100 µl of TE buffer. Every sample had DNA that could be amplified using PCR. Gel electrophoresis was used to assess the amount and quality of genomic DNA. The DNA quality was estimated using a 1% gel electrophoresis. A spectrophotometer was used to verify purity and concentration at A260/280.

The 1%Concentration of Agarose Gel: DNA presence was verified through agarose gel electrophoresis. A 0.5% agarose gel was prepared using 10× TBE buffer and stained with ethidium bromide. DNA samples mixed with bromophenol blue were loaded into the wells, and electrophoresis was performed at 100 V for 30 minutes. The DNA bands were visualized using a gel documentation system.

The primers applied for amplification of desired product: In this study, the loci ITS and rbcL were selected as suitable candidates for DNA barcoding. Primer-3 (version 0.4.0) software was used to generate sequencing primers for the Bergenia species' ITS and rbcL genes, as shown in Table 1.

Table 1. The sequence of barcoding loci for PCR amplification.

Name of Gene	Primer pair	Annealing temperature
rbcLaF	5'-**TGTAAAACGACGGCCAGT**ATGTCACCACAAAC AGAGACTAAAGC-3'	55-60
rbcLaR	5'-**CAGGAAACAGCTATGAC**GTAAAATCAAGTCCACCRCG-3'	55-60
nrITS2-S2F	5'-**TGTAAAACGACGGCCAGT**ATGCGATACTTGGTGTGAAT-3'	50-55
nrITS2-S3R	5'-**CAGGAAACAGCTATGAC**GACGCTTCTCCAGACTACAAT-3'	50-55
matk-3F	5'-**TGTAAAACGACGGCCAGT**CGTACAGTACTTTTGTGTTTACGAG-3'	60-65
matk-1R	5'-**CAGGAAACAGCTATGAC**ACCCAGTCCATCTGGAAATCTTGGTTC-3'	60-65

Fig. 3. Extracted DNA and amplified products of rbcL and ITS.

The polymerase chain reactions or PCR: A polymerase chain reaction (PCR) was conducted using a thermal cycler device known as SimpliAmp. In order to get a DNA amplification that can be reproduced reliably, it is necessary to work with a final volume of 25 µL. A total volume of 25 µL was prepared by combining the following components in 0.2 mL PCR tubes: 4 µL of DNA + 2.5 L of taq buffer + 2.5 µL of MgCl2 + 0.5 µL of Fermentas dNTPs (10 mM) + 1 µL each of forward and reverse primers + 0.5 µL of taq DNA polymerase + 13 µL of PCR water. In order to achieve homogenization, the reaction mixture was centrifuged at the speed of 8,000 rpm.

The following were the reaction conditions: 39 cycles of amplification come after 3 minutes of 95°C denaturation of the DNA template. Three steps made up each cycle: denaturing the DNA into single strands for one minute at 95 degrees Celsius, ligation of the primers to their template DNA for 1minute at 55–63 degrees Celsius and elongating the complementary DNA strand from each primer for one minute at 72 degrees Celsius. Additionally, taq polymerase was allowed to amplify the remaining DNA strands for ten minutes at 72ºC. The final held was then performed at 4ºC. Ultimately, the PCR amplification was verified by using 2% agarose gel electrophoresis.

Agarose Gel Electrophoresis: 2% agarose gel was prepared using 10× TBE buffer and stained with ethidium bromide. DNA samples mixed with bromophenol blue were loaded into the wells, and electrophoresis was performed at 100 V for 30 minutes. DNA bands were visualized using a gel documentation system.

The purification of amplicons: The amplified products, potentially containing primer dimers and other contaminants, were subjected to purification and clean-up using the "FavorPrepTM PCR Clean-Up Mini Kit and the FavorPrepTM Gel Purification Mini Kit".

The process of sequencing: The PCR products were sequenced at various facilities, namely the Centre for Applied Molecular Biology (CAMB) in Lahore, as well as the at 1st BASE and the Korean Macrogen Company.

Sequence analysis: The software BioEdit, available at http://www.mbio.ncsu.edu/BioEdit, was utilized to modify nucleotide variants present in the sequences. By using The Clustal W in the MegAlign tool nucleotide sequences were compared with known global sequences using from the LASERGENE package developed by DNASTAR Inc. in Madison, WI, USA. The primary origin of global sequences utilized in this study was the NCBI Genbank database, which may be accessed at the following URL: http:// www.ncbi.nlm.nih.gov/genomes. Moreover, after edition consensus sequences were matched against sequences in the NCBI database using BLAST Analysis. Sequences showing a similarity of over 98% were then stored on the BOLD platform, accessible at (www.barcodinglife.org), along with preliminary data summaries.

