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PHYLOGENETIC ANALYSIS OF SOME ENDEMIC MOSSES OF NORTHERN PAKISTAN

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Abstract

The study has been focused on morpho-anatomical and molecular characterization of some taxa from the families Fissidentaceae and Grimmiaceae in district Dir Upper, Khyber Pakhtunkhwa Pakistan. A sequence and the phylogeny of *Fissidens dubius, Fissidens grandifrons*, and *Grimmia ovalis* was performed for the first time from Pakistan. Nuclear Internal transcribed spacer (ITS1) region was used to find out the phylogenetic position of these taxa. Phylogenetic analyses sequences of the nrDNA ITS1 (BMBC-LS4) region of collections (IM25, IM27, IM54 and IM31) were obtained with both the primers. Evolutionary history was inferred by using the software Maximum Likelihood method based on the Tamura-Nei model. *Fissidens dubius* and *Fissidens grandifrons* are described as new report from the study area. Our findings describe the potential of ITS1 in identification and classification of the species. Description of these species along with local photographs and phylogenetic tree are provided. The current attempt is the first one of its natures to describe the molecular characterization of mosses from district Dir upper. It will help the researchers for further taxonomic studies in exploring moss flora of the study region and will lead a way forward for more research in ecological and phytochemical fields.

Key words: District Dir; *Fissidens*; *Grimmia*; ITS1, Mosses; Phylogeny

Introduction

There are about 107 families, 880 genera and 14728 accepted species of mosses. They are further classified into 77,456 scientific names of infraspecific rank for the Bryophyta (The Plant list, 2024). The mosses, either separately or combine with liverworts, form the sister group to Tracheophytes (Lewis *et al*., 1997; Nickrent *et al*., 2000).

Fissidentaceae and Grimmiaceae arformarpous and placed among 30 haplo-lepideous families. About 4000 species distributed in 30 families and 232 genera (Frey & Stech, 2009), haplo-lepideous mosses (Dicranidae) form the 2nd largest sub-class among mosses. These families are characterized with the help of arthrodontous–haplolepideous peristome, which consists of a single row of teeth's present around the mouth of capsule. All molecular phylogenetic evidence so far support the monophyly of Dicranidae, and a position nested within the arthrodontous–diplolepideous mosses, as sister to the largest subclass of mosses, the diplo-lepideous-alternate Bryidae (Stech & Frey, 2008; Cox *et al*., 2010). The molecular data therefore indicates that the haplo-lepideous peristome developed from diplo-lepideous ancestor (Stech, 1999).

Morphological features are used to find out relationships between different species (Pelser *et al*., 2004). The beginning of molecular systematics provides a baseline to use DNA data to check out morphological assumptions about these relationships (Felsenstein, 2012). Next-generation sequencing technologies provide opportunities to construct phylogenetic trees based on data from thousands of genes consuming a very small amount of tissue taken from natural specimens (Folk *et al*., 2021). Morphological lessening has been shown to be rampant across mosses, particularly for structures such as the leaf costa, Capsule, seta length, peristome, and has been hypothesized to accompany shifts in their habitat e.g., movement from aquatic to terrestrial or to epiphytic (Vitt, 1981), but on the basis of morphoanatomical and habitat characters among moss species in some cases are not highly recommended (Huttunen *et al*., 2018). Evolutions in gametophytes of mosses have been used as a describing feature for clades of mosses (McDaniel & Perroud, 2012). Investigating the association between microhabitat and morphological features in a phylogenetic context can allow us to recognize homoplasious and homologous morphologies and assess correlations between these features in light of evolution (Huttunen *et al*., 2018).

