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GENETIC DIVERSITY AND DNA BARCODING OF PTERIDACEAE FERNS OF HINDUKUSH-HIMALAYAN REGION: SWAT VALLEY, PAKISTAN

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Abstract

Ferns of the Swat region represent a rich component of Pakistan's flora, yet they remain unexplored at molecular and phylogenetic level. Specimens representing several fern species were collected from four areas of Swat Valley located in the Hindukush-Himalayan mountain range of Pakistan. The specimens, initially identified based on morphology, were subjected for the first time to molecular and phylogenetic analyses for DNA barcoding using sequences of the *rbcLa* marker (a segment of *rbcL* gene). This chloroplast marker was successfully amplified and sequenced using Sanger sequencing method. Genetic identification or DNA barcoding at the species level was conducted using three DNA based datasets approaches: BLASTn-based sequence similarity searches, genetic distance, and maximum likelihood (ML) phylogenetic reconstruction. All three methods yielded congruent and reliably authenticated results for genetic diversity and DNA barcoding of sampled fern taxa. The *rbcLa* barcode exhibited strong discriminatory power and clearly resolved species within their respective clades, which was statistically supported by the Mann-Whitney U test ($Z = -7.615$, $p<0.001$) and ROC analysis (AUC = 0.991). This study authenticated three species of *Adiantum* and two species of *Pteris* through *rbcLa*-based molecular identification. It also provides molecular evidence for taxonomic status of *Onychium lucidum* (D. Don) Spreng and *O. cryptogrammoides* Christ, which united in phylogenetic tree suggesting that *O. contiguum* should be treated as *O. cryptogrammoides*. Also, the presence of *Aleuritopteris acrostica* (Balb.) Windham & Schuettp. [*Hemionitis acrostica* (Balb.) Mosyakin] and *A. nitidula* (Hook.) Windham & Schuettp. [*H. nitidula* (Hook.) Christenh] has been uncertain in Pakistan, our results provided the first DNA-based confirmation of their presence in the country and in fern flora of Swat. Overall, our findings highlighted the effectiveness of *rbcLa* for authentication of species of Pteridaceae ferns and provided the first molecular insight into fern flora of the Swat Valley lacking DNA-based systematic documentation, while also suggesting that incorporation of additional plastid or nuclear markers may further improve species level resolution in future studies.

Key words: DNA barcoding; *rbcLa*; Pteridaceae; Ferns; Hindu Kush-Himalaya; Phylogenetic analysis

Introduction

The Swat Valley of Khyber Pakhtunkhwa Province covers an area of approximately 5337 km² in Malakand Division of Pakistan. The region is exceptionally biodiversity-rich due to its unique geographical position and broad elevational gradients, ranged from about 680 to 7,000 meters above sea level (Ahmad *et al.*, 2024). The sampling sites, the upland areas of Madyan, Malam Jabba, Miandam, and Marghazar with temperate climate are rich in fern flora with a strong Hindu Kush-Himalayan biogeographic signature. These sites lie within a transitional zone where the floristic elements of the Western Himalayas and Hindu Kush converge, creating a mosaic of habitats supporting diverse pteridophyte and other fern assemblages.

The "Flora of Pakistan" documents approximately 6000 plant species and is largely based on morphological taxonomy, whereas the taxonomic treatment of Pteridophytes is still in progress (Nasir & Ali, 1971-1989; Ali & Nasir, 1989-1991; Ali & Qaiser, 1993-2021). However, most of these plant species, particularly Pteridophytes have not been investigated using molecular data. Irfan *et al*., (2022), through a chorotype analysis, reported 168 morphologically distinct pteridophyte taxa from Pakistan, while Shah *et al*., (2022) documented the number to 202 species of ferns and lycophytes. Both studies emphasized that pteridophytes, the second largest group of the vascular plants of the country, remained insufficiently explored across many regions of Pakistan, including Swat. Like other parts of the country, the pteridophytes of Swat Valley require authentic identification to support their conservation and increasing anthropogenic pressure such as habitat fragmentation, land-use change, pollution and over harvesting of economically and medicinally valuable species (Iqbal & Hamayun, 2004). Previous floristic surveys of Swat Valley, based on morphological and micromorphological characteristics, documented pteridophyte richness of the region (Attaullah *et al*., 2017; Khan *et al*., 2017; Attaullah *et al*., 2019; Irfan *et al*., 2021; Fraser-Jenkins *et al*., 2023). The molecular and genetic techniques, however, have not been used to identify and differentiate the members of the fern family at species and ecotype levels.

Pteridaceae is comprised of 950 species with worldwide distribution, represents one of the two most abundant fern families of mountainous range of Pakistan, the other being Dryopteridaceae. Although members of Pteridaceae have been extensively investigated at the molecular level globally

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(Chao *et al.*, 2014; Huiet *et al.*, 2018; Prado *et al.*, 2007; Schuettpelz *et al.*, 2007; Zhang *et al.*, 2015), DNA-barcoding studies for this family from Pakistan, however, have not been conducted or reported. DNA marker-based identification relying on the sequencing of short standard regions of genomic DNA (Purty & Chatterjee, 2016) is a robust and extensively adopted approach for delimitation of taxa. Anon., (2009), after evaluating multiple chloroplast markers, recommended the use of two-locus barcodes (*rbcL* and *matK*) for delimiting species of land plants. However, *matK* is not applicable on ferns due to its insufficient resolution and low amplification rate (de Groot *et al.*, 2011; Ma *et al.*, 2010; Wang *et al.*, 2016). Various researchers have used only *rbcL* markers to study molecular phylogeny and taxonomy of Pteridaceae (Bouma *et al.*, 2010; Prado *et al.*, 2007). Although *rbcL* is highly conserved plastid gene in ferns yet several studies showed that its 5′ region, known as *rbcLa*, exhibits comparatively greater sequence variability and provides better species-level resolution (Trujillo-Argueta *et al.*, 2021; Yameen *et al.*, 2025). Therefore, the present investigation was designed and conducted for the functional gene *rbcLa* segment for barcoding the species of Pteridaceae in molecularly unexplored region of the Swat.

