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BACIDIA SUFFUSA (FR.) A. SCHNEIDER (RAMALINACEAE), AN ADDITION TO THE LICHEN BIOTA OF PAKISTAN CONFIRMED BY MOLECULAR PHYLOGENY

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Abstract

Bacidia suffusa, a member of lichenized family Ramalinaceae is reported on the bark of *Alnus nitida*, from Himalayan moist temperate forest of Pakistan, based on critical morpho-anatomical and ITS-based phylogenetic analysis. Its occurrence here indicates that it is the first report of this lichen from Pakistan and South East Asia as well. The taxonomic characters of the taxon are given, along with an ITS-based phylogenetic tree and notes on its ecology and distribution.

Key words: Himalaya, Koza Gali, Khyber Pakhtunkhwa, New record

Abbreviations: ITS: Internal Transcribed Spacer; CTAB: Cetyl Tri-methyl Ammonium Bromide; PCR: Polymerase Chain Reaction; MAFFT: Multiple Alignment using Fast Fourier Transform; MEGA: Molecular Evolutionary Genetic Analysis.

Introduction

The Himalayan belt is the most prominent and distinguished structure due to its complex geologic structure, series of elevational zones, and diversified phytogeographic stocks (Singh & Singh, 1987). It lies within the West and North of the Pakistan and South to the Indus basin. The Himalayan range encompasses the southern part of the Hazara, Murree hills and Khanspur and is considered as a lichen rich landscape of Asia (Aptroot & Iqbal, 2012).

Bacidia De Not. is one of the species rich, cosmopolitan genus with approximately 230 species of lichenized fungi in the Lecanorales (Lücking *et al*., 2017)**.** The genus was first described by Giuseppe De Notaris in 1846. Many species placed under '*Bacidia*' belong to other genera or even other families, so the *Bacidia*, in its strict sense, is estimated to consist of 60–90 species (Ekman, 1996, 2001; Coppins & Aptroot, 2009; Vondrak *et al*., 2018). Lichen species within *Bacidia* occur in almost all substrates, habitats and climatic zones of the world, but are abundant and diverse in sheltered habitats, such as tree bark, in moist areas of boreal, temperate and especially tropical regions (Kirk *et al*., 2008). *Bacidia* in the strict sense is consequently characterized by acicular spores and a well-developed, prosoplectenchymatic proper exciple composed of radiating, abundantly furcate and rarely anastomosed hyphae with heavily gelatinized cell walls and cell lumina that become compressed and narrower with age (Gerasimova *et al*., 2018; Kistenich *et al*., 2018). Species of this genus also produce one or more pigments in the apothecia. These pigments provide the single most important character set to distinguish between species of *Bacidia* (Ekman, 1996). *Bacidia suffusa* produces atranorin as the principal secondary metabolite (Culberson & Culberson, 1969; Ekman, 1996), which is among compounds possessing antibacterial, antifungal, antiviral, anti-inflammatory, analgesic, wound healing and immunomodulatory activities (Studzińska-Sroka *et al*., 2017).

Four species of *Bacidia* have been reported from Pakistan viz., *B. fraxinea* Lönnr., from Baragali, Khanspur, Murree Hills and Shogran; *B. laurocerasi* (Delise ex Duby) Zahlbr., from Murree; *B. rosella* (Pers.) De Not., from Rawlakot and *B. rubella* (Hoffm.) A. Massal., from Sharan (Aptroot & Iqbal, 2012). During field survey of Himalaya moist temperate forests in Pakistan, KP, Koza Gali near Ayubia National Park (Fig. 1), we collected a sample twice which is assigned to genus *Bacidia.*

Materials and Methods

Morphological and chemical studies: The survey of the study area and lichen sampling were carried out during summer 2018. Morphological characters were observed under a stereomicroscope (Meiji Tech., EMZ-5TR, Japan). For anatomical analyses, free hand sections of the apothecia were cut, mounted on glass slides using water and 5% KOH as mounting media and examined under compound microscope (MX4300H, Meiji Tech. Co., Ltd., Japan). Spot tests were performed by applying suitable reagents to a lichen fragment and the color changes were observed under the stereomicroscope (Hale, 1979). Secondary metabolites were detected by thin-layer chromatography using Solvent System C, following standard methods (Orange *et al*., 2001). The terminology and description followed here is that of Smith *et al.*, (2009). The examined materials are deposited in LAH herbarium, Institute of Botany, University of the Punjab, Lahore.