The bryofloristic studies in Pakistan for a long time have been ignored and stay behind in past without any apparent reason. Few workers have put in their efforts in this regard. The Northwestern Himalayan Hindukush region was the source of very few European bryologists' initial collections (Brotherus & Duthie, 1898; Bartram, 1955; Froehlich, 1963; Stormer, 1954; Dixon, 1926; Blatter & Fernandez, 1929; Herzog, 1938). Later on, some Japanese contributors made collections from Karakorum and Himalayan regions (Noguchi, 1956, 1959, 1964; Higuchi, 1992; Nishimura *et al*., 1993a; Nishimura, 1993b; Nishimura & Higuchi, 1994; Higuchi & Nishimura, 2003). A few European bryologists have recently raised concerns about the bryoflora of Pakistan and investigated it (Solman, 2008; Gruber & Peer, 2010. The collective results of these previous efforts led to the discovery of approximately 450

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species of bryophytes in Pakistan (Islam *et al.*, 2015). Previously 45 Bryophytes species have been reported from Sheringal, District upper Dir Pakistan (Hazrat *et al.*, 2020). It's noteworthy that these workers mainly focused on the North-Western Himalayan and Hindukush regions of Pakistan and published there finding on the basis of morphological data. According to available literature no molecular studies of mosses have been conducted, having potential in this neglected division of plants from Pakistan. The present attempt was made to explore moss flora with the help of morpho-anatomical and molecular studies. Due to vast diversity in mosses and lack of previous relevant literature, the current study has been focused on these 2 haplo-lepideous families with a focus on molecular characterization. Therefore, further extensive field studies in different sites of the study area are important in order to explore the diversity and distribution of Bryoflora.

Material and Methods

Study area and collection period: This study was carried out in District upper dir, northern areas of Pakistan. Total area of the study area is about 3,699 square kilometers. It is located at Latitude 35º06'51" N and Longitude 72º01'59"E. Geographically, it share boundaries with district Chitral north, on western side it bounded with Afghanistan, district dir lower is located on the southern side and district Swat on east. Average rainfall in the study area is about 700 to 750 mm and the temperature contrasts from -6°-38°C (Khan *et al.*, 2015). Mosses Data were collected in spring and autumn season of 2022-23.

Sampling site: After a thorough review of existing literature and previously examined specimens valuable data was gathered regarding the exact locality, elevation, maturation, and capsulation period of mosses. Various characteristics of taxa were recorded, and each specimen was assigned a unique collection number. Following collection, the specimens were properly washed to remove soil and other debris. The dried specimens were then packed in standard size 4x6 inch paper envelops. Morphological data such as plant height color, capsulation, and habitat were examined (Islam *et al.*, 2015).

Morpho-anatomical characterization: Macro morphological characters of gametophyte and sporophytes were studied under the stereoscope (Model-Optika microscope Italy). The specimen were plunged into water and left for 10-15 minutes to return to its normal shape before being examined under a stereoscope. The first step was to separate the leaves from the gametophytes using needles. Leaves length-width and pairs of leaves were noted. Details of minute characters were taken with the help of compound microscope (Model-OlympusBX51). For anatomical description, leaves were pasted on the slides and treated with alcohol-acetic acid and lectophenol to remove wastes. Different characters like marginal, apical, medial, basal, alar cells, coastal length, and measurement of cells were examined under the different microscopic lens. In some cases cross sections of leaves were also done (Islam *et al.*, 2015).

Molecular characterization: Fresh and dried samples were taken for genomic DNA extraction. The first step was to crush the samples in liquid nitrogen and extract DNA using a modified version of the D-Neasy Plant Mini kit (Thermo-scientific). Internal Transcribed Spacer (ITS1) region was amplified using ITS-BMBC-F and ITS-LS4-R primers (Shaw, 2000), PCR conditions as given by Gardes & Bruns (1993) were followed. Forward-reverse sequences were taken and consensus were assembled by using Software Bio Edit ver. 7.2.5 (Hall, 1999) and BLAST at NCBI to cross match with other online DNA sequences (https://www.ncbi.nlm.nih.gov/); for Phylogenetic analysis was following (Bull *et al.*, 1993).

