

New Records of Saxicolous Lichens from Pakistan

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Abstract—Two saxicolous species viz., *Bagliettoa calciseda* and *Diploschistes actinostomus* are reported as new to country. Among which, *Bagliettoa calciseda* also makes a new generic record for Pakistan. Complete morpho-anatomical descriptions along with ecology, distribution and ITS-based phylogenetic analyses are also discussed.

Keywords: Tirah Valley, Kallar Kahar, Margalla Hills, phylogeny, taxonomy

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INTRODUCTION

Pakistan is located in western South Asia between 24°–37° N latitude and 62°–75° E longitudes. The country is well known for its geographical and climatic variations which are linked with rich biodiversity (IUCN 2006). So far, up to 400 lichens have been reported from Pakistan from various localities (e.g. Aptroot and Iqbal, 2012; Habib et al., 2017, 2019, 2021; Zulfiqar et al., 2020; Fatima et al., 2021; Kousar et al., 2021; Fayyaz et al., 2022; Nadeem et al., 2022). Surveys of the several areas still left unexplored will definitely contribute further to our knowledge of the lichen flora of Pakistan.

During present investigation of lichens from different localities of Pakistan, we found two species viz., *Bagliettoa calciseda* and *Diploschistes actinostomus* as new records. From Pakistan, there is no previous record of genus *Bagliettoa* whereas three species of genus *Diplochistes* have been previously reported so far, viz: *D. diacapsis* (Ach.) Lumbsch, *D. muscorum* (Scop.) R. Sant. Lumbsch, *D. scruposus* (Schreb.) Norm. Lumbsch (Aptroot and Iqbal, 2012). The genus *Bagliettoa* also makes a new generic record for the country, described for the first time from Pakistan on the basis of morpho-anatomical and ITS based phylogenetic analysis, which further made an addition to the lichen flora of Pakistan.

MATERIALS AND METHODS

Morphological and Chemical Studies

Collections were made during a lichen survey of different localities of Pakistan including Tirah Valley, Kallar Kahar and Margalla Hills in year 2021. Morphological characters were observed under a stereomicroscope (Meiji Techno, EMZ-5TR, Japan). Stan-

dard microscopy and spot tests (Hale, 1979) were used for identification. Secondary chemistry was analysed by thin-layer chromatography using Solvent System C, following Orange et al. (2001). Measurements were made from free hand section of apothecia mounted in water on a glass slide. The sections were observed using a compound microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Minimum twenty measurements in water were made for each diagnostic feature.

DNA Extraction and PCR Amplification

We used thallus material along with apothecial material to extract fungal DNA using a 2% CTAB protocol (Gardes and Bruns, 1993). The primer pair ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) was used to amplify the internal transcribed spacer (ITS) region under PCR conditions. PCR products were visualized in a 1% agarose gel using ethidium bromide (Sambrook and Russell, 2001). PCR products were sequenced from BGI, China.

Phylogenetic Analysis

The ITS regions of all specimens were amplified and sequenced. Bio-edit sequence alignment editor was used to reassemble forward and reverse sequences (Hall, 2005). The multiple sequence alignment was performed using MAFFT v7 with all parameters set to default values (Katoh and Standley, 2013). The ends of the alignment were trimmed to nearly an equal number of sites for all sequences. All gaps were treated as missing data. Maximum Likelihood analysis was performed with MEGA X using a GTR model for bootstrapping (Kumar et al., 2018). One thousand rapid bootstrap replicates were run to infer the evolutionary history of each species.