Molecular characterization: Genomic DNA was isolated from dried thallus and apothecia using the 2% CTAB protocol (Gardes & Bruns, 1993). The ITS region was amplified using the ITS1F/ITS4 primer pair following the amplification protocol of Khan *et al*., (2018). The amplified DNA fragments were observed in 1.2% Agarose gel (Sambrook & Russell, 2001) and PCR products were sequenced by BGI, Hong Kong.

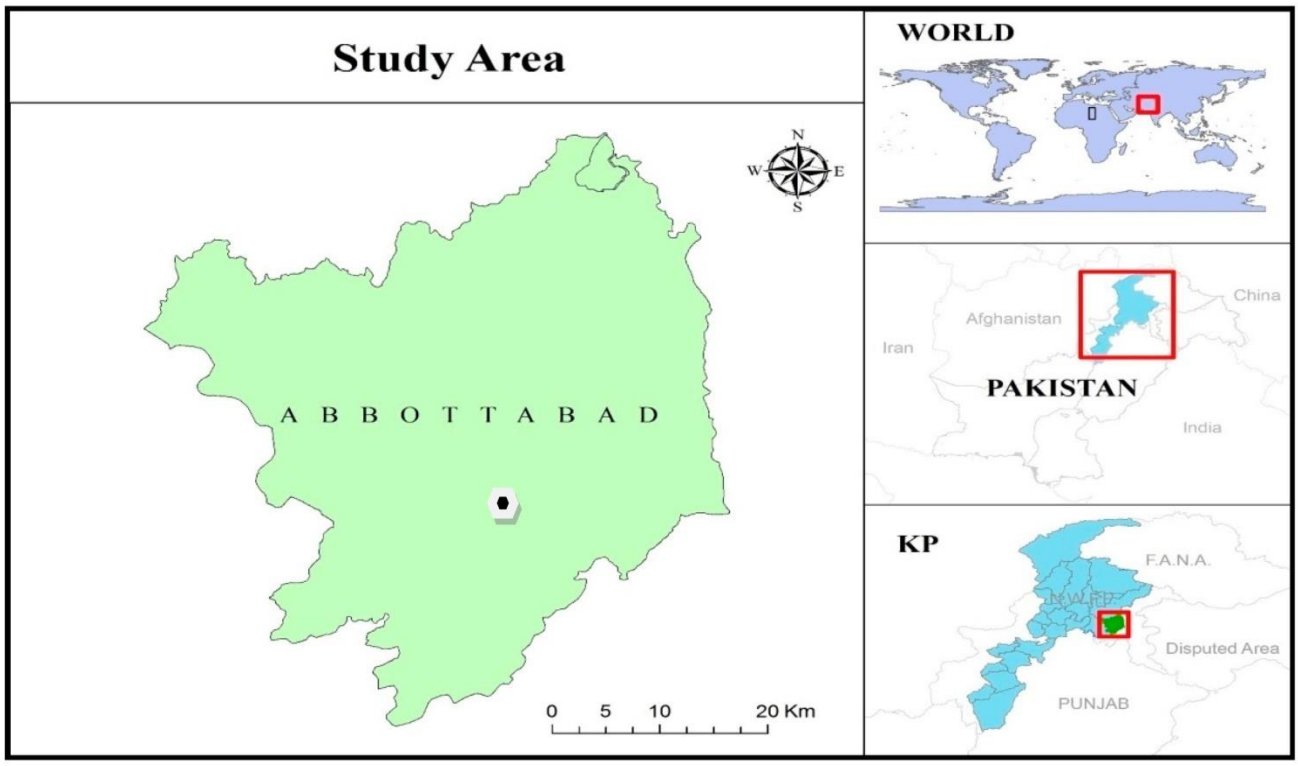

Fig. 1. Map of the study area.