Phylogenetic analysis: The present investigation involved the utilisation of the Barcode of Life Data System search engine and the Basic Local Alignment Search Tool (BLAST) accessible on Genbank/NCBI for the purpose of identifying Bergenia species. Multiple sequence alignments were conducted to assess the sequence divergence among Bergenia species in Azad Jammu and Kashmir (AJ&K). Phylogenetic trees were constructed using various methods, including neighbor-joining (NJ), maximum-likelihood (ML), with Kimura 2-parameter techniques. To evaluate the reliability of the phylogenetic trees, bootstrap tests with 1000 repetitions were performed using the MegAlign program in the LASERGENE software, following the approach outlined by (Saitou & Nei, 1987. Jiang *et al.,* 2014; Khan *et al.,* 2016).

Additionally, the MEGA 11.0 software was utilized to calculate the transition/transversion bias (R), following the principles established by (Baldwin, 1992). Furthermore, to gain insights into genetic variation, DnaSP 5.0 was employed to compute the average number of pairwise nucleotide differences, as proposed by (Rieseberg, & Soltis, 1991) as well as nucleotide substitutions per site, based on the method by (Nei & Kumar, 2000).

Results

In the present study, all *Bergenia stracheyi* sequences were successfully deposited in the GenBank database and assigned unique accession numbers (ON721698, ON721699, ON423296, ON427377, ON712827, ON712828) as shown in Table 2. DNA barcoding proved to be an effective approach for species identification. However, its success largely depends on the researcher's expertise and involves several essential steps such as polymerase chain reaction (PCR), cloning, sequencing, and alignment with GenBank. The process typically requires 2–4 days for completion. For global recognition, DNA barcodes must contain easily amplifiable and variable regions that enable reliable species-level identification (Meyer & Paulay, 2005). DNA barcoding based on specific genetic markers serves as a reliable tool for accurate species discrimination (Cowan & Fay, 2012). In addition to the plastid rbcL gene, the nuclear ribosomal internal transcribed spacer (ITS) region is also employed for analysis (Lunt *et al.,* 1996).

Sequencing and alignment of the *Bergenia* primers were performed using the Clustal W program. The alignment process facilitated the classification of species into distinct groups. Comprehensive information regarding accession numbers, sequences, trace files, collection localities and dates of collection were available in NCBI Genbank. Furthermore, all sequences of *B. stracheyi* were successfully barcoded using the rbcL and ITS genes (Baldwin, 1992). The absence of nuclear pseudogene co-amplification was confirmed by the lack of stop codons and the presence of distinct peaks, consistent with findings from previous studies (Muellner *et al.,* 2014).

The alignment of *B. stracheyi* with the ITS gene (Table 3) was composed of a total of 513 base pairs. Out of 513 sites 487(94.93%) were conserved and 26(5.06%) were variable characters with 18 parsimony informative and 8 singleton variables having Best fit model: JC 1195.470 BIC and 1239.676 BIC values correspondingly.

The degree of divergence among sequences varied slightly. ITS region divergence values varied from 0.00 to 0.70. Among all the sequence pairs under study, the average evolutionary divergence was 0.008±0.003. According to (Table 4). The average percentage divergence (K2P) distance for *B. ciliata* was 0.01 percent, whereas for *B. stracheyi* it measured 0.03 percent.

Table 2. List of barcoded samples with accession numbers.

No of species	Name of species	Gene name	Genbank accession no.	Collection locality
1.	*B. stracheyi*	ITS	ON427377	Upper Brithware
2.	*B. stracheyi*	ITS	ON712827	Leswa Neelum
3.	*B. stracheyi*	ITS	ON712828	Bedori Leepa
4.	*B. stracheyi*	rbcL	ON721698	Upper Brithware
5.	*B. stracheyi*	rbcL	ON721699	Leswa Neelum
6.	*B. stracheyi*	RbcL	ON423297	Bedori Leepa

Table 3. Nucleotide Composition of *B. stracheyi* through ITS gene.

	T%	C%	A%	G%	Total
B. stracheyi (KY986459.1)	26.1	24.8	23.6	25.5	330
B. stracheyi (KY986455.1)	26.1	24.8	23.6	25.5	330
B. stracheyi (ON712828)	24.6	23.4	27.1	25.0	513
B. stracheyi (ON712827)	24.6	23.8	26.8	24.8	512
B. stracheyi (ON427377.1)	24.6	23.4	27.0	25.0	512
B. stracheyi (KY986458.1)	26.1	24.8	23.6	25.5	330
B. stracheyi (KY986457.1)	26.1	24.8	23.6	25.5	330
B. stracheyi (KY986456.1)	26.3	23.4	24.3	26.0	688
B. stracheyi (KU524084.1)	26.3	25.1	23.9	24.8	331
Avg.	25.5	24.1	25.1	25.3	430.7

Table 4. The details of the paired intra-specific and inter-specific distances in barcode loci of *Bergenia* species.