Material and Methods

In this study 5 spatially separated individuals per species of family Pteridaceae were collected from each of four regions in Swat Valley: Madyan, Malam Jabba, and Miandam in the northeast, and Marghazar in the south of Mingora, the main city. Fresh pinnules from young fronds were excised and carefully placed in polythene zip-lock bags containing silica gel to ensure rapid dehydration and preservation for DNA extraction. Additionally, photograph of each plant species was captured in the field, (Fig.). The collected plant samples were pressed, dried, and mounted onto the standard herbarium sheets. The specimens were morphologically examined, identified using standard taxonomic literature (Kramer and Green, 1990; Fraser-Jenkins *et al*., 2023). The taxonomic names adopted in this study were standardized by cross-checking the sampled taxa and reference sequences against major taxonomic backbones, including Plants of the World Online (Anon., 2019), World Flora Online (Anon., 2026), (Anon., 2026) and Flora of China (Zhang *et al*., 2013). All examined specimens were subsequently deposited in the Dr. Sultan Ahmed Herbarium, Government College University, Lahore, Pakistan. Voucher numbers were assigned and received for record and future reference after completion of documentation.

DNA extraction, PCR amplification, and gel electrophoresis: The pinnules of young fronds were stored at -20°C for 2 days in dark. Then DNA extraction was performed using standard GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fischer Scientific, Cat. No. K0791) following recommended protocol. The extracted DNA was quantified by spectrophotometer at A260/A280 by adopting the method of Doyle (1991).

The *rbcLa* segment of DNA extracted from each sample was amplified through PCR technique using DreamTaq Green PCR Master Mix (2X) kit (Cat. No. K1081) and GeneAmp PCR System 9700 thermocycler. The set of reverse and forward primers to amplify *rbcLa* region was: 5`-ATG TCA CCA CAA ACA GAG ACT AAA GC-3`F and 5`-GTA AAA TCA AGT CCA CCR CG-3`R (Levin *et al.*, 2003; Kress and Erickson, 2007). The cpDNA region of all samples were amplified using 35µL volume of reaction mixture: 18µL master mix, 1.5µL forward primer, 1.5µL reverse primer, 4µL extracted DNA and 10µL of injection water. The optimized PCR conditions used for amplification of *rbcL*a were: 94°C for 3 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 40sec; final extension 72°C for 10min. The PCR products were separated by gel electrophoresis (Fig. 2) on (1%) agarose gel using a standard 1kbp DNA ladder following Lee *et al*., (2012). The isolated bands of ~550 kb were cut and processed for purified *rbcLa* amplicons (Fig. 2) which were submitted for Sanger sequencing at Apical Scientific Malaysia (formerly known as First Base Laboratories). The nucleotide sequence data received was subjected to downstream analyses.

Genetic analyses: The chromatograms of partial gene (rbcLa) sequences were visually inspected in Chromas version 2.6.6. (Technelysium Pvt. Ltd., 2023); sequences were trimmed at both upstream and downstream ends. Basal Local Alignment Search Tool for nucleotides (BLASTn) of NCBI was used to find out homologous gene sequences, from GenBank database. The BLAST results were then analyzed by considering various metrices. The sequences of accession numbers of top matches with some other closely matching sequences were selected for phylogenetic analysis. The purified rbcLa sequences of 8 specimens, sequences of their "correct matches," and other close sequences preferably from top ten BLASTn results were imported into MEGA12 software for sequence alignment and downstream analysis. Outgroup sequences from members of Aspleniaceae (Schuettpelz *et al*., 2007) were added and multiple sequence alignment (MSA) was conducted using MUSCLE tool. The final dataset of 473 columns and 32 rows was analyzed for pairwise genetic distances and phylogenetic inferences. The visual presentation for the metrics of pairwise genetic distances was generated in EXCEL software as a histogram (Fig. 3). Furthermore, the SPSS statistical software package was used to perform Mann-Whitney U test followed by calculation of effect size (Table 2), to determine whether a significant difference exists between intraspecific and interspecific genetic distances and to evaluate the magnitude of this difference. In addition, Receiver Operating Characteristics (ROC) curve analysis and Area Under the Curve (AUC) were calculated (Fig. 4) using the same software to assess how effectively K2P genetic distance separates interspecific and intraspecific classes.

Phylogenetic analysis by the maximum likelihood method: The phylogenetic inference derived from Maximum likelihood tree was (Fig. 5) constructed using K2P, Kimura 2-parameter (Kimura, 1980) of nucleotide substitution. The phylogram with highest log-likelihood (-1,625.30) and bootstrap value 1000 was used to construct ML tree while the evolutionary rate differences among sites were modeled by a discrete Gamma distribution across 5 categories (+*G*, parameter = 0.2023). The analytical procedure covered 35 *rbcLa* sequences (coding) using 1st, 2nd, and 3rd positions. The option of partial deletion was checked to exclude all positions with < 95% site coverage resulting in a final data set comprising 473 positions. The evolutionary analyses were conducted in MEGA12 applying up to 3 parallel computing threads (Kumar *et al.*, 2024).