Results

***Fissidens dubius* P. Beauvois, Prodr. Aethéogam. 57. 1805:** (Fig. 1). Plant height; 5.5-06 cm, turf. Stem mainly branched or in some cases unbranched, light green-yellowish green in color. Rhizoids dark brown. Leaves 30-35pairs on stem,10-11pairs on a branch, branch,3-4mm long,1-2mm width, when dry leaves tip become incurved, when hydrate become straight-undulate, erect to patent, distichous, lanceolate to ovate-oblong, acute-obtuse, oblong to lanceolate. Dorsal lamina long up to, 4mm, less decurrent; Vaginant lamina cover up to 65-75% of leaf, unequal distally. Ventral lamina cover 25-35%, margins serrate-crenulate, serrulate and less serrate at apex, basal margin smooth, limbidia absent; Costa curve upper, end at cell before apex, coasta apex, percurrent; laminal cells slightly mamillose in ventral & dorsal lamina, plane, swollen, round-less hexagonal. Basal cells rectangular quadrate to hexagonal, 3-4 rows of marginal cells thinner, dorsal lamina form pale marginal bands, Sporophyte was not observed. During the field survey, this species is found the most diverse in the study area.

Specimens examined: Pakistan, Khyber Pakhtunkhwa, Dir upper, Gulibagh 1700m, a.s.l; October-16-2023, *Imran Ahmad* and *Nadeem Khan* (IM27, accession no, PV290437, IM54, accession no, PV290438).

***Fissidens grandifrons* Brid., Muscol. Recent. Suppl. 1:170. 1806:** (Fig. 2). Plant short to medium dark greenish color, firm, when matured become brownish, creeping. Stem usual simple, 3.5-04 cm long. Leaves in 20-25pairs, fixed even when dry, the lowest leaves small, lanceolate- ovate, 4-6mm long, 1-2 mm wide, distichous arrangement, apex less acute to obtuse; dorsal lamina not decurrent; Vaginant laminae round about 50-70% of the whole leaf; form bands shape, Costa-wide, ending 4cells before apex, percurrent; margins smooth, marginal cells small compare to medial cells, , cells of apical and dorsal laminae round-quadrate, cells become large toward base, Sporophyte was not observed. This plant is hydrophytic in nature and dark greenish color.

Specimens examined: Pakistan, Khyber Pakhtunkhwa, Dir upper, Gulibagh 1700 m a.s.l., 16-10-2023, *Imran Ahmad* and *Nadeem Khan* (IM25, accession no, PQ069029).

General distribution: Mexico, Central North America, Europe, India, Pakistan.

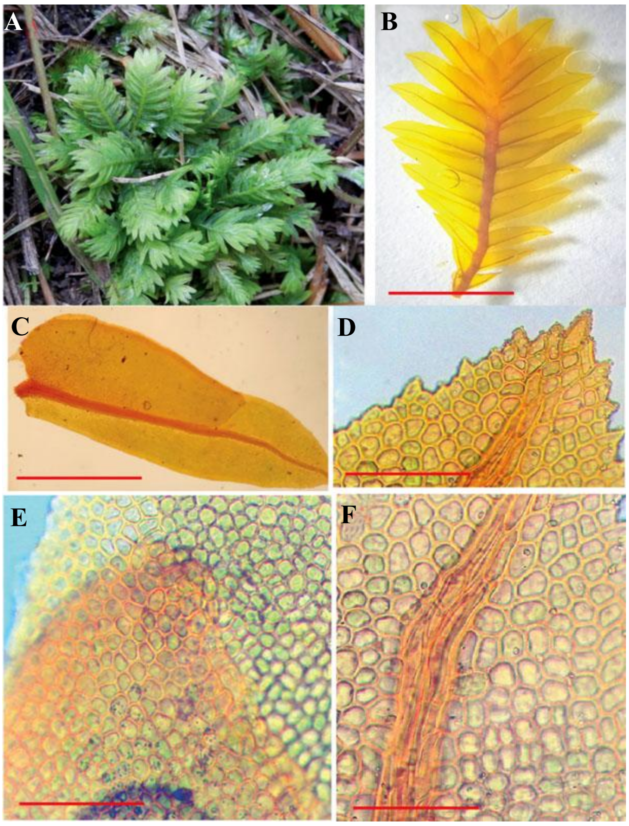

Fig. 1. *Fissidens dubius*: **A.** Plant habitat**; B.** Stem Branch; **C.** Leaf: **D.** Apex: **E.** Junction of Lamina; **F.** Medial cells**.** Scale bar**;** A=06cm**,** B=10mm, C=4mm, D=25µm, E=10µm, F=15 µm, F=10 µm.