Species name	Summary of Intra-specific distances (%)		
	Minimum	Maximum	Mean
B. ciliate	0.00	0.02	0.01
B. emeiensis	0.00	0.00	0.00
B. ligulate	0.00	0.70	0.25
B. purpurascens	0.00	0.06	0.01
B. scopulosa	0.00	0.00	0.00
B. stracheyi	0.00	0.08	0.03

Phylogenetic investigations of *Bergenia* species: *Bergenia stracheyi* is present in sub alpine zone ranges from 3200m to 4300m. In current research, the plant was collected for the first time from remote areas of Azad Jammu & Kashmir and barcoded with universal primers. By using the Clustal W programme, the ITS gene of the *Bergenia* species was sequenced and matched with every global sequence found in the GenBank and BOLD databases. Because of this alignment, species could be grouped into a distinct cluster.

Evolutionary trees have been constructed using two methods: maximum likelihood (ML) and neighbor joining (NJ), which reflect discrete character and distance methods respectively. Depending on the type of investigation, there are two methods for creating a phylogenetic tree (Distance Methods and Discrete Methods). In order to produce pairwise distance matrices, distance methods use matched nucleotide sequences. In the distance methods section, phylogenies, UPGMA, and NJ approaches are listed. However, different studies demonstrated that these approaches produced remarkably similar results when the data had little noise and strong signals (Saitou & Nei, 1987; Gogoi & Bhau, 2018).

The investigation aimed to identify *Bergenia* using ITS and rbcl gene loci barcodes. The neighbour joining analysis demonstrated that the ITS and rbcl genes are trustworthy markers for identification of species (Felsenstein, 1985). The research utilised the Neighbor-Joining technique to deduce the evolutionary lineage, employing the methodology introduced by (Saitou & Nei, 1987). The provided tree is the best tree with a total branch length of 0.02314032.

Additionally, a bootstrap analysis with 1000 replicates was performed to evaluate the robustness of the phylogenetic tree. Branch support values, representing the percentage of replicate trees in which associated taxa clustered together, were calculated following the method proposed by (Felsenstein, 1985) are presented adjacent to the corresponding branches. The Jukes-Cantor approach, as originally proposed by (Jukes & Cantor, 1969) was employed for the computation of evolutionary distances. The data set utilized in this analysis comprised nucleotide sequences, resulting in a final dataset consisting of 506 positions in total. The entire evolutionary analysis was conducted using MEGA 11, as outlined in the study by (Stecher *et al*., 2018).

Bergenia stracheyi is inhabitant of sub-alpine zone. In Azad Jammu & Kashmir the collection of this plant could be possible during June, July and August. For present study the species of *B. stracheyi* were collected from Leswa (Neelum Valley), Upper Brithware (Leepa Valley), and (Bedori) the highest track of brithware with an altitude of (3400m). In phylogenetic analysis the samples collected from Azad Jammu and Kashmir constructed a separate cluster with 91% discrimination rate whereas samples reported from India formed another branch of tree with 95% bootstrap value (Fig. 4). Sample of Bedori (ON712828) and Upper Brithware (ON427377) branched into same cluster. Both samples have been collected from two different sites of same valley (Leepa Valley), have less genetic differences because of same environmental condition.

Species in the trees are indicated by their names and their accessions numbers.The sequences of *Bergenia stracheyi* of Azad Jammu & Kashmir formed a separate cluster with 86% and 100% bootstrap values followed by Indian samples with 59% to 90% bootstrap value as shown in Fig. 5.

The intraspecific divergence between all above samples of *Bergenia stracheyi* species ranged from 0.000 to 0.081 (Table 5). The interaspecific divergence between the Indian species and the species of Azad Jammu & Kashmir revealed the highest ratio with 0.005 to 0.057.

Investigations on the phylogenetics of *Bergenia* species using rbcL: The primary aim of the present study was to analyse the suitability of the rbcL barcode for distinguishing between various species within the genus *Bergenia*, alongside the analysis of the ITS region of nuclear DNA. The Neighbor-Joining analysis demonstrated the validity of the rbcL (Fig. 6) gene as a marker for species identification.

The alignments of *Bergenia stracheyi* showed the consensus length of 724 sites with 709(97.9%) conserved sites and 15(2.02%) variable characters having 12 parsimony informatives, out of which 3 were singleton as shown in (Table 6). The best fit model values were 3034.060 and 2259.433 BIC respectively.

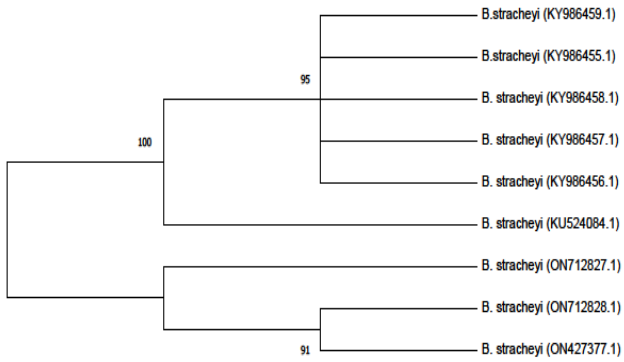


Fig. 4. The maximum likelihood method based ITS gene phylogenetic analysis of *Bergenia stracheyi* species on the Tamura-Nei Model with MEGA 11.