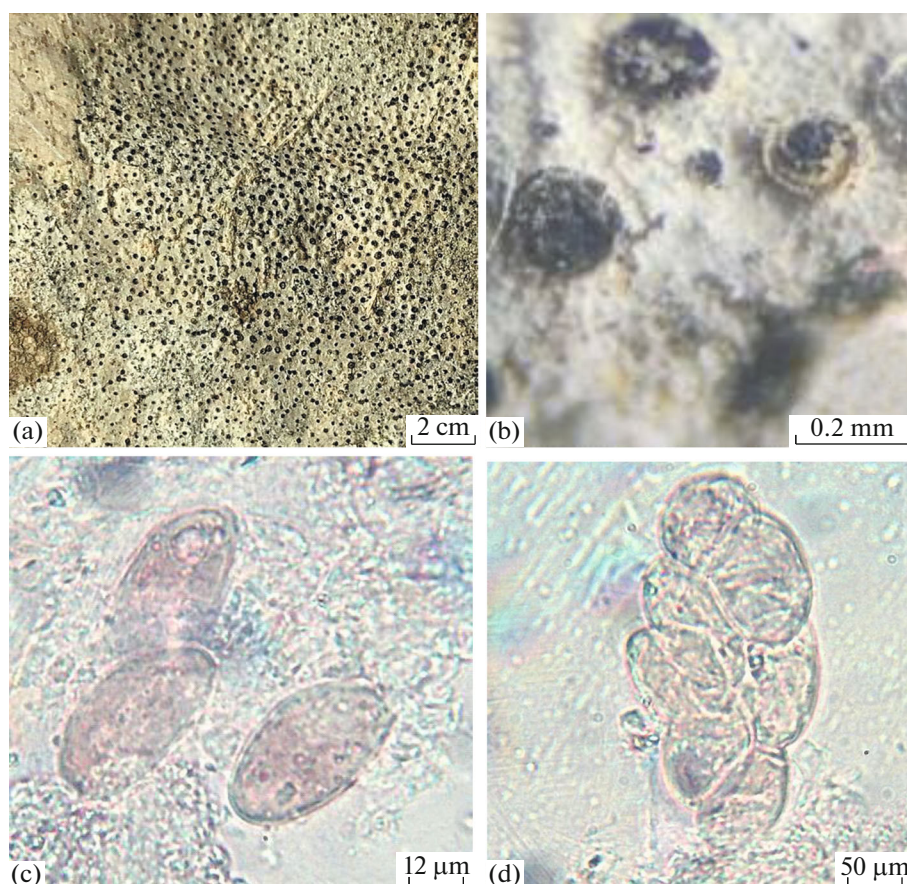


Fig. 1. *Bagliettoa calciseda* (DC.) Gueidan and Cl. Roux, (a) Crustose thallus. (b) Perithecia. (c) Ascospores. (d) Ascus.

RESULTS

A total of 23 sequences were used in the phylogenetic analysis of *Bagliettoa* spp. including three Pakistani collections and one sequence of *Dermatocarpon miniatum* (L.) W. Mann (MH027413) as outgroup. The final data matrix included 526 nucleotides, of which 345 sites were conserved, 177 were variable, 126 were parsimony informative and 51 were singleton sites.

The sequences used for phylogenetic analysis of *Diploschistes* spp. were downloaded from GenBank based on the similarity. A total of 25 sequences were used in the phylogenetic analysis including four Pakistani collections and one sequence of *Thelotrema gomezianum* Lücking (NR 120283) as outgroup. The final data matrix included 549 nucleotides, of which 363 sites were conserved, 151 were variable, 78 were parsimony informative and 73 were singleton sites.

TAXONOMY

Bagliettoa calciseda (DC.) Gueidan and Cl. Roux,
Bull. Soc. linn. Provence 58: 187 (2007)

Thallus: continuous, endolithic, 2 cm across, smooth, dull, slightly rugose, often cracks are present, epruinose. Color: whitish grey to grey.

Perithecia: numerous, dark black, completely immersed in the thallus, 0.1–3 mm in diameter, globose, convex, leaving empty pits behind. Ostiole: indistinct. Invollucellum: absent. Periphyses: 15–25 µm long. Asci: clavate, hyaline, 55–75 × 15–20 µm. Ascospores: ellipsoid, simple, hyaline, 15–23 × 9–11 µm. Pycnidia: not found.

Chemistry: all negative. TLC: no substance detected.

Substrate and ecology: saxicolous, found in sub-tropical semi-arid environment of district Chakwal at an altitude of 5.54 m a.s.l., temperature varying between 15–40°C with an annual rainfall 350–500 mm, while other collections was found in humid sub-tropical forests of Margalla Hills, fully exposed to sunlight and rain, at an altitude of 1604 m a.s.l., temperature varying 34–3°C with an annual rainfall 1200 mm.