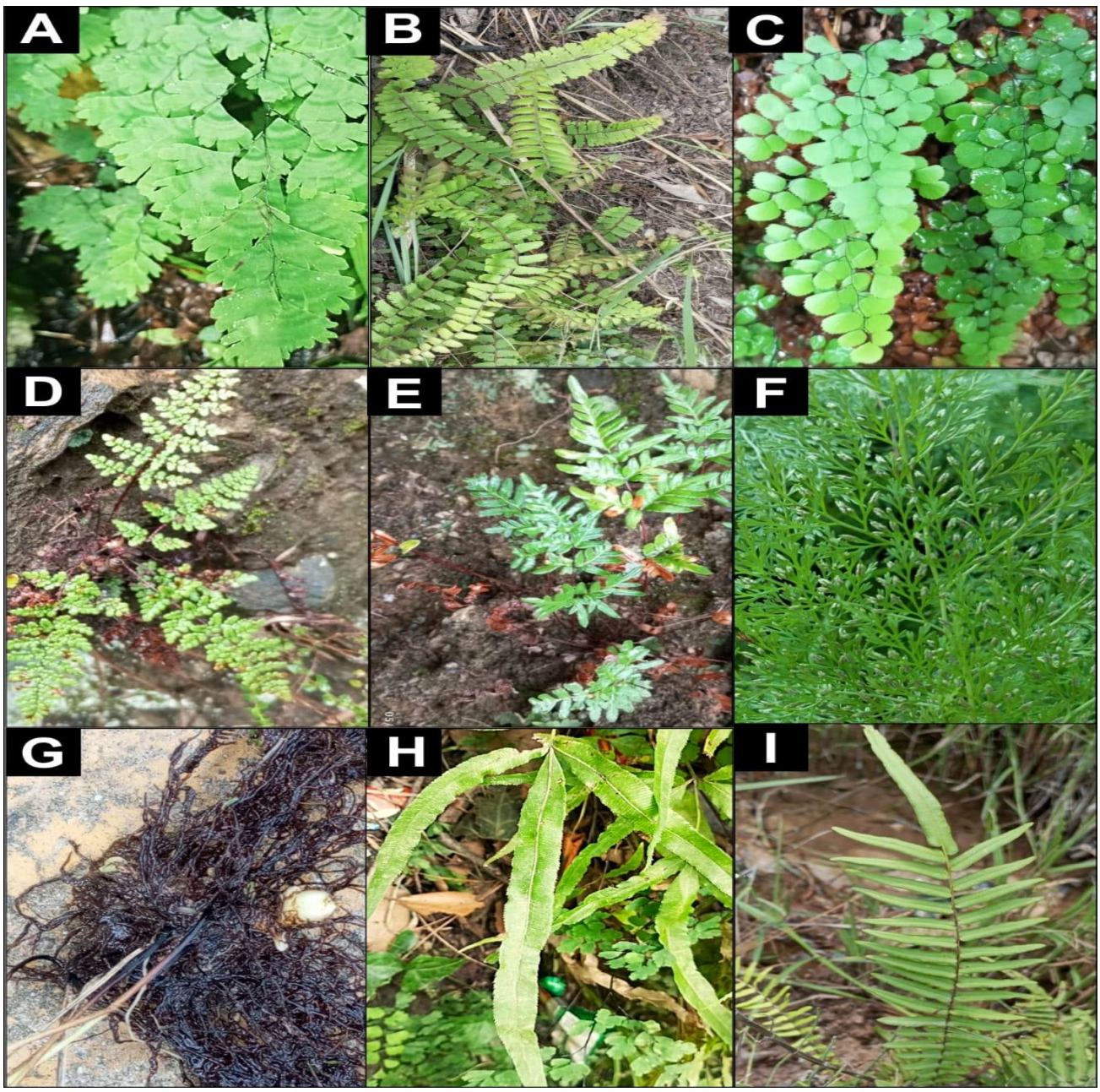

Fig. 1. Fern species collected from Swat, morphologically identified and sequenced for *rbcLa*. A, Fronds of *Adiantum capillus-veneris;* B, *A. incisum;* C, Fronds of *A. venustum;* D, *Hemionitis acrostica;* E, *H. nitidula;* F, Abaxial portion of fertile pinnae of *Onychium cryptogrammoides;* G, Stipe with black base arising from rhizome*;* H, Fronds of *Pteris cretica;* I, *Pteris vittata.*

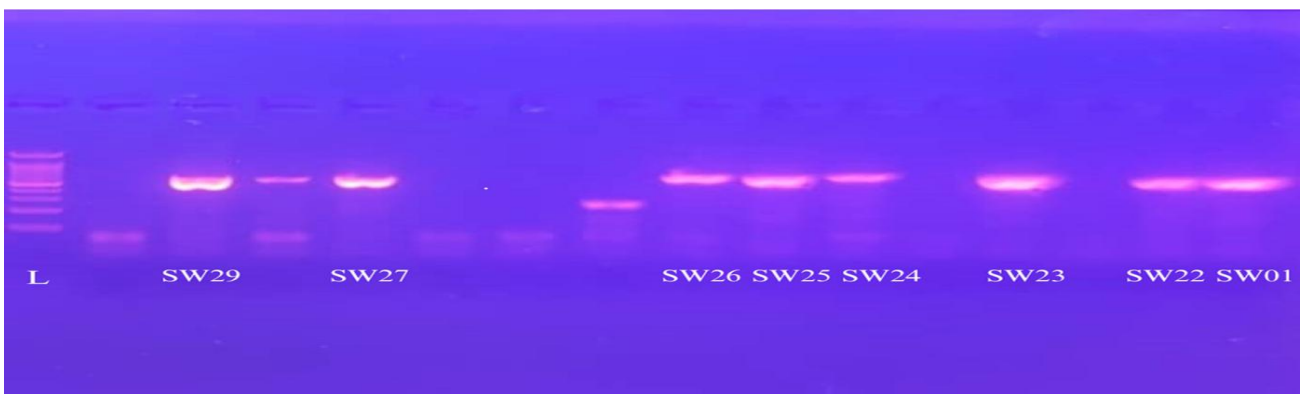

Fig. 2. PCR products of *rbcLa* segment of Pteridaceae fern samples collected from Swat (SW) and separated at ~550 bp on a 1% agarose gel. Legends for the DNA segments are: SW01(*Adiantum capillus-veneris*), SW22 (*A. incisum*), SW29 (*A. venustum*); SW23 (*Aleuritopteris acrostica*), SW24 (*A. nitidula*); SW25 (*Onychium cryptogrammoides*); SW26 (*Pteris cretica*); SW27 (*Pteris vittata*); L (leftmost lane) = 1 kb DNA ladder. Lanes with negative or very faint amplification were not part of this study.