Phylogenetic analysis: Bidirectional sequences (ITS1 and ITS4) were reassembled by using BioEdit software (Hall, 1999). For phylogenetic analysis ITS sequences were retrieved using Basic Local Alignment Search Tool (BLAST) network service of the National Centre for Biotechnology Information (NCBI) (Altschul *et al*., 1990). Closest matching sequences were downloaded from GenBank for subsequent phylogenetic analysis (Table 1). Online MAFFT v 7.0 with default setting was used to align (Katoh & Standley, 2013). Our new sequences with the sequences retrieved from NCBI GenBank. The alignments were trimmed from both 5' and 3' ends to the borders of ITS using BioEdit software. Phylogenetic tree was constructed by software MEGA X (Kumar *et al*., 2018). The evolutionary history was retrieved with constructing Maximum Likelihood (ML) methods using Kimura-2 parameter model. The model was selected by searching best DNA model for ML analysis in MEGA X (Kumar *et al*., 2018**).** One thousand rapid bootstrap replicates were run to test the robustness of the resulting phylogenetic hypothesis.

Results

***Bacidia suffusa* (Fr.) A. Schneider (Fig. 2: A-E)**

Thallus: Crustose, ± thin, warty granular, continuous to partly rimose, rarely scattered, dark green to dark olive green. **Photobiont:** chlorococcoid, algal cells ±subglobose, 8–16 µm in diam. **Apothecia:** frequent, 0.8–1.2 mm wide, scattered to crowded, sometimes fused, rounded, strongly constricted at base. **Disc:** orange yellow to brown, weakly pruinose, flat to convex, smooth, glossy. **Margins:** whitish grey, continuous, slightly raised when young, thin when mature, thick pruina especially well developed on the margins of young apothecia. **Proper exciple:** 25–40 µm wide, edges greyish brown, hyaline inner, (along the edge with a 4–6 cell layers thick). **Epihymenium:** 10–15 µm high, light brown to brown. **Hymenium:** 90–115 µm high, hyaline. **Hypothecium:** 80–110 µm tall, hyaline to light brown. **Paraphyses:** septate, branched, hyaline, 2–4.2 µm wide. **Asci:** cylindrical-clavate, 8-spored, 60–100 × 10–22 µm. **Ascospores:** acicular, hyaline, transversely 15–20 septate, 55–85 × 2–5 µm. **Pycnidia:** not found.

Spot test: Thallus K+ (light yellow), C–, KC–, P–; **TLC:** *atranorin.*

Specimen examined: PAKISTAN. Khyber Pakhtunkhwa: Khanspur, Koza Gali, at 2,250 m a.s.l, 34° 1' 16 N; 73° 25' 40 E, on bark of *Alnus nitida* (Spach) Endl., April 30, 2019, A.N. Khalid and K. Habib: LAH36839; GenBank–MW728313; LAH36838; GenBank–MW788561.

Ecology: *Bacidia suffusa* is corticolous, with a preference for deciduous trees (Ekman, 1996). Collections were made in mixed temperate coniferous forests at an altitude of about 2,250 m a.s.l., found on the bark of *Alnus nitida*. The region has hilly topography with mean maximum and minimum temperature 21.5°C and -2°C, respectively, and receives annual rainfall about 600 mm.

Distribution: Worldwide, it is widespread in the eastern temperate region of North America (Ekman, 1996). In Eurasia, *B. suffusa* is reported only from the Russia (northwest Caucasus), but rare or absent in Europe (Otte, 2007). This study reports it as a new record to the lichen biota of Pakistan.

Table 1. GenBank accession numbers of sequences used in phylogenetic analyses.