Distribution: widely distributed in the Mediterranean phytogeographic region of Turkey (Guvenc et al., 2020), Armenia (Gasparyan et al., 2015), Los Angeles (Kocourková, 2009), Germany, France, UK and England (Yuzon et al., 2014), Sweden, Norway, Finland (Santesson et al., 2004), New Zealand (McCarthy, 1991), Central and southern Europe, Africa, Arizona and Asia (Nash et al., 2007).

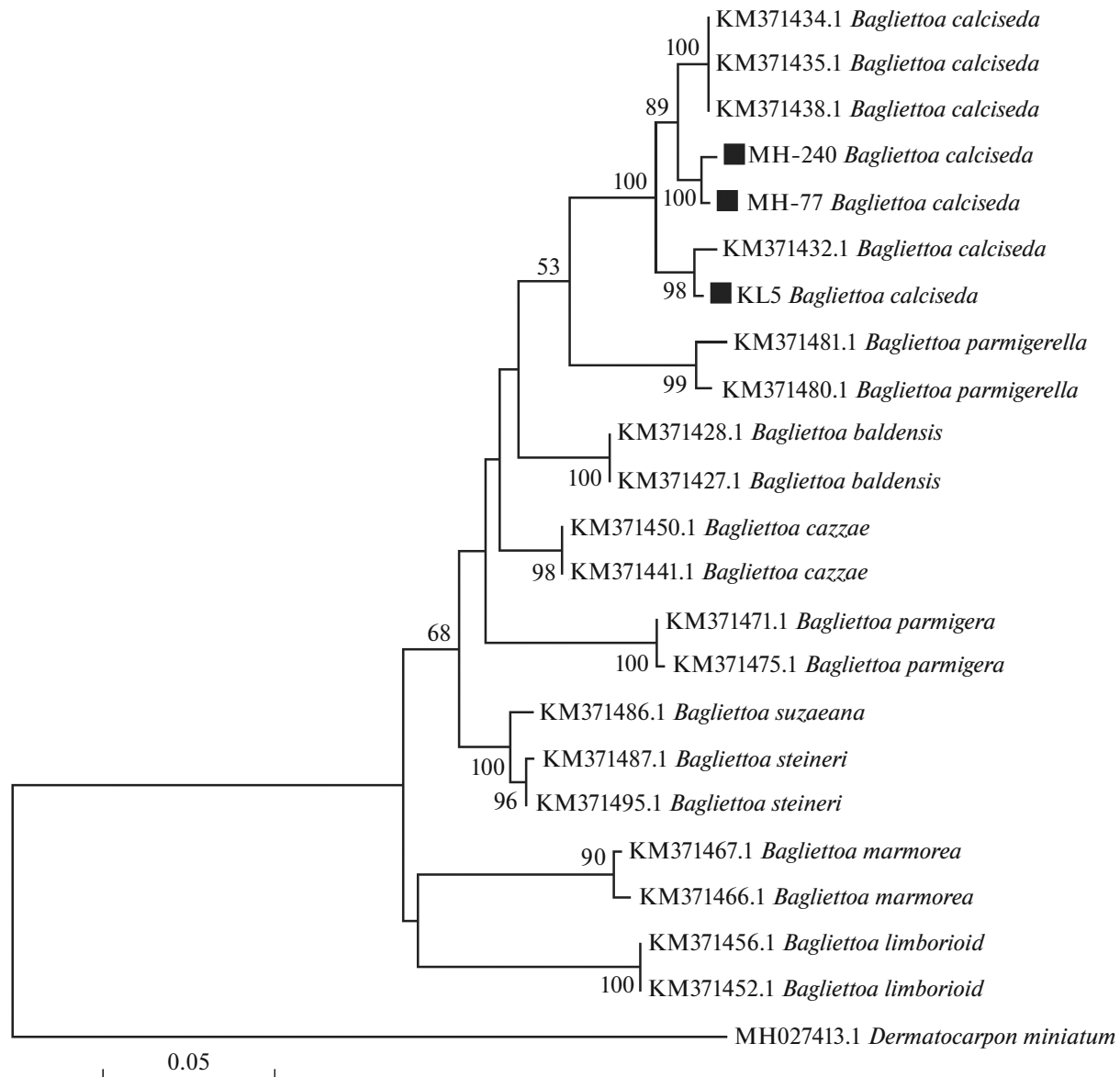


Fig. 2. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Sequences generated from local collection are represented with a black box.