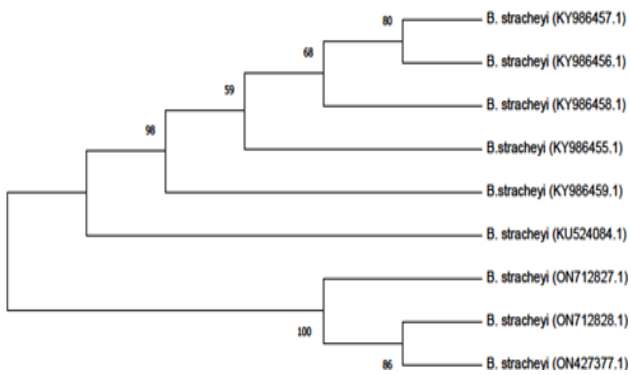


Fig. 5. *Bergenia stracheyi* species phylogenetic study based on ITS gene sequence using MEGA 11 neighbor-joining method (NJ).

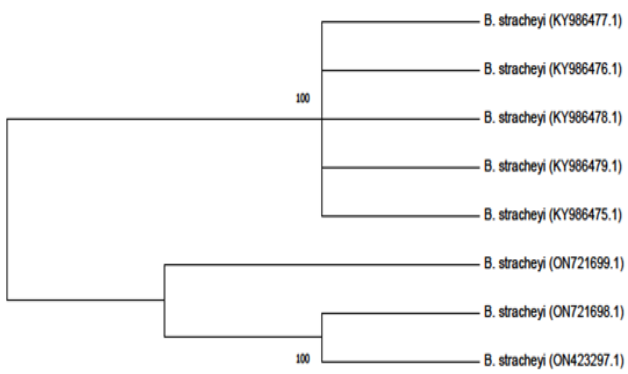


Fig. 6. The study of the evolutionary links of *B. stracheyi* with rbcL by NJ method.

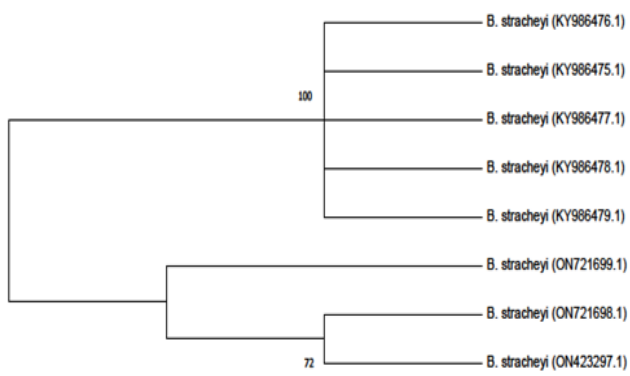


Fig. 7. The study of the evolutionary links of *B. stracheyi* with rbcL by maximum likelihood method.

Table 5. Unrooted phylogenetic tree based on current study nucleotide sequences and international *B. stracheyi* sequences (with accession numbers).

	Name of species	1	2	3	4	5	6	7	8	9
1.	*B. stracheyi* (KY986459.1)									
2.	*B. stracheyi* (KY986455.1)	0.000								
3.	*B. stracheyi* (ON712828)	0.057	0.057							
4.	*B. stracheyi* (ON712827)	0.050	0.050	0.004						
5.	*B. stracheyi* (ON427377.1)	0.057	0.057	0.000	0.004					
6.	*B. stracheyi* (KY986458.1)	0.000	0.000	0.057	0.050	0.057				
7.	*B. stracheyi* (KY986457.1)	0.000	0.000	0.057	0.050	0.057	0.000			
8.	*B. stracheyi* (KY986456.1)	0.000	0.000	0.081	0.074	0.081	0.000	0.000		
9.	*B. stracheyi* (KU524084.1)	0.009	0.009	0.047	0.040	0.047	0.009	0.009	0.009	

Table 6. Nucleotide composition of *B. stracheyi* through rbcL gene.

	T %	C %	A %	G %	Total
B. stracheyi (ON721699.1)	28.9	20.2	28.3	22.7	724
B. stracheyi (ON721698.1)	29.0	20.2	28.3	22.5	724
B. stracheyi (ON423297.1)	29.1	20.0	28.0	22.8	724
B. stracheyi (KY986479.1)	28.6	20.4	28.4	22.5	714
B. stracheyi (KY986478.1)	28.6	20.4	28.4	22.5	714
B. stracheyi (KY986477.1)	28.6	20.4	28.4	22.5	714
B. stracheyi (KY986476.1)	28.6	20.4	28.4	22.5	714
B. stracheyi (KY986475.1)	28.6	20.4	28.4	22.5	714
Avg.	28.7	20.3	28.3	22.6	714

The Neighbor-Joining method was employed to reduce phylogenetic relationships. The optimal tree is presented, whereby the total sum of branch lengths is precisely 0.02170624. Adjacent to the branches, the percentages of duplicate trees are presented, indicating the extent to which the associated taxa were clustered together in the bootstrap test (1000 repititions). The evolutionary distances were quantified in terms of base substitutions per site and were computed utilizing the Maximum Composite Likelihood method. The ultimate dataset comprised a total of 724 positions. The evolutionary history was inferred using the Maximum Likelihood (Fig. 7) approach and the Kimura 2-parameter model.