Sr. No.	ITS GenBank Accession no.	Specimen	Country	Voucher
01.	MH048630	*Bacidia rubella*	Russia	M-0182581
02.	JQ796852	*Bacidia rubella*	Switzerland	LG DNA 578
03.	AF282088	*Bacidia fraxinea*	Sweden	Johansson 1620 (BG)
04.	AF282086	*Bacidia rosella*	Sweden	Ekman 3117 (BG)
05.	AF282085	*Bacidia absistens*	Norway	Ekman 3223 (BG)
06.	MG925955	*Bacidia squamulosula*	Ecuador	O:L 113543
07.	FR799126	*Bacidia arceutina*	United Kingdom	EDNA09-01507
08.	JQ796853	*Bacidia sipmanii*	Spain	LG DNA 361
09.	AF282084	*Bacidia scopulicola*	Sweden	Ekman 3106 (BG)
10.	AF282082	*Bacidia lutescens*	USA	Ekman L1161 (LD)
11.	NR163772	*Bacidia subareolata*	Thailand	MFLU 18–1817
12.	MH048614	*Bacidia areolata*	Russia	M–0182592
13.	NR160612	*Bacidia areolata*	Russia	M:M–0182592
14.	MH048615	*Bacidia suffusa*	Russia	M–0182601
15.	MN264753	*Bacidia sp.*	USA	JCL48883
16.	MH048618	*Bacidia suffusa*	USA	M–0289887
17.	MH048619	*Bacidia suffusa*	USA	M–0289888
18.	KX151766	*Bacidia schweinitzii*	USA	Lendemer 31230A (NY)
19.	KX151761	*Bacidia schweinitzii*	USA	Lendemer 30548 (NY)
20.	KX151771	*Bacidia sorediata*	USA	Lendemer 31527 (NY)
21.	KX151774	*Bacidia sorediata*	USA	Lendemer 38909 (NY)
22.	KX151772	*Bacidia sorediata*	USA	Lendemer 33787 (NY)
23.	AF282078	*Bacidia laurocerasi*	USA	Wetmore 74318 (MIN)
24.	MH048609	*Bacidia laurocerasi*	Russia	Galanina 424
25.	AF282079	*Bacidia biatorina*	Sweden	Knutsson 94–148 (Knutsson priv. herb.)
26.	MH048612	*Bacidia kurilensis*	Russia	M-0182622
27.	MH048611	*Bacidia kurilensis*	Russia	M-0182621
28.	MH048610	*Bacidia kurilensis*	Russia	M-0182620
29.	AF282083	*Bacidia arceutina*	Norway	Ekman 3110 (BG)