Materials examined: Pakistan. Kallar Kahar: district Chakwal; 32°46' N and 72°42' E; 554 m a.s.l., on rocks; 15 Oct., 2021; A. Naseer, KL-5 (LAH37532) (GenBank no. OP002273); Pakistan. Islamabad, Margalla hills, 1604 m a.s.l., on rocks; 33°49' N 73°16' E, 4 Oct., 2021; A. Ashraf; MH-77 (LAH37580) (GenBank no. OP002274); MH-240 (LAH37581) (GenBank no. OP002275).

Diploschistes actinostoma (Ach.) Zahlbr.
[as "*actinostomus*"], *Hedwigia* 31: 34 (1892)

Thallus: rimose-areolate, up to 1.5 cm across, cracked, continuous, dull, 100–150 µm thick in sec-

tion. Areoles: plane, pruinose 0.5–0.8 mm in diameter, rounded to irregular. Color: white to whitish gray.

Upper cortex: 15–20 µm thick, cells rounded, 6–8 µm in diameter. Algal layer: 30–40 µm thick, photobiont chlorococcoid, 5–8 µm in diameter. Medulla: prosoplectenchymatous, 90–120 µm thick, hyaline hyphae, 2–3 µm wide.

Apothecia: frequent, immersed in areoles. Disc: black, pruinose, rounded, plane, 0.2–0.3 mm in diameter. Paraphyses: simple, not swollen apically, 1–2 µm wide. Epithymenium: dark brown, 15–20 µm tall. Hymenium: hyaline, 90–150 µm tall. Hypothecium: dark brown, 10–20 µm tall. Asci: clavate, 55–65 × 10–15 µm, Ascospores: muriform, ellipsoid, brown,

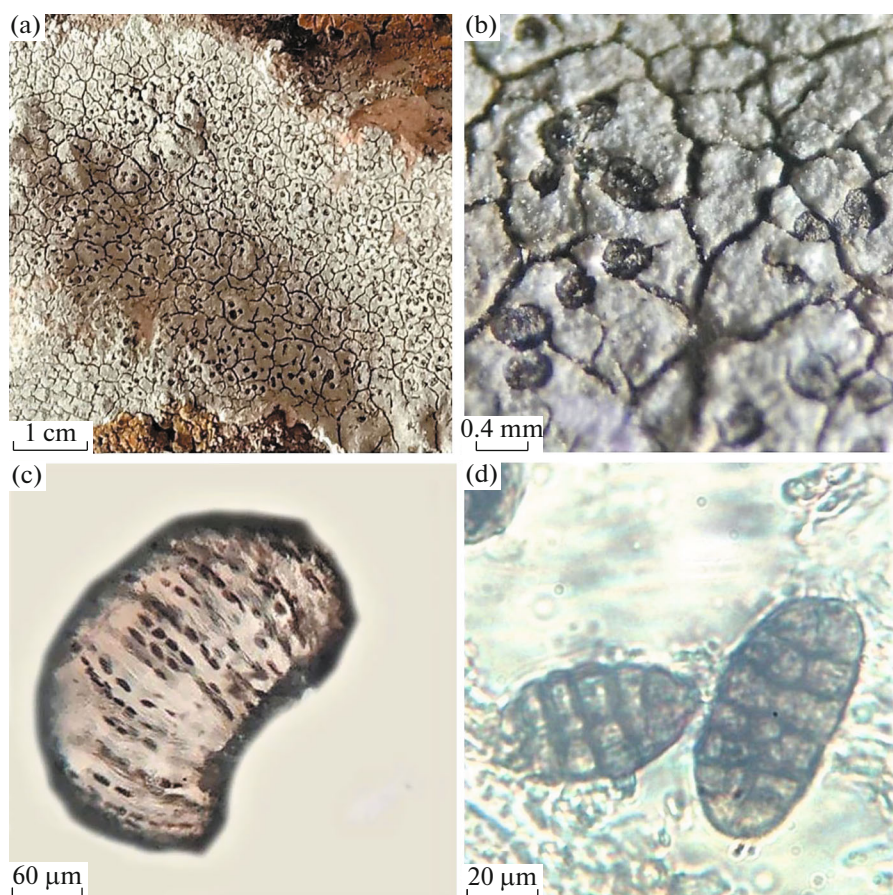


Fig. 3. *Diploschistes actinostomus* (Ach.) Zahlbr: (a) Thallus, (b) apothecia closer view. (c) Cross section of apothecium, (d) asco-spores.