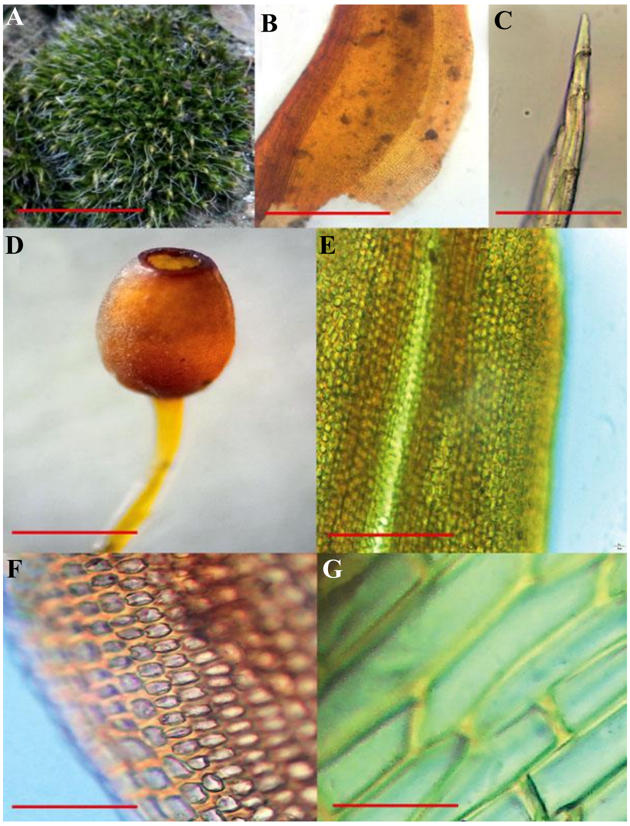

Fig. 3. *Grimmia ovalis*: A. Plant Habitat, B. Keel shape leaf; C. Leaf Awn; D. Capsule; E. Medial cells; F. Marginal cells; G. Sheath cells. Scale bar; A=02cm, B=5mm, C=2mm, D=4mm, E=10 µm, F=15 µm, G=40 µm.

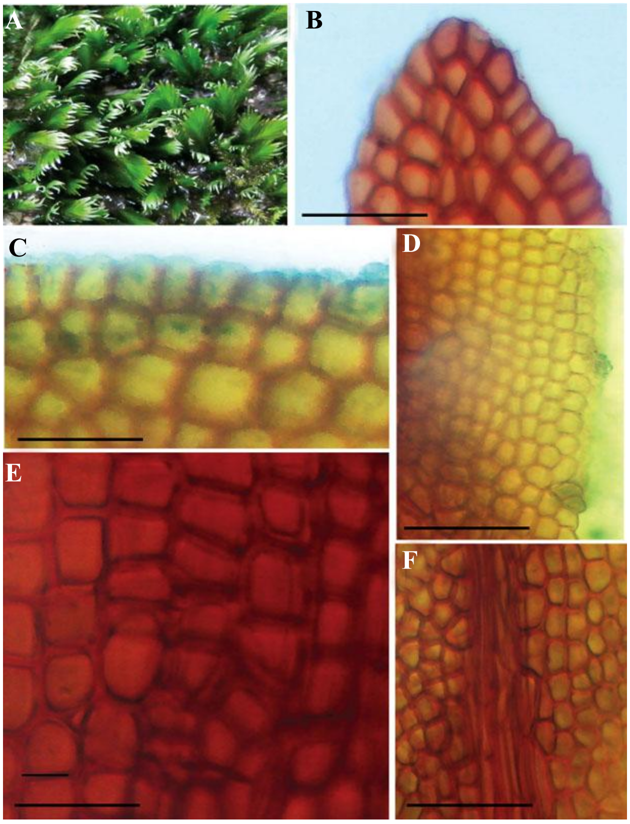

Fig. 2. *Fissidens grandifrons*: A. Plant habitat; B. Leaf apex; C. Lower margin: D. upper marginal cells: E. Lower medial cells; F. Justacoxial cells. Scale bar; A=04cm, B=25 µm, C=30 µm, D=15 µm, E=25 µm, F=20 µm.