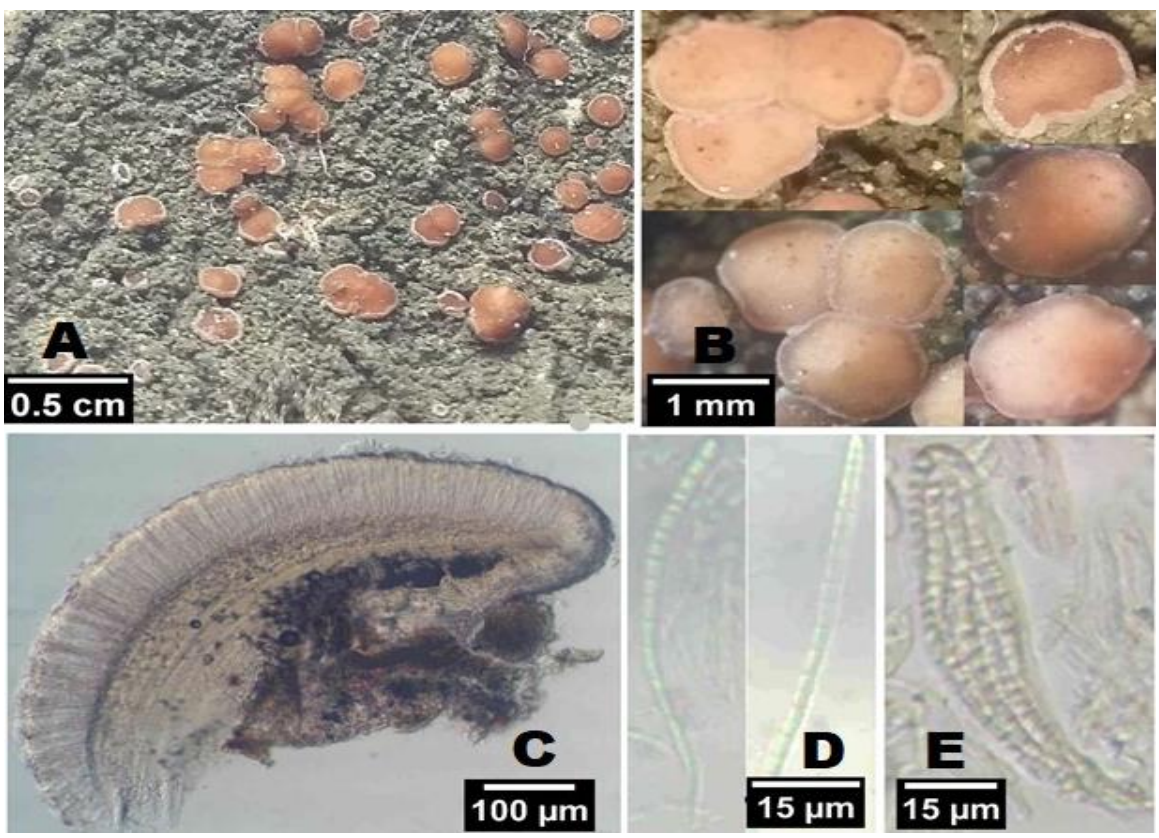

Fig 2. (A–E) *Bacidia suffuse*; A: Thallus, B: Apothecia, C: Section of apothecium, D: Acicular ascospores, E: Ascus.