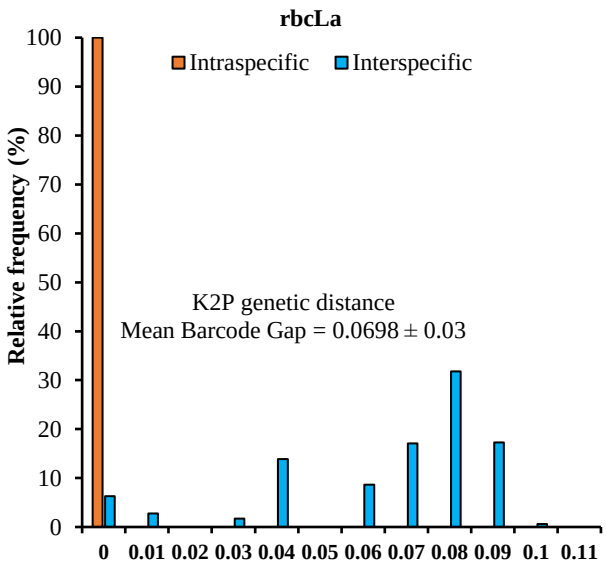

Fig. 3. Distribution of K2P genetic distances for all pairwise intraspecific (orange bar) and interspecific (blue bars) comparisons of *rbcLa* query sequences and reference sequences (including outgroup) retrieved from GenBank. Total pairs = 496 including intraspecific pairs =21; interspecific pairs= 475 and number of interspecific pairs showing 0 genetic distance (extreme left blue bar) due to synonymous names = 8.

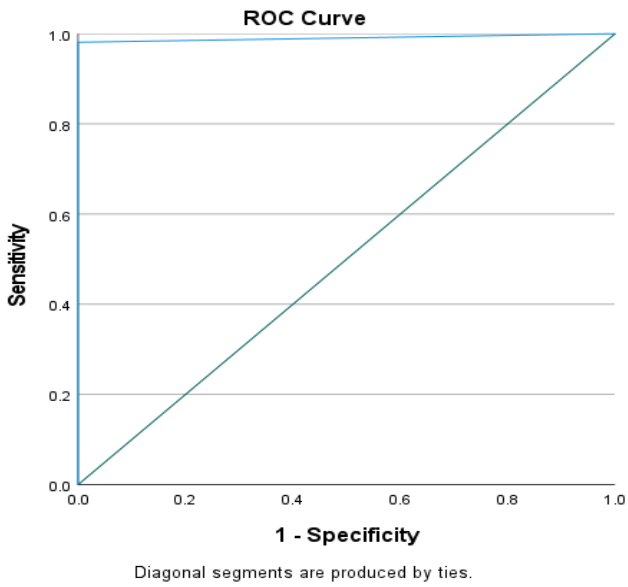

Fig. 4. ROC curve depicting the classification performance of K2P genetic distances for distinguishing intra- and interspecific comparisons in Pteridaceae ferns from Swat Valley. The high AUC value (0.991) demonstrates strong discriminatory power of the marker. Sensitivity (x-axis) represents the proportion of correctly identified interspecific comparisons, whereas specificity (y-axis) represents the proportion of correctly identified intraspecific comparisons.

Table 1. BLASTn data based genetic alignment and analyses of rbcLa (partial gene) sequences of Pteridaceae fern specimens collected from four localities (Madyan, Miandam, Marghazar and Malam Jabba,) of Swat Valley situated in mountainous range of Hindukush-Himalayan region, Pakistan.

Sample ID	Morphological Identification	Previous GenBank records	GenBank accession	Percent identity (PI)	Query cover (QC)	BLASTn Identification
SW01	*Adiantum capillus-veneris* L.	40	MN207129	100	100	Correct
SW22	*Adiantum incisum* Forssk.	4	MH019584 JF935300	100	100	Ambiguous
SW23	*Aleuritopteris acrostica* (Balb.)Windham & Schuettp. [*Hemionitis acrostica* (Balb.) Mosyakin]	One	MK632705	100	100	Correct
SW24	*Aleuritopteris nitidula* (Hook.) Windham & Schuettp. [*Hemionitis nitidula* (Hook.) Christenh.]	4	DQ432638	100	100	Correct
SW25	*Onychium cryptogrammoides* Christ	One	KP903749 AY266416 KM008137	100	100	Ambiguous
SW26	*Pteris cretica* L.	43	MF972806	100	100	Correct
SW27	*Pteris vittata* L.	58	MH500228	100	100	Correct
SW29	*Adiantum venustum* D. Don	11	EF452136	99.82	100	Correct

Results

The *rbcLa* sequences generated from the fern samples queried against the NCBI nucleotide database using BLASTn, and the results strongly supported and confirmed the prior morphological identification of the Pteridaceae taxa at species level (Table 1). All the sequences showed high query cover (99.82-100%) and high percent identity (100%) with conspecific NCBI database. The *rbcLa* query sequences of samples SW01, SW23, SW24, SW26, SW27, and SW29 were identified as *Adiantum capillus-veneris*, *Aleurotopteris acrostica*, *A. nitidula*, *Pteris cretica*, *P. vittata* and *Adiantum venustum*, respectively. The sequences of remaining two samples apparently produced ambiguous results. The sample SW22 showed best hit with *A. incisum* (accession, MH019584) and *A. sinicum* (QC=100; PI=100) while SW25 matched with reference sequences of *Onychium cryptogrammoides* (Accession, KP903749) and *O. contiguum* (accessions, AY266416 and KM008137).