16–30 × 15–20 μm. Pycnidia: black, immersed, conidia bacilliform, 3–8 × c. 1 μm.

Chemistry: K-, C+(red), KC-. TLC: lecanoric acid, diploschistesic acid detected.

Substrate and ecology: saxicolous in sub-tropical forests of district Kohistan and Tirah Valley, at an altitude of 841 m a.s.l., temperature varying between 30–10°C, with an annual rainfall 200–400 mm, while other collections was found in humid sub-tropical forests of Margalla Hills, fully exposed to sunlight and rain, at an altitude of 1604 m a.s.l., temperature varying 34–3°C with an annual rainfall 1200 mm.

Distribution: widely distributed in maritime and oceanic Mediterranean regions (Lumbsch 1989), sub-cosmopolitan in relatively open habitats in arid, semi-arid and Mediterranean areas found in California (Nash et al., 2002), USA (Brime et al., 2013), Portugal (Sipman and Aptroot, 2020), South Africa (Plata et al., 2012) and Asia (China and India) (Pant and Upreti, 1993). It is first time reported from Pakistan.

Materials examined: Pakistan, Tirah Valley, Khyber Pakhtunkhwa Province; N 33°48' E, 70°44', on calcareous rocks, 22 Sep., 2021, K. Habib and A.N. Khalid, TV-8 (LAH37541) (GenBank number:

OP022339); Pakistan. Khyber Pakhtunkhwa Province, Kohistan: Dasso, on calcareous rocks, 841 m a.s.l., 35°35' N, 73°37' E, Sep. 9, 2020; K. Habib and A.N. Khalid; KOH-80 (LAH37540) (GenBank no. OP022338); Pakistan. Islamabad, Margalla hills, on rocks; 33°49' N 73°16' E, 1604 m a.s.l., 4 Oct., 2021; A. Ashraf; MH-165-1 (LAH37579) (GenBank no. OP022341); MH-105 (LAH37578) (GenBank number: OP022340).

DISCUSSION

During present investigation of lichens from Tirah Valley, KP, Kallar Kahar and Margalla hills, Pakistan, we found two species viz., *Bagliettoa calciseda* and *Diploschistes actinostomus* as new records for Pakistan.

Bagliettoa calciseda is characterized by light grey thallus prominent around perithecia, sunken in pits with no secondary metabolite detected (Yuzon et al., 2014).

The ITS lineage of Pakistani *B. calciseda* species (KL-5, MH-77, MH-240) is related to *B. calciseda* species (KM371432, KM371434, KM371432) reported from Australia with a strong bootstrap support and formed a sister branch relation with *B. par-*