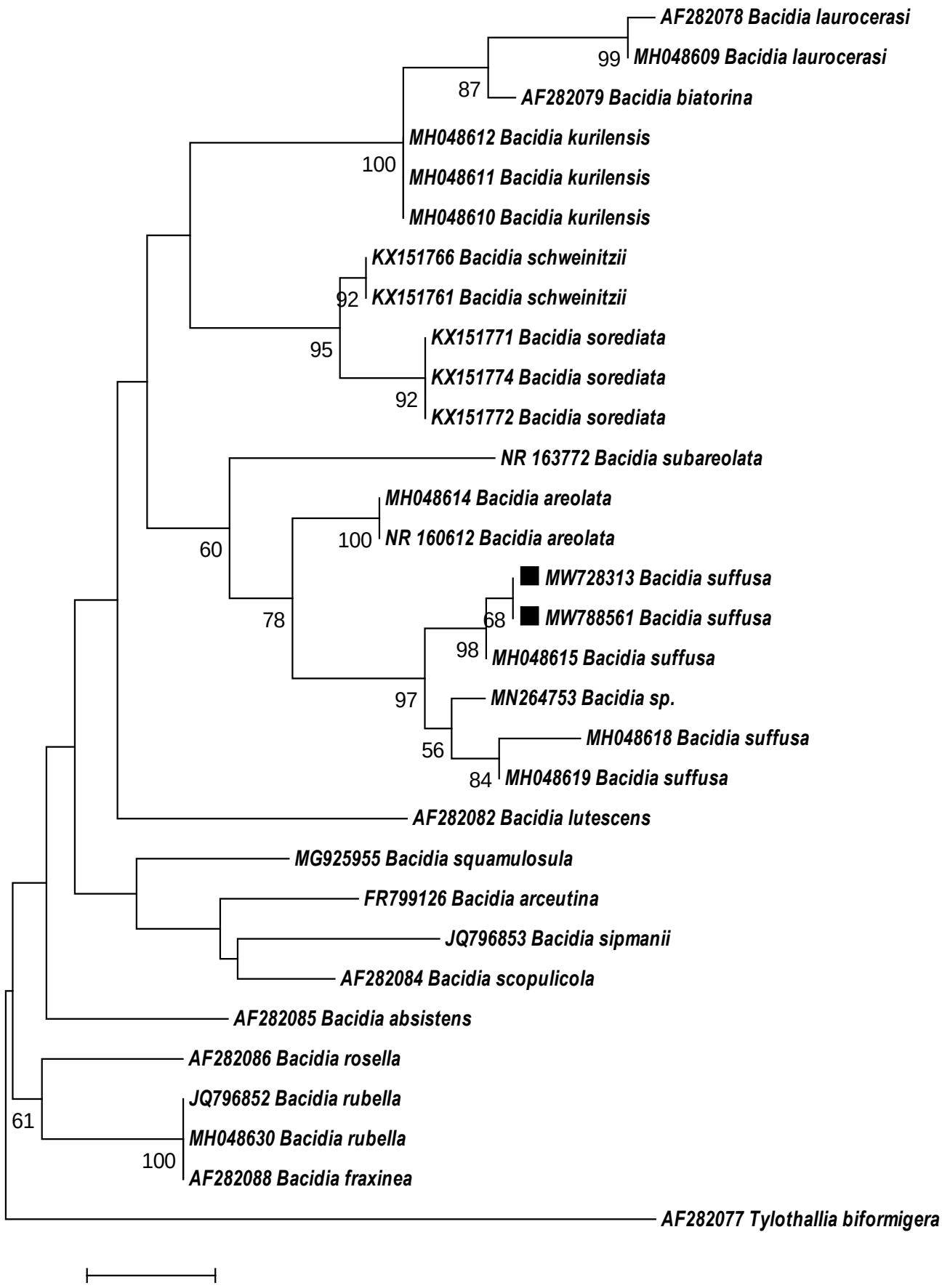

Fig. 3. Phylogram depicting the best maximum likelihood tree of *Basidia suffusa* based on rDNA sequences, including ITS1, 5.8S and ITS2. Numbers below branch node represent ML bootstrap (>50%) based on 1000 replicates. Sequences generated from Pakistani collections are marked with square box.

Phylogram analysis: Length of final alignment matrix was 544 nucleotides, which included 285 conserved, 225 variable and 171 parsimony informative sites. *Tylothallia biformigera* (Leight.) P. James & H. Kilias (AF282077) was selected as outgroup (Gerasimova *et al.,* 2018). In the phylogram, newly generated sequences from Pakistani collections clustered with *B. suffusa* specimens and formed a sister group relationship with *B. areolata* Gerasimova & A. Beck (Fig. 3). The branch representing the *B. suffusa* specimens shows the bootstrap value of 97%.

Discussion

So far, 383 lichens have been reported from Pakistan (Ahmad, 1965; Aptroot & Iqbal, 2012; Habib *et al.,* 2017; Khan *et al.,* 2018; Habib & Khalid, 2019; Habib *et al.,* 2020; Fatima *et al.,* 2020; Zulfiqar *et al.,* 2020; Habib *et al.,* 2021a,b). In the past, lichens of this region were identified on basis of morphology and anatomy. Recently, 22 lichen species have been identified using molecular technique (Habib *et al.,* 2017; Khan *et al.,* 2018; Habib & Khalid, 2019; Habib *et al.,* 2020; Fatima *et al.,* 2020; Zulfiqar *et al.,* 2020; Habib *et al.,* 2021a,b).

In ITS based phylogenetic analysis, the Pakistani collection of *B. suffusa* clustered with specimen of same taxon from Russia, Far East (MH048615). The sequences of the Pakistani and Russian *B. suffusa* differ in 3 nucleotides in their ITS-rDNA locus. Morphological comparison also confirms its identity as *B. suffusa* (Kuznetsova *et al.,* 2013). It is the frequently pruinose species having long ascospores. In the phylogram, *Bacidia suffusa* made sister branch to *B. areolata* reported form Russia (Gerasimova *et al.,* 2018), which is morphologically different from Pakistani specimen in having smooth, cracked to areolate thallus and small ascospores.

Bacidia suffusa was hitherto an eastern North American endemic taxon (Ekman, 1996). It occurs mostly on the trunks of hardwoods, hardly on twigs, rocks and bryophytes and differs from other members of the genus in substrate preference. The other species of the genus occur on *Juniperus* with some consistency. *Bacidia suffusa* has been collected on different varieties of hardwoods of *Aesculus*, *Alnus*, *Asimina*, *Carya*, *Celtis*, *Diospyros*, *Fraxinus*, *Juglans*, and *Quercus* (https://www.nybg.org/bsci/lichens/ozarks/Bacidia.html#3.). Its occurrence here indicates that it is first report from South East Asia.

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