Moreover, reference sequence availability in GenBank varied substantially among the taxa. *Pteris vittata* has comparatively many *rbcL* records (58); whereas *Aleuritopteris acrostica* and *Onychimu cryptogrammoides* each one is represented by single submission only. The number of GenBank accessions for *rbcL* gene sequences of remaining six samples occur between 1 and 58. Presence of high number of reference sequences of a species in database more strongly supported the results (Table 1).

Overall, the BLASTn analyses corroborated the field and herbarium-based identifications for 6 of the 8 specimens and highlighted SW22 and SW27 as a case where *rbcLa* alone provides strong generic placement but warrants cautious interpretation at the species level.

Hemionitis vice Aleuritopteris

The genetic distance analysis of *rbcLa* gene sequences by pairwise K2P distances showed the clear separation within-and-between-species. All intraspecific distances were zero and interspecific were non-zero positive integers (Fig. 3). The conspecific accessions shared identical *rbcLa* haplotypes in the dataset. The differences between interspecific distances fell between 0.002 to 0.198, with most comparisons clustering between 0.04 and 0.10 and consecutively fewer pairs occurred towards the upper end of the distribution (0.15-0.19). A fraction of interspecific comparisons (n=9) also showed zero distance (Fig. 3), indicating situations in which *rbcLa* did not distinguish closely related species but also reflecting nomenclatural issues of synonymy. The value of strict barcode gap (minimum interspecific minus maximum intraspecific K2P distance) is extremely low, 0.002119 (Fig. 3; also see discussion). However, the value of mean barcode gap = 0.0698 ± 0.03, indicating substantial average divergence between species relative to within-species variation and supporting the overall discriminatory capacity of the rbcLa marker in the sampled taxa.

Statistical validation of species discrimination: The Mann-Whitney U test revealed a highly significant difference between intraspecific and interspecific K2P genetic distances (U=94.50, Z = -7.615, p<0.001; Table 2). The mean rank of interspecific distances (258.80) was substantially higher than that of intraspecific distances (15.50), indicating clear separation between the two groups. Subsequently, the calculated effect size (r = 0.44) indicated a moderate to strong separation between intra and interspecific distances. consistent with these results the mean barcode gap was 0.0698 ± 0.03, reflecting substantial divergence between species relative to within-species variation. Furthermore, receiving operating characteristic analysis (Fig. 4) demonstrated excellent discriminatory performance of the marker (rbcLa), with an area under curve (AUC) of 0.991 (95% CI: 0.983-0.998, p<0.001), indicating that K2P genetic distances can reliably distinguish intra and interspecific comparisons within the sampled Pteridaceae taxa.

Table 2. Results of the Mann–Whitney U test comparing intra- and interspecific K2P genetic distances.

Distance type	Number of pairs (N)	Mean rank	Sum of ranks
Intraspecific	21	15.50	325.50
Interspecific	275	258.80	122930.50
Test statistics: U=94.50, Z = -7.615, p<0.001			

Maximum Likelihood (ML) phylogram of the *rbcLa* dataset recovered well-resolved clades representing phylogenetic affinity of genera and species (Fig. 5). The bootstrap values robustly supported *Adiantum* clade sister to *Aleuritopteris* clade, *Pteris* clade to *Onychium* clade corresponding to the major genera of Pteridaceae with *Asplenium* forming a distinct outgroup (Fig. 5). The four major representative genera of the fern family, *Adiantum*, *Aleuritopteris*, *Onychium* and *Pteris* exhibited monophyletic groups.

All query sequences from Swat represented by suffix "SW" clustered with their morphologically identified conspecifics. *Adiantum* specimens identified as *Adiantum capillus-veneris* (SW01), *A. incisum* (SW22) and *A. venustum* (SW29) were nested in *Adiantum* clade with sequences bearing the same species names. *A. capillus-veneris* (SW01) sequence formed a strongly supported clade with two *A. capillus veneris* accessions (MN207129, D14880) whereas *A. venustum* (SW29) clustered with multiple *A. venustum* sequences and closely related congeners. In contrast, SW22 sequence was placed within a subclade comprising *A. incisum* (MH019584) and *A. sinicum* (KP637237, JF935300) supported by 100 bootstrap, indicating close affinity and incomplete resolution between these taxa at *rbcLa* locus.

In *Aleuritopteris* clade, SW23 and SW24 stabilized strongly with their conspecifics *A. acrostica* (MK632705) *A. nitidula* (DQ432638), respectively; the deeper nodes, however, showed weak support. The rbcLa haplotypes of *Pteris* placed *Pteris vittata* (SW27) and *P. cretica* (SW26) within well-supported conspecific clades comprising multiple GenBank accessions of each species. The noticeably short internal branches within these clades indicate low intraspecific divergence in *rbcLa* among the *Pteris* samples analyzed.

In comparison to other genera, species-level resolution within *Onychium* was less clear and weak. The sequence of *Onychium cryptogrammoides* (SW25) clustered in a strongly supported *Onychium* clade that included *O. cryptogrammoides*, *O. continguum*, *O. japonicum* and *O. lucidum*. Within this group, the immediate relationships between two subclades, i.e., *japonicum-lucidum* and *contiguum-cryptogrammoides* received high bootstrap support. The query sequence SW25 was consistently placed in *contiguum-cryptogrammoides* subclade representing its ambiguous identity.

Overall, phylogenetic analysis, genetic distance and BLASTn results congruently identified six species (75%) and showed equivocalness in discrimination of remaining two (25%) taxa i.e., *Adiantum incisum* and *Onychium cryptogrammoides*.

DATA submission to GenBank: All newly generated sequences of eight samples after bioinformatic analysis (BLASTn, Genetic Distance and ML tree) and molecular identification were finally submitted to the GenBank database using the BankIt protocol. Accession numbers were obtained for all eight submissions (Table 3).