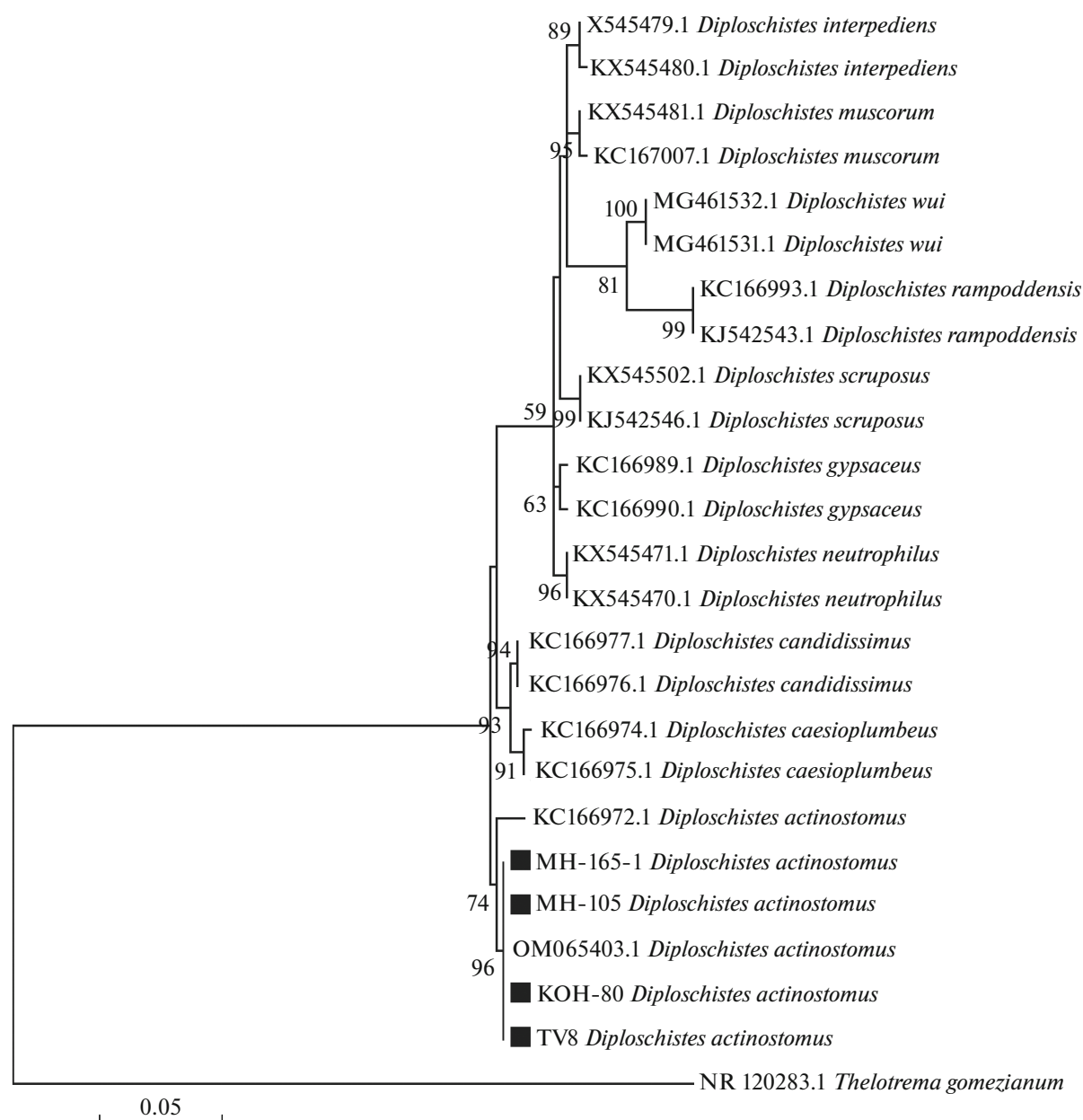


Fig. 4. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura-2 parameter model. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGAX Sequence of local collection is marked with black box.

migerella (KM371480, KM371481) within the same clade (Fig. 2).

Morphologically our specimens are similar to Australian *B. calciseda* in having the same thallus coloration, identical size of ascospores and the absence of invollucellum (Yuzon et al., 2014). This species is being reported for the first time from Pakistan, also makes a new generic record for the country.

The second collection, *Diploschistes actinostomus* is characterized by the perithecioid ascomata, the presence of lecanoric acid and the whitish gray to gray thallus (Nash et al., 2002). The ITS sequences of Paki-

stani *Diploschistes actinostomus* (TV-08, KOH-80, MH-165-1, MH-105) branched with *Diploschistes actinostomus* (OM065403) reported from China with strong support (BS 96%) (Fig. 4), whereas a three nucleotide difference was found between Pakistani and USA *Diploschistes actinostomus* (KC166972).

Morphologically, Pakistani collections are similar to Italian collection of *D. actinostomus* in having similar thallus coloration, perithecioid apothecia and nearly identical size of ascospores except the presence of pruinose disc of apothecia in Pakistani collection (Nimis, 2016). It is a new record for Pakistan.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

The authors declare no competing interests to disclose. This article does not contain any studies involving animals or human participants performed by any of the authors.

ADDITIONAL INFORMATION

Samples analysed during this study has been deposited in the LAH Herbarium, University of the Punjab, Lahore. (<https://vymaps.com/PK/LAH-Herbarium-Department-of-Botany-University-of-the-Punjab-Pakistan-334248940395482/>).

Sequences generated during this study has been deposited in NCBI, GenBank data repository (<https://www.ncbi.nlm.nih.gov/genbank/>).

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