Grimmia ovalis **(Hedw.) Lind., Acta Soc. Sci. Fenn. 10: 75 1871:** (Fig. 3). Plant height; 1.5-2.0cm, cushions-turf. Dark when dry, become greenish dark when wet. Stem straight to curve. Leaves lanceolate-ovate, having awn, margins mainly smooth, incurved distally, form keel shape, upper laminal cells round, at median portion round to quadrate, quadrate-rectangular toward margins, allar cells rectangular, basal cells oblong rhomboidal to quadrate. Capsule exserted, 3-4×1.5-1.8 mm, ovoid shape, annulus prominent, calyptra missing, operculum missing, Peristome were degraded during collection. Spores were not observed. Majority member of this genus is found on stones and rocks.

Specimens examined: Pakistan, Khyber Pakhtunkhwa, Dir upper, Gulibagh 1700m a.s.l., 16-10-2023, *Imran Ahmad* and *Nadeem Khan* (IM3, accession no, PV400746).

General distribution: Mexico, Central North America, Europe, India, Pakistan.

Phylogenetic analysis: (Fig. 4). For Phylogenetic analyses sequences of the nrDNA ITS-BMBC and ITS-LS4-R primers region of collections (IM27 (PV290437), IM54 (PV290438), IM25 (PQ069029), and IM31 (PV400746) were obtained with both the primers. ITS sequences are tend to be relatively variable and are mainly used in low level systematic studies of Plants (Baldwin, 2014). The evolutionary history was inferred by using the soft wear Maximum Likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 63 nucleotide sequences, and a total of 2591 positions in the final dataset, out of which 815 were conserved, 1021 variable, 789 parsimony informative and 211 singletons. Evolutionary analyses were conducted by MEGA6.

Bootstrap analysis recovered four distinct sub clades and *Tortula muralis* (AF144125) was chosen as the out-group. The ITS sequences generated from Pakistani collections (IM25) showed 100% similarity and clustered with USA collections *Fissidens grandifrons* (OL457752). Second Pakistani collection (IM27) clustered and showed 100% similarity with Norway sequence *Fissidens dubius* (KC333208) and the collection (IM31) showed similarity with Spain sequence *Grimmia ovalis* (EU343785). *Grimmia ovalis, Fissidens grandifrons* and *Fissidens dubius* was recorded phylogenetically for the first time in Pakistan. Therefore, the three species collected in this study support the positions in ITS phylogeny.