Discussion

DNA barcoding in the field of molecular taxonomy emphasizes the use of nucleotide sequences of DNA segments for validating the morphological identities of plants. Among the molecular markers being used in industry, the official core plant barcode *rbcL* is preferred due to universality, primer success, short amplicon, better sequence quality, and low sequencing cost. Although *rbcL* gene sequence is relatively conserved, the 5′ region (*rbcLa*) contains enough informative sites to delimit species of plants. Many DNA barcoding and phylogenetic studies of vascular plants including ferns recommended the use of

rbcLa marker (Kress & Erickson, 2007; Trujillo *et al*., 2021, 2022; Yameen *et al*., 2025); this explains our selection of *rbcLa* for barcoding Pteridaceae ferns of Swat Valley. Moreover, the statistical analyses of *rbcLa* sequences of Pteridaceae taxa used in present study indicate that this marker provides strong discriminatory power (Table 2; Fig. 4). The clear separation between intra and interspecific genetic distances suggests that sequence variation in this marker is generally sufficient for species-level identification.

The BLASTn homology searches provided unambiguous matches for 6 of 8 Swat species, while two specimens SW22 and SW25 showed ambiguous top hits. In both cases, the ambiguity is best interpreted as a nomenclatural and reference database issue, rather than a contradiction of morphology. Specifically, *Adiantum sinicum* Ching treated as a synonym of *Adiantum incisum* Forssk. (Anon., 2026) explains why SW22 can return equally strong hits under either name. Likewise, *Onychium contiguum* C. Hope, is currently placed as a synonym of *O. lucidum* (D. Don) Spreng. in the major taxonomic backbone (Anon., 2026) which can propagate inconsistent naming among deposited sequences and published identifications. Moreover, Zhang *et al*., (2013) noted that the name *O. contiguum* is misapplied to *O. cryptogrammoides* in Asian literature of pteridophytes and is a superfluous name of *O. japonicum* var. lucidum. However, morphologically, *O. cryptogrammoides* differs from *O. japonicum*, among other traits, in black stipe bases, consistent with diagnostic character state of our specimen SW25 (Fig. 1). Altogether, *rbcLa* was a sufficient marker for species-level assignment of Swat specimens when BLASTn evidence was interpreted in the light of current nomenclature and corroborated by morphology.