The tree with the greatest log likelihood value is denoted as (-1072.65). The provided data illustrates the clustering of linked taxa within a subset of trees that are adjacent to the branches. The heuristic search's starting tree(s) were automatically constructed by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances. These distances were assessed using the Maximum Composite Likelihood (MCL) approach. The topology with the highest log likelihood value was then selected. To characterize changes in evolutionary rates among different locations, a discrete Gamma distribution with a parameter value of 0.1062 (+G) was employed. Certain sites were deemed to be evolutionarily constant according to the rate variation model, namely those designated as [+I] with a prevalence of 0.00% sites. The current analysis utilized a final dataset that contained 704 positions in total.

Discussions

Numerous plant species have the potential to be used medicinally, industrially, or economically. Unfortunately, insufficient attention is devoted to the conservation, correct use, and long-term flow of these species. A wide range of ecological, social, and economic factors are all contributing to the extremely significant danger to the medicinal plant population. Because these plant species are considered as public assets, conservation is ignored in the interest of maximising personal benefits and many people are unaware of their importance due to lack of education. The local community uses valuable plants for animal grazing, firewood, and fodder. Despite being the source of many significant synthesised pharmaceuticals, the number of non-productive uses for these species is rising along with their population. These species are vital for the preservation of biodiversity. The extinction of species of vulnerable plants and animals, loss of natural habitat, and land degradation are just a few of the detrimental effects that may await future generations if the population of these plant species remain below a sustainable level. The results of the present research supported the taxonomic placement of the genus Bergenia and the effectiveness of the rbcL gene and the ITS region as genetic markers for distinguishing plant species was confirmed, highlighting their potential as reliable candidates for plant DNA barcoding (Feng *et al.,* 2016; Yu *et al.,* 2021). Progress in the use of DNA barcodes in the identification and classification of medicinal plants.

High-identity consensus sequence from Bergenia confirmed consistent identification, supporting the ITS region as a reliable marker despite occasional sequencing challenges. Recent studies have highlighted its growing importance in plant identification (Bafeel *et al.,* 2012; Feng *et al.,* 2016).

Comparably, it would be possible to prove species adulteration in the majority of traded raw drugs using the conserved rbcL marker, which is primarily capable of genus level taxonomic discrimination but also has good PCR amplification and sequencing success (Parmentier *et al.,* 2013; Newmaster *et al.,* 2013; Chen, *et al*., 2015). But as a useful core or supplemental marker, ITS is developing to improve raw drug authenticity and species resolution (Seethapathy *et al.,* 2015; Saha *et al.,* 2020).

In the present work, ITS, matK, and rbcL were used as candidate DNA barcoding loci for the identification of the *Bergenia* species. The results of PCR amplification demonstrated that the two DNA barcode regions had 100% amplification and sequencing efficiency, and suitable to identify *Bergenia* species. These all studied barcoding loci showed reasonably good results except matK which showed worse performance in amplification. Previous literature, had described the PCR and sequencing problems with matK gene. The NJ tree based on ITS and rbcL barcodes yielded bootstrap values above 50% on key branches, supporting the reliability of these markers for barcoding *Bergenia* species with medicinal relevance and broad distribution (Prince, 2002; Tripathi *et al.,* 2013).

The conservation rates observed for the ITS locus in this study, ranging from 94% to 98.9%, align with previously reported findings. Notably, the ITS region exhibits a relatively higher evolutionary rate compared to the matK and rbcL loci, as demonstrated in earlier studies (Aziz *et al.,* 2015; Vassou *et al.,* 2015; Zhao *et al.,* 2015; Xiong *et al.,* 2018).

Moreover, DNA barcoding has been suggested as a reliable approach for species identification, capable of detecting both known and novel species (Chen *et al.,* 2022). Initially, rbcL and *matK* were proposed as universal barcode regions for plant DNA barcoding, while *trnH-psbA* and *ITS* were employed as supplementary markers in the previous study (Fernandes *et al.,* 2021).

Similarly, our findings support the use of DNA barcoding for species identification, which is essential for conservation. PCR and sequencing success rates are key criteria for selecting an effective barcode (Wu *et al.,* 2019).

In this study, two universal barcodes (matK and rbcL) along with the supplementary barcode ITS were analyzed to determine the most effective marker for identification of *Bergenia* species. The evaluation was based on primer universality, species identification success rate, and phylogenetic tree analysis.