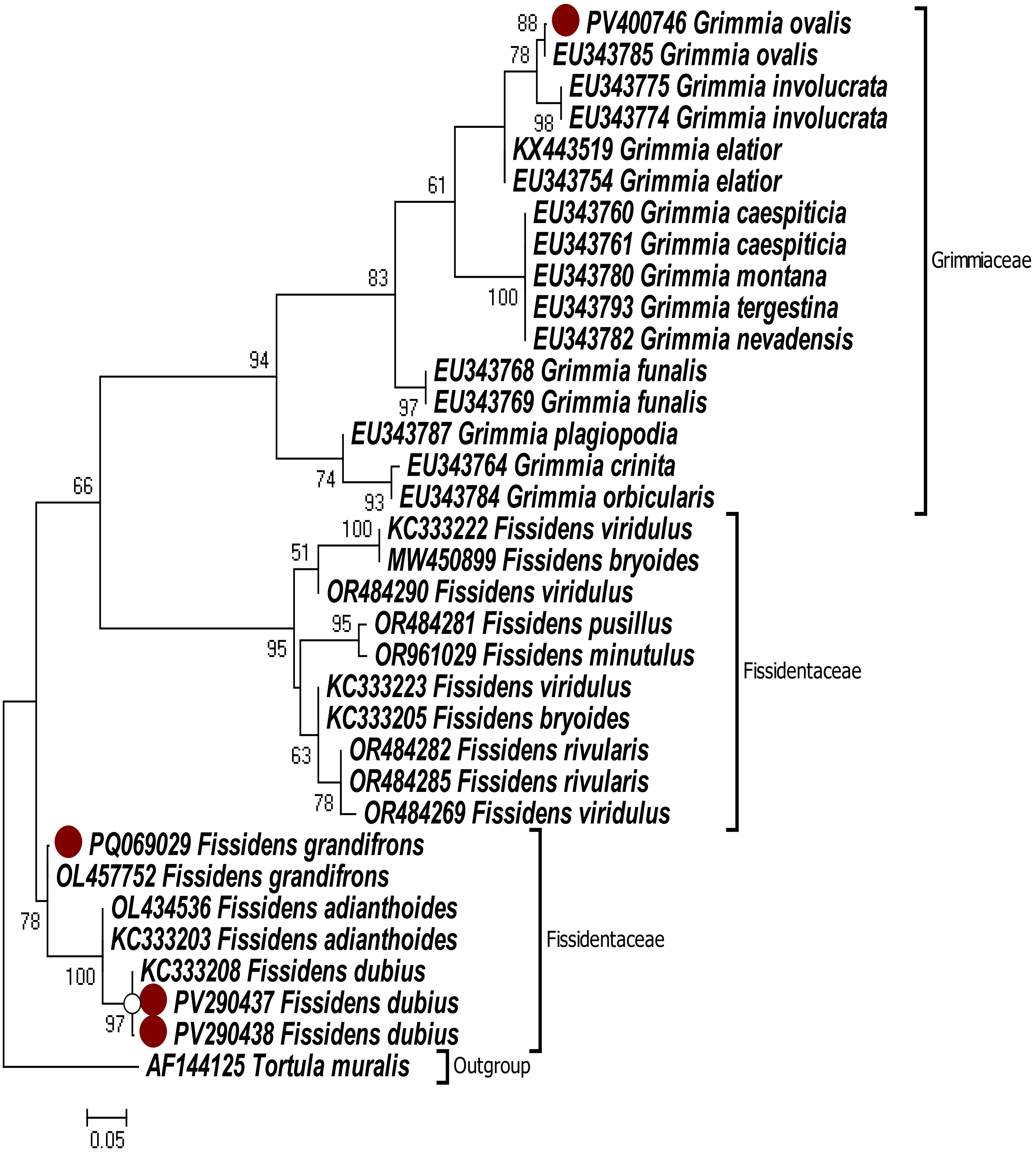

Fig. 4. Molecular phylogenetic analysis by maximum likelihood (ML) method based on ITS sequences. The evolutionary history was inferred by using the RAxML-HPC2 v 8.1.11 and conducted in MEGA 6. Bootstrap values >60% based on 1000 replicates are shown at the branches and sequences generated during this study are in bold.

Discussion

Phanerogams are documented in the Flora of Pakistan, however, lower plant flora are still neglected and a very little information about the Bryoflora of Pakistan is known so far (Islam *et al*., 2016a). Previously available literature just provides simple checklists (Nishimura & Niguchi, 2003; Sollman, 2008; Gruber & Peer, 2010, Islam *et al*., 2016c). Hazrat *et al*., (2020) recently published a checklist of 45 mosses from Sheringal district upper dir only on morphological analysis but no molecular characterization of mosses has conducted. In current investigation, a detailed morpho-anatomical and molecular study of mosses of Fissidentaceae and Grimmiaceae have been carried out. In current investigation the District upper dir has been chosen for detailed morpho-anatomical and molecular study of this musci group. Previously, thirteen taxa of Fissidentaceae have been reported from Pakistan, but the studies are very preliminary and focus on morphological characteristics (Islam *et al*., 2016b). According to available literature, no member of genus Fissidens taxa are reported from study area. Our results confirm the collection of two taxa from study area as new record.

