



Original Scientific Paper

Aspicilia nigromaculata sp. nov. (Megasporaceae, Pertusariales, Ascomycota) from Azad Jammu and Kashmir, Pakistan, evidence from morphology and DNA sequencing data

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ABSTRACT:

A new species of Megasporaceae, *Aspicilia nigromaculata* sp. nov. is described and illustrated from Pakistan. A comparative morpho-anatomical study and ITS-LSU-based molecular analyses confirmed its position within the genus *Aspicilia*. It differs from the other species of the genus by the following set of features: a well-developed, grey to greenish grey frequently black spotted thick thallus, well-developed thicker peripheral and flat to slightly concave areoles, and small conidia $10\text{--}15 \times 0.5\text{--}1 \mu\text{m}$. Its positioning in a separate branch in the phylogenetic tree also makes it distinct from the other known species of the genus.

Keywords:

ITS nrDNA, nrLSU, phylogeny, saxicolous lichens

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INTRODUCTION

The currently accepted taxonomy of the lichenized family Megasporaceae (Pertusariales) includes eight genera i.e. *Aspicilia* A. Massal., *Circinaria* Link (SOHRABI *et al.* 2013a), *Lobothallia* (Clauzade & Cl. Roux) Hafellner (PAUKOV *et al.* 2019), *Megaspora* (Clauzade & Cl. Roux) Hafellner & V. Wirth, *Sagedia* Ach. (NORDIN *et al.* 2010), *Teuvoa* Sohrabi & S. Leavitt (SOHRABI *et al.* 2013b), *Aspiciliella* M. Choisy (ZAKERI *et al.* 2017) and *Oxneriaria* S.Y. Kondr. & Lőkös (HAJI MONIRI *et al.* 2017). The lichen-forming ascomycete genus *Aspicilia* A. Massal. has a worldwide distribution and covers a miscellaneous collection of 200–250 species (SOHRABI *et al.* 2013c). It presents a considerable range of morphological variations (SOHRABI *et al.* 2011). Within the genus a number of species have radiating thalli and elongate, often \pm diverging and \pm branched marginal areoles, closely attached to the substratum (NORDIN *et al.* 2011). *Aspicilia nigromaculata* is described from Pakistan, based on a detailed morphological and anatomical characterization. The ITS and nrLSU based phylogenetic analyses also support the identity of this taxon as a novel species.

MATERIAL AND METHODS

Collection and preservation. The survey of the Jhelum Valley and the Peer Chinasi area, in the Muzaffarabad district of Azad Jammu and Kashmir, Pakistan, undertaken in 2020, focused on increasing the biotic knowledge of the lichens of Pakistan. Azad Jammu and Kashmir, which lies in the north-eastern part of Pakistan, comprises an area of 13297 km², with mountains ranging from 360 to 6325 m in altitude, and is characterised by rocky and undulating terrain (DAR *et al.* 2012). The collected samples were deposited in the herbarium of the Institute of Botany, University of the Punjab, Lahore (LAH).

Morphological characterisation. The specimens were examined macro and micromorphologically under a stereomicroscope (Meiji Techno, EMZ-5TR, Japan) and compound microscope (SWIFT M4000-D), with a 9MP camera system, respectively. For anatomical investigation, sections of the apothecia were cut manually and mounted in water, KOH. A minimum of twenty measurements in water were made for each diagnostic feature.

Chemical characterisation. The secondary chemistry was analysed using spot tests which were performed using KOH (10%; K), and a sodium hypochlorite solution (C). Thin layer chromatography was carried out using Solvent System G, following standard methods (ORANGE *et al.* 2010).

DNA extraction, PCR amplification and sequencing. Genomic DNA was extracted directly from a portion of the thallus with apothecia from each specimen using a modified 2% CTAB method (GARDES & BRUNS 1993). The ITS-nrDNA region (Internal Transcribed Spacer of the nrDNA) was amplified using the primer pair ITS1F (forward primer) (GARDES & BRUNS 1993) and ITS4 (reverse primer) (WHITE *et al.* 1990). The nrLSU region was also amplified by using the LROR as the forward and LR5 as the reverse primer for the nrLSU region (VILGALYS & HESTER 1990), following the amplification protocol of KHAN *et al.* (2018). The PCR products were visualized on a 1% agarose gel with ethidium bromide (SAMBROOK & RUSSEL 2001). The PCR products were then sent to Tsingke, China for sequencing. The sequences were aligned using BioEdit (HALL 1999).