In this regard, the rbcL beat all other studied barcode regions (Pei *et al.,* 2017). The findings of the present study agree with earlier research reporting a 90% success rate for rbcL (Pei *et al.,* 2017; Khan *et al.,* 2019). While some studies have documented a sequencing success rate of up to 100% (Fernandes *et al.,* 2021).

Conversely, amplification and sequencing of the matK region have proven challenging, and similar difficulties were encountered in this study, where successful amplification of this region was not achieved (Umer *et al.,* 2022). The diversity of medicinal plant species is particularly important, as it contributes to local livelihoods. Investigating these species allows researchers to gain insights into their traditional therapeutic use (Chen *et al.,* 2022). DNA barcoding is recommended as an effective tool for species identification, as it can accurately detect both known and novel species (Gao *et al*., 2010).

In the present study, both the Internal Transcribed Spacer (ITS) and the Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) genes proved to be effective DNA barcodes for identifying *Bergenia* species. The amplification success rate for both markers was 100%, while the sequencing success rate for *B. stracheyi* ranged from 86% to 100%. These results are consistent with previous studies (Tamura *et al.,* 2007; Sijimol *et al.,* 2020; Dhivya *et al.,* 2020). which have also identified ITS as a reliable barcode for various plant species due to its high genetic variability, making it particularly useful for distinguishing closely related taxa. The rbcL and ITS were tested as novel barcodes for identification of *Bergenia* species in Pakistan. Accurate identification requires at least three reference sequences and a 2–3% genetic match with the query Wong & Hanner, 2008; Sharma & Kala, 2022).

Phylogenetic analysis revealed that all *Bergenia* samples from AJK (Pakistan) constituted a monophyletic group with bootstrap values more than 50%. Samples may be categorised into their species using both rbcL and ITS barcodes. However, in the current study, a comparison of the two primers revealed that rbcL has 44.4% variability whereas, 98.9% of the ITS region is retained. Despite this heterogeneity, the plant from the subalpine zone, *B. stratcheyi*, produces the greatest results. These findings are consistent with Sharma and colleagues research (Sharma & Kala, 2022). Their research reveals that rbcL produces the greatest results for higher altitudinal medicinal plants.

Generally, the loss of plant populations, also known as population decline or genetic erosion, can result from a combination of genetic and environmental factors. Genetic factors play a significant role in this process, contributing to reduced genetic diversity and the potential for inbreeding depression. As a result, the phylogeny of *Bergenia* species is very helpful for taxonomy and the investigation of progressive patterns and processes in AJK, Pakistan.

Present investigations examine the evolutionary connection of *Bergenia* species in AJK, Pakistan. In addition to the monophyletic origin of the Saxifragaceae family, its members may be traced back to shared ancestral lineages. The *Bergenia* species exhibit a clear clustering pattern, with no instances of sharing or overlapping seen with species outside of the outgroup. To align a monophyletic group, morphological features must be evaluated, resulting in the natural categorization of species, but DNA data supplied unbiased characters. This is consistent with the findings of (Hörandl & Emadzade, 2012).

The similarity between marker genes and morphological traits provided evidence supporting the monophyletic origin of Saxifragaceae. Current work offered first significant information on DNA barcoding of genus *Bergenia* from Azad Jammu & Kashmir. The *Bergenia* species treated with different primers that are closely related and form a single cluster. The results of our study also provide information for the detailed relationship among *Bergenia* species found in different regions of AJK, Pakistan.

Historically, the inference of phylogenetic connections and speciation in organisms mostly relied on the analysis of morphological features. Distinguishing between *Bergenia* species poses a challenge because to the striking resemblance in their exterior shape. Similarly, to overcome the adulteration the barcoding of all natural vigor is necessary. The objective of this investigation was to assess the discriminatory ability of nuclear (ITS) and chloroplastic (rbcL) DNA barcodes in distinguishing between different species. The present findings highlighted the accuracy of ITS and rbcL genes and their applications for resolution of query sequences at species level.

Although the genus *Bergenia* (Saxifragaceae) is native to central Asia and encompasses 32 known species in all over the world. Among these, nine are of pharmacological relevance. But in Pakistan only two species of *Bergenia* are found in flora of Pakistan (Ali & Qaiser, 1986). This plant is located in remote areas with very risky habitat. Mostly this plant lies on cliff or high rocks so its collection is very difficult. In comparison with *Bergenia ciliata* the *Bergenia stracheyi* is distributed in limited areas of Azad Jammu and Kashmir. Although the plant is not in endanger plant according to IUCN list but in Azad Jammu & Kashmir the rhizome of this plant is overexploited not only by human beings but also by goats and monkeys during drought conditions.