Fissidens grandifrons; can be found everywhere, but the sporophytes are scarce and have only been described and illustrated in Asia collections, the probable center of distribution for the species (Pursell, 2007). *Fissidens grandifrons* are collected from various localities of Pakistan by different contributors, Dixon (1926-29) first time do collection from Gilgit and Waziristan, Blatter & Fernandez (1931) from Waziristan, Froehlich (1963) from swat, Highuchi (1992) from Swat, Kaghan & Nishimura (1993a) from Baltistan, Sindh and Islam *et al*., (2016b), from district Mansehra. During morphoanatomical comparison our specimens show little differences with Islam *et al*., (2016b); coasta was thick and clear, leaf base was not decurrent, margin smooth, and leaves were 20-25 in pairs. Sequencing of the nr ITS region of our collection (IM25) yielded fragments of 870 base pairs. Based on the molecular phylogenetic analysis, our collection close relative to USA collection (OL457752) with 100%bootstrap values and placed in Fissidentaceae super clade.

Fissidens dubius; is considered longest species among Fissidens and have collected from different localities of Pakistan by Noguchi (1946), Nishimura (1993a), Islam *et al*., (2016a) from Baltistan, Kaghan district Mansehra, Swat. Our finding is slightly different from Islam *et al*., (2016a) by; Plant height 3-5.7cm long, leaves pairs 30-35, leaf base-less decurrent, coasta end two cells before apex. According to nr ITS sequencing local specimen (1M27, IM54) form clustered with Norway sequence (KC333208 and KC33208). Our Pakistani sequence was 850 base pair long and placed in Fissidentaceae super clade, showing 100% bootstrap values.

Grimmiaceae generally include 20 Genera and 758 species distributed worldwide, 18 species and 2 genera are reported from Pakistan (Islam *et al*., 2016), while 1 genera and 1 species from study area (Hazrat *et al*., 2020). Grimmia ovalis become dark black after dehydration which attract collectors by this unique character. Grimmia ovalis were documented from various site of Pakistan. Brotherus & Duthie (1898) were first to collect it from Himalaya and Chitral, followed by Dixon (1926) from Gilgit, Blatter and Fernandez (1931) from Waziristan, Herzog (1938) from Hindukush, Noguchi (1959) from Hindukush, Townsend (1994) from Rawalpindi and Kaghan, Mazhar *et al*., (2016) from District Mansehra, Hazrat *et al*., (2020) from Dir upper. Our finding was disagreeing with already published specimen from district Mansehra by; Capsule was not covered by calyptra, operculum and peristome were degraded during collection. Local specimens (IM31) sequence was generated successfully by amplifying ITS region of nr DNA. Total number of base pair fragments was 850 and clustered with Spanish (EU343785) sequence and placed in Grimmiaceae super clade. All these above-mentioned contributors only focused on morpho-anatomical characters and documented their findings in the form of checklist. According to the phylogenetic study, from ITS sequences of world collection, this is the first attempt where molecular data has been applied to name and confirm the nomenclature of these mosses.

Conclusion

The present study was focused on morpho-anatomical and molecular characterization of *Fissidens dubius, Fissidens grandifrons*, and *Grimmia ovalis*. Nuclear Internal transcribed spacer (ITS1) region was used to find out the phylogenetic position of these taxa. Among these *Fissidens dubius* and *Fissidens grandifrons* have been described as new report from the study area. Our findings described the potential of ITS1 in identification and classification of the species. The study is very rich in mosses but very limited literature with preliminary morpho-anatomical description is available. The current attempt is the first one of its natures to describe the molecular characterization of mosses from district Dir. It will help the researchers for further taxonomic studies in exploring moss flora of the study region and will lead a way forward for more research in ecological and phytochemical fields.

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