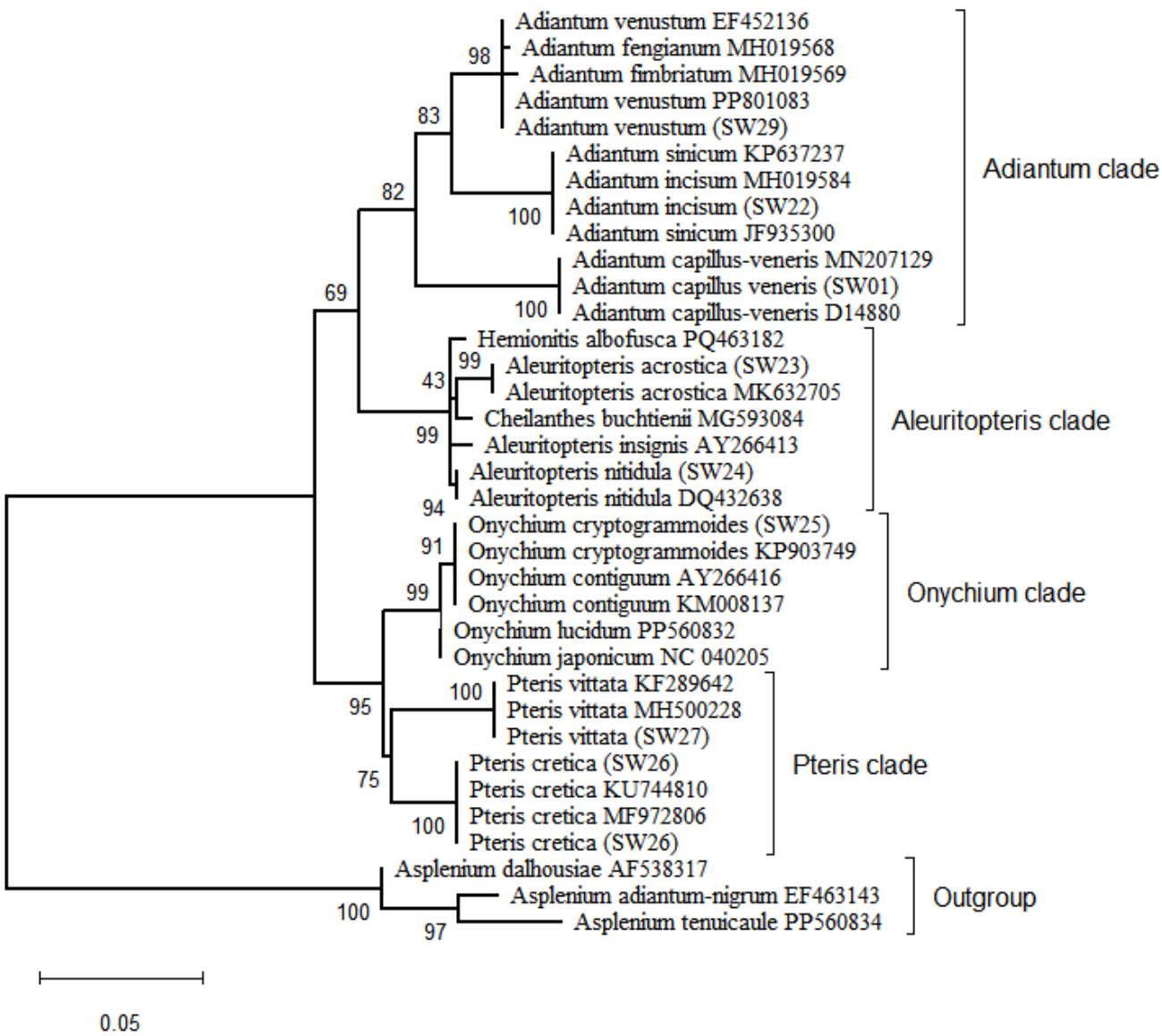

Fig. 5. Maximum Likelihood phylogram of *rbcLa* sequences of 35 species including our *eight* query sequences (names with suffix; SW01, SW22, SW23, SW24, SW25, SW26 and SW27 and SW29) matching sequences of BLASTn results retrieved from GenBank with outgroup taxa of *Asplenium*. The phylogram shows four clades: Adiantum, Aleuritopteris, Onychium and Pteris rooted with outgroup *species*. Branch lengths are drawn to scale; the scale bar represents 0.05 substitutions per site. The few deeper nodes show relatively weak support likely as the dataset includes only a subset of Pteridaceae species rather than a comprehensive representation of the family. Taxon names in the phylogenetic tree follow the names used for the corresponding NCBI reference sequences. The Swat specimens identified as *Aleuritopteris acrostica* and *A. nitidula* clustered with reference sequences of *Aleuritopteris*. It should be noted that these taxa are treated as *Hemionitis acrostica* and *H. nitidula*, respectively, in POWO/Kew.

Table 3. Pteridaceae ferns collected from Swat Valley (Pakistan) and DNA barcoded using the chloroplast rbcLa marker, showing sample IDs, morphological identifications, voucher specimen numbers (GC Herbarium, Department of Botany, GCU Lahore), and GenBank accession numbers for newly generated sequences.

Sample ID	Species name	Voucher specimen	GenBank accession obtained
SW01	*Adiantum capillus-veneris* L.	GC. Herb.Bot-4377	PV983596
SW22	*Adiantum incisum* Forssk.	GC. Herb.Bot-4892	PX694026
SW23	*Aleuritopteris acrostica* (Balb.)Windham & Schuettp. [*Hemionitis acrostica* (Balb.) Mosyakin]	GC. Herb.Bot-4894	PX694027
SW24	*Aleuritopteris nitidula* (Hook.) Windham & Schuettp. [*Hemionitis nitidula* (Hook.) Christenh.]	GC. Herb.Bot-4895	PX694028
SW25	*Onychium cryptogrammoides* Christ	GC. Herb.Bot-4896	PX694029
SW26	*Pteris cretica* L.	GC. Herb.Bot-4897	PX694030
SW27	*Pteris vittata* L.	GC. Herb.Bot-4893	PX694031
SW29	*Adiantum venustum* D. Don	GC. Herb.Bot-4378	PV983595

The genetic-distance histogram provided the same message. A small subset of interspecific comparisons at K2P=0.00 indicates that *rbcLa* does not separate all closest species pairs in the reference set, producing overlap between intra and interspecific distances and compressed the barcode gap (Fig. 3). Such zero-distance interspecific pairs are expected when (i) sequences are deposited under synonymous or inconsistently applied names (ii) closely allied taxa share very similar plastid haplotypes (Anon., 2009). In the present dataset, the observed overlap is best explained by scenario (i), as the affected comparisons involve taxa represented in public databases under synonymous or superfluous names. Additionally, misidentifications have been pointed out in publicly available reference sequence databases by a number of researchers (Schneider & Schuettpelz, 2006; Burgess *et al.*, 2011; Abdullah, 2017). Conversely, the high-distance tail (0.03–0.1) is most parsimoniously attributed to comparisons involving more distantly related lineages and therefore reflects deep divergence rather than improved resolution among the closest focal taxa.

Considering phylogenetic placement of query sequences, even a single locus approach of *rbcLa* was enough to confirm identities of Pteridaceae samples as *Adiantum capillus-veneris* L., *A. incisum* Forssk., *A. venustum* D. Don, *Aleuritopteris acrostica* (Balb.) Windham & Schuettp, *A. nitidula* (Hook.) Windham & Schuettp, *Onychium cryptogrammoides* Christ, *Pteris cretica* L. and *P. vittata* L. The approach, thus suitable and reflected both the relatively good representation of the taxa in public databases and the moderate interspecific divergence within these genera by *rbcLa* DNA barcoding. The phylogenetic cladding, clade cohesion, and overall placement of Pteridaceae species from Swat are consistent with previous studies on the phylogeny of the family (Prado *et al.*, 2007; Schuettpelz *et al.*, 2007; Bouma *et al.*, 2010; Lu *et al.*, 2012; Chao *et al.*, 2014), especially when combined with other plastid marker (Schuettpelz *et al.*, 2007). For basal polypod ferns and *Adiantum*, marker combinations such as *rbcL + psbA-trnH* or *rbcL+ trnL-F* have been combined and applied to maximize discriminatory power (Wang *et al.*, 2016). Placement of *Adiantum sinicum* to *Adiantum incisum*, and *A. contiguum* to *Onychium cryptogrammoides* in phylogenetic tree (Fig. 5) also underscores the synonymy of taxa.

The genetic based methods of analysis of gene sequence data sets with morphological characteristics are useful and provide a high level of congruence between traditional sole morphology-based identities and *rbcLa* gene sequence similarities for vascular plants including Pteridophytes (Kuo *et al.*, 2024a; Liu *et al.*, 2024). BLASTn, K2P and ML methods applied for genetic identification using DNA barcoding for *rbcLa* gene sequences of *Adiantum*, *Aleuritopteris*, *Onychium* and *Pteris* validated the morphological identification and provided an authentic and reliable phylogenetic relationships at genera and species levels within and with each of fern taxa collected (sampled) from Hindukush-Himalayan region of Swat. Moreover, the results indicated that same species of a genus share identical *rbcLa* sequences while different species are typically separated by moderate to high levels gene sequence divergence. Such a distance profile is consistent with earlier reports (Trujillo-Argueta *et al.*, 2021).