Similarly, *B. stracheyi* with ITS yielded a 91% bootstrap value with 0% error, but the international sequence of Indian *B. stracheyi* yielded a 95% bootstrap value with 2e- error. The samples obtained from two different sites of Leepa Valley i.e., Bedori ON712828 and upper brithware ON427377 form the same cluster. Because, both have smaller altitudinal differences, With the NJ technique, both Bedori and Brithware demonstrated 86 to 100% bootstrap value, whereas ON722827 demonstrated 100% bootstrap value. In some plant genera, it has been determined that the most optimal choice is to utilize a mix of plastid loci and ITS. Based on our findings, it was observed that ITS and rbcL exhibited a higher number of parsimony informative sites and more discriminatory capability compared to the other hypothesized barcode loci, namely ITS, rbcl, and matK. The findings of this study are consistent with the outcomes reported in prior research conducted by (Jin *et al.,* 2014). Analysis of pairwise genetic distances suggests that species exhibiting higher inter-specific than intra-specific divergence tend to have greater genetic distinctiveness. However, each *Bergenia* species shows a degree of geographic specificity or restriction.

Therefore, DNA barcoding serves as an effective tool for resolving species identification challenges, particularly in cases involving illegal trade and verification of geographic origin. DNA barcoding technologies have shown to be reliable techniques for the accurate identification of medicinal plants, as well as their replacements and adulterants, namely at the genus and species level. This methodology yields consistent and dependable outcomes irrespective of the sample's age, plant component, or environmental variables. This study aims to assess the potential use of DNA barcoding in the taxonomic identification and origin determination of endangered and endemic ethno taxa that are subject to illegal trafficking. Based on the findings of this study, it can be inferred that the authenticity of DNA barcode identification may be enhanced by the utilization of an integrated strategy that incorporates both prior and posteriori date.

Phylogenetic trees, diagnostic nucleotides, and inter- and intra-specific divergence collectively affirm that the ITS region and rbcL gene represent the most robust DNA barcoding loci for precise delineation of *Bergenia* species. These two markers exhibit high discriminatory power, rendering them effective tools for taxonomic resolution. Conversely, the matK locus yielded suboptimal results. However, with more specifically designed primers, they could provide better results.

Moreover, the overexploitation of medicinal plants leads to adulteration and species authentication challenges. Other techniques, including as chemotaxonomy, chromatography, and microscopy, have had little efficiency in identifying species due to complex chemical structures, a deficiency of distinctive chemicals, environmental factors, plant age, and regional variations. DNA barcoding can alleviate these problems by utilising tissue from any part of the plant at any point of its life cycle, whether living or dead.

Furthermore, there are various potential issues that may impede the reliability of DNA barcoding as a method for plant identification. The first challenge is creating reference DNA sequences on a regular basis. Secondary compounds in medicinal plants, such as by co-precipitating or attaching to DNA, tannins, alkaloids, and polysaccharides may inhibit DNA extraction and amplification. Proper primers are required to amplify the specified gene region after acquiring clean genomic DNA. Certain gene sections such as rbcL, trnL, and the trnL-F intergenic spacer, have been found to be very efficient in amplifying DNA sequences in a wide range of plant species. Additional sites, such as matK, can exhibit greater variability and so require a customized primer design. Primers play a crucial role in ensuring successful amplification during the polymerase chain reaction (PCR); however, they may lack the necessary specificity for efficient performance during cycle sequencing, which is required for synthesizing fluorescently labeled DNA. Cycle sequencing can fail despite successful PCR amplification if there is insufficient complementarity between the primer and its target region. Various chloroplast markers have been employed in plant DNA barcoding efforts (Sucher & Carles, 2008; Techen *et al.,* 2014) with the Plant Working Group of the Consortium for the Barcode of Life recommending rbcL and matK as core barcode regions for plant species identification (Plant, C.B.O.L., 2009).

As a result, the fundamental aims of DNA barcoding are to identify universal sequence sections containing conserved sequences and to increase species diversity. The variable barcoding sections' flanking sites and conserved sequences are made to complement the universal primers' design (Ali *et al.,* 2014). Therefore, it is imperative that barcode sequences display both intraspecific consistency and interspecific variance (Rai *et al.,* 2000).

In addition, the *Bergenia ciliata* is predominantly found in the Himalayan regions of India and is extensively utilized in the trade of raw medicinal materials. Several DNA barcoding studies have addressed the issue of species adulteration in medicinal plant raw materials and natural health products (Bruni *et al.,* 2010; Kool *et al.,* 2012; Wallace *et al.,* 2018).

Although, numerous studies have shown that DNA barcoding is an excellent way of species identification. DNA barcoding, according to (Bruni *et al.,*2010) is a valuable approach for quickly and precisely detecting harmful plant components. Similarly (Tiwari *et al.,* 2015) reported the genetic variability and population structure of *Bergenia* species (Saxifragaceae) in the western himalaya by using DAMD and ISSR markers and found the genetic diversity and population structure in 41 accessions representing three populations of *B. stracheyi*. Besides genetic diversity DNA barcoding is an advanced tool of identifying and authenticating species in plant systematics. Because it can

aid in identifying species boundaries, providing a deeper comprehension of biodiversity, and establishing priorities for habitat conservation in several vulnerable plant groups (Aubriot *et al.,* 2013; Fernandes *et al.,* 2021).

Moreover, the utilization of plant DNA barcoding in the detection of species adulteration in commercially available raw medications is considered to be a highly beneficial application. In the past, the primary method for determining the phylogenetic studies of plants and understanding their speciation was to utilise physical characteristics. It is challenging to distinguish between distinct species of *Bergenia* only by looking at their outward appearance. The limited dependability shown in reconstructed phylogenetic trees that rely on morphological characteristics can be attributable to the intricate evolutionary dynamics including morphological and physiological components. This phenomenon has undergone changes due to recent breakthroughs in the field of molecular biology. Hence, the utilization of brief DNA sequences derived from either the nuclear or organelle genomes represents a sophisticated methodology known as DNA barcoding, which serves as an advanced means of identifying biological materials. With the help of DNA technology, phylogenies could be inferred and genuine, genealogically based classifications could be proposed. According to (Goldstein & DeSalle, 2011) DNA barcoding is useful for defining species borders, highlighting new species, and delineating existing species in addition to aiding in species identification.

However, prior to this study from Pakistan, (Jamil & Ashfaq, 2016) attempted to improve rural living standards in Pakistan by biochemical profiling and DNA barcoding of medical plants in northern and desert areas. They successfully barcoded 34 plants out of 36 plants from the Swat Valley, with the exception of *Bergenia ciliata,* which gave 0% results despite using all three of their designed primers, ITS, Matk, and rbcL. Whereas present study successfully barcoded the *Bergenia* species with two ITS and rbcL barcoding loci. Present results showed deviation from their study. This study aimed to evaluate the efficacy of selected DNA markers for the first-time barcoding of the *Bergenia* genus. The primers used for most markers were suitable; however, the *matK* primer pairs demonstrated limited amplification efficiency, suggesting the need for improved primer specificity.

Conclusion

This work is the inaugural investigation into the evaluation of DNA barcode candidates in *Bergenia* species originating from the Himalayan area of Azad Jammu & Kashmir (Pakistan). The present investigation demonstrated that every species under the genus *Bergenia* constituted a monophyletic assemblage. The current results were compared with those retrieved from BOLD and NCBI databases and the species name was determined based on the sequences that exhibited a similarity of 99 to 100 percent with the reference sequences. So this work has great importance with its novelity. These findings may be useful in the future for distinguishing the species of other therapeutic plants besides *Bergenia*, and hence may be successful as barcodes for this genus.

Challenges and limitations of barcoding: DNA barcoding faces challenges like incomplete databases, taxonomic issues, intraspecific variation, hybridization, DNA sample quality, pseudogenes, convergence, legal and ethical considerations, technical hurdles, and interpretation difficulties. Despite these challenges, DNA barcoding remains valuable for species identification and biodiversity research, with ongoing efforts to improve its effectiveness. NGS technologies have the potential to revolutionize the process by providing more extensive genomic data. Obtaining purified, high molecular weight DNA is crucial for molecular methods in medicinal plant research, but it's challenging because of DNA degradation and the presence of secondary metabolites. A number of techniques, including both commercially available kits and traditional approaches, are employed to isolate high-quality DNA from medicinal plants. DNA barcoding, a traditional technique, is effective for plant identification, but next-generation sequencing (NGS) offers advanced possibilities.

Recommendations

Based on the findings of the present study, several important recommendations are proposed to address the decline of medicinal plants, particularly in light of the absence of regulatory frameworks and pricing mechanisms necessary to protect this inestimable natural resource.

Here are some recommendations;

- Encourage the cultivation and promotion of significant therapeutic plants at the farm level.
- Promote the utilization of a scientific approach to advance herbal medicine.
- Carry out molecular documentation, particularly DNA barcoding, for significant species of medicinal plants found in the whole country.
- Prefer the biochemical profiling of medicinal plants in order to increase their economic worth, especially the species of utmost value should be emphasized.
- Raise awareness among local communities about the economic significance of medicinal plants and provide training in sustainable collection methods.
- Facilitate the establishment of a unified medicinal plant industry within the private sector, supported by well-organized and standardized local markets, to ensure a regular and sustainable availability of plant-based products.
- Make a comprehensive set of government policies concerning the preservation and price of medicinal plants.
- Establish and implement export policies for medicinal plants, overseen by a regulatory body.
- Promote collaboration between plant research institutions and industry to effectively harness the industrial potential of medicinal plant resources. Support educational and research institutions in the creation of medicinal plant libraries for knowledge dissemination.
- Designate areas rich in medicinal plant species as protected areas to safeguard them from wildlife and nomadic activities.

- Establish seed banks to ensure the availability of high-quality medicinal plant seeds.
- Preserve Kashmir's biodiversity by generating appropriate barcodes for medicinal plants and registering them in databases like NCBI and BOLD.
- Initiate molecular characterization of key medicinal plant species to promote organic living and support the development of the plant-based pharmaceutical sector in Azad Jammu and Kashmir (AJK).

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