Importantly, the Swat specimens often matched multiple conspecific accessions that had been generated in independent studies from geographically distant populations. Such matches strengthen confidence that our identifications are not artifacts of a single mislabeled reference but instead link the Swat flora to a broader global barcode framework for Pteridaceae. In this sense our sequences help fill our biogeographic gap by adding authenticated *rbcLa* records for Pakistani populations to the worldwide reference library of Asian ferns (Kuo *et al.*, 2024b).

Aleuritopteris acrostica and *A. nitidula* have been morphologically reported from Pakistan in previous works, often under alternative generic placements/synonyms (Gul *et al*., 2016; Irfan *et al*., 2021, 2022). However, the records remain scattered and nomenclaturally inconsistent across references; therefore, their status has been treated as requiring confirmation. In our study the two species of genus *Aleuritopteris* showed perfect BLASTn matches and formed tight *rbcLa* clades with their respective conspecific references, indicating clear molecular separation between *A. acrostica* and *A. nitidula* in our sampling. The *Pteris* accessions behaved in the same way: *P. cretica* (SW26) and *P. vittata* (SW27) each grouped with multiple conspecific sequences, with very short internal branches, reflecting low intraspecific divergence and relatively high between-species differences at *rbcLa* within this genus.

This overall pattern supports strong congruence between morphology and *rbcLa* for most taxa but reduces resolution in a minority of problematic groups — mirrors broader fern barcoding studies, where *rbcL* often performs well for many lineages yet struggles in species complexes with recent divergence, hybridization, or polyploidy (Sigel, 2016; Wang *et al.*, 2016).

From a regional perspective, this work provides one of the first *rbcLa*-based assessments of Pteridaceae in Swat, a mountainous district that lies within a transition zone where elements of the western Himalayas and Hindu Kush overlap and which has been recognized as part of a broader Himalayan biodiversity hotspot (Alam *et al.*, 2024; Myers *et al.*, 2000; Qasim *et al.*, 2011). The recent checklists (Fraser-Jenkins *et al*., 2023) document the fern flora of this area primarily on morphological grounds, and pteridophytes remain under-represented in molecular datasets relative to angiosperms (Jamil & Ashfaq, 2016; Morajkar *et al.*, 2022). By linking field identifications and herbarium vouchers to curate *rbcLa* sequences and depositing them in public databases, our study adds verifiable molecular records that can be used in future taxonomic revisions, population level studies, and environmental DNA surveys.

From a conservation standpoint, reliable species identification is crucial for assessing distribution ranges, endemism, and threat status. DNA barcoding is an efficient means of verifying field identifications of pteridophytes; therefore, many molecular investigations have been conducted on Asian pteridophytes to improve the quality of biodiversity inventories (Ebihara & Kuo, 2012; Wei *et al.*, 2021; Kuo *et al.*, 2024b). The confirmation of common widely distributed species such as *P. vittata* and *A. capillus veneris* alongside less frequently documented taxa such as *A. nitidula* and *O. cryptogrammoides* help to anchor conservation and ecological studies in a secure taxonomic framework and contribute to improved documentation of biodiversity in the Hindukush-Himalayan region. The availability of voucher-linked DNA barcode sequences from this study will facilitate future biodiversity monitoring, species verification, and conservation planning for fern flora in Swat Valley and other parts of northern Pakistan.

Limitations and future directions: Despite its usefulness, this study has several limitations. We analyzed a single plastid locus, *rbcLa*, and a limited number of species and individuals focusing on eight Pteridaceae taxa from a single region (Swat Valley). *rbcLa* alone is known to be insufficient for resolving many closely related plant species and multi-locus barcoding approaches that combine *rbcL* with more variable regions such as *psbA-trnH*, *trnL-F* or nuclear markers generally show higher species level discrimination (Wang *et al.*, 2016). Expanding the marker set, increasing sampling across populations and including additional families of Swat pteridophytes will be essential to build a more comprehensive barcode library for Pakistan's fern flora.

Moreover, the reliability of any barcode-based identification depends on the taxonomic accuracy and completeness of reference databases. Our findings in *Adiantum and Onychium* underline the need to critically evaluate GenBank records considering modern classifications such as Pteridophyte Phylogeny Group I for ferns (Anon., 2016). Future work should therefore couple barcode generation with targeted sequencing of well-curated voucher specimens from under sampled regions and contribute these sequences to public repositories to improve the reference dataset available for fern barcoding.

Conclusion

The strong agreement between morphological identification and *rbcLa* sequence for most species suggests that traditional fern taxonomy in Swat is largely robust for the Pteridaceae examined. At the same time, the ambiguous signal in *Adiantum* and *Onychium* illustrates how DNA barcoding can reveal hidden taxonomic problems rather than simply confirming existing identifications. In this way, barcodes serve both as a practical tool for routine identification and as a starting point for recognizing species complexes that warrant detailed systematic investigations.

The *rbcLa* sequence-based study validates the presence of eight species of family Pteridaceae: three species of *Adiantum* (*A. capillus-veneris*, *A. incisum* and *A. venustum*), two species of *Pteris* (*P. cretica* and *P. vittata*), two species of *Aleuritopteris* (*A. acrostica* and *A. nitidula*) and one species of *Onychium* from Swat Valley, Pakistan. The Swat specimens were generally placed with their respective conspecific sequences in BLASTn searches and phylogenetic analyses, supporting their morphological identification. The study confirms the occurrence of *Aleuritopteris acrostica* and *A. nitidula* in Pakistan and provides molecular support for the placement of sequences labelled as *Onychium contiguum* within the *O. cryptogrammoides* clade, suggesting that this taxon may be better treated under *O. cryptogrammoides*. Together, these findings add verifiable molecular records for the fern flora of Swat and provide a baseline for future taxonomic, ecological, and conservation studies in this underexplored region.

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Conflict of Interest: The authors declare no conflict of interest.

Data Availability Statement: The vouchers specimens and sequence dataset used in this study are available and can be provided on reasonable request. Furthermore, the *rbcLa* sequence of species studied has been submitted in GenBank database and accession numbers assigned.

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