BLAST analysis was used to retrieve highly similar sequences of both the ITS and nrLSU regions. Maximum query coverage and percent identity between related taxa were noted. The sequences retrieved from Genbank and proposed in the literature were used for initial alignment, which was trimmed and then realigned using web-PRANK with default settings (LÖYTYNOJA & GOLDMAN 2010). On the CIPRES Portal (MILLER *et al.* 2010) the HYK+G+I model was selected using jModelTest (POSADA 2008). Maximum likelihood analysis was also conducted using RAXML-HPC2 v. 8.1.11 on the CIPRES web server (STAMATAKIS 2014). 1000 bootstraps were used for rapid bootstrapping. FigTree v 1.4.3 (RAMBAUT *et al.* 2014) was used to display the phylogeny from the ML analysis.

RESULTS

An ITS sequence was successfully obtained from two specimens of the new species, described as *Aspicilia nigromaculata*. The sequences for the holotype of the new species, and a second collection (JV-07) were identical. An additional eighteen sequences of selected species of

Table 1. Specimens used in the ITS phylogenetic analyses of the *Aspicilia* species. New sequences are in bold

| Name of species | Country/ Origin | Collection number | Genbank no. (ITS) | Reference |
|---------------------------------------|--------------------|------------------------------|----------------------|---|
| <i>Aspicilia abbasiana</i> | China | Ismayil et Abbas 20111154 | KM609324 | ISMAYIL <i>et al.</i> 2015; KONDRATYUK <i>et al.</i> 2016 |
| <i>Aspicilia blastidiata</i> | Russia | Paukov AGP20111009-01 | KX129963 | PAUKOV <i>et al.</i> 2015, 2017 |
| <i>Aspicilia blastidiata</i> | Russia | Paukov AGP20120801-01 | KX159286 | PAUKOV <i>et al.</i> 2015, 2017 |
| <i>Aspicilia cinerea</i> | Sweden | Hafellner 37308 | AF332111 | IVANOVA & HAFELLNER 2001 |
| <i>Aspicilia cinerea</i> | Austria | Kocourkova & Hafellner 46364 | AF332112 | IVANOVA & HAFELLNER 2001 |
| <i>Aspicilia cuprea</i> | USA | Owe-Larsson 9112 | EU057902 | NORDIN <i>et al.</i> 2007 |
| <i>Aspicilia dudinensis</i> | Sweden | Nordin 6036 | EU057906 | NORDIN <i>et al.</i> 2007 |
| <i>Aspicilia epiglypta</i> | Sweden | Nordin 6303 | EU057907 | NORDIN <i>et al.</i> 2007 |
| <i>Aspicilia epiglypta</i> | Sweden | Nordin 6305 | HQ259261 | NORDIN <i>et al.</i> 2011 |
| <i>Aspicilia fluviatilis</i> | Sweden | Nordin 6188 | HQ259264 | NORDIN <i>et al.</i> 2011 |
| <i>Aspicilia goettweigensis</i> | Austria | Vondrák 14026 | KX159289 | PAUKOV <i>et al.</i> 2017 |
| <i>Aspicilia goettweigensis</i> | Russia | Paukov AGP20120513-03 | KX159292 | PAUKOV <i>et al.</i> 2017 |
| <i>Aspicilia granulosa</i> | Sweden | Nordin 6174 | HQ259265 | NORDIN <i>et al.</i> 2011 |
| <i>Aspicilia nigromaculata</i> | Pakistan | LAH36940 | MW969628 | This paper |
| <i>Aspicilia nigromaculata</i> | Pakistan | LAH36941 | MW969629 | This paper |
| <i>Aspicilia subdepressa</i> | France | Roux 24653 | JF703123 | ROUX <i>et al.</i> 2011 |
| <i>Aspicilia subepiglypta</i> | Korea | 100857 KoLRI | KY249607 | KONDRATYUK <i>et al.</i> 2016 |
| <i>Aspicilia subepiglypta</i> | Korea | 110495 KoLRI | KY249608 | KONDRATYUK <i>et al.</i> 2016 |
| <i>Aspicilia subradicans</i> | Sweden | Nordin 5984 | HQ259267 | NORDIN <i>et al.</i> 2011 |
| <i>Aspicilia subradicans</i> | Finland | Nordin 6370 | HQ259268 | NORDIN <i>et al.</i> 2011 |
| <i>Circinaria esculenta</i> | Kazakhstan | Lvanov s. n. (UFU L-1743) | MK347507 | PAUKOV <i>et al.</i> 2019 |
| <i>Circinaria fruticulosa</i> | Russia | Paukov 3074 (UFU L-3256) | MK347508 | PAUKOV <i>et al.</i> 2019 |
| <i>Ochrolechia parella</i> | Antarctica | Park PCH080112-32 | KJ607905 | PARK <i>et al.</i> 2015 |
| <i>Oxneriaria dendroplaca</i> | Finland | Nordin 6366 | HQ259260 | NORDIN <i>et al.</i> 2011 |
| <i>Oxneriaria mashiginensis</i> | Sweden | Nordin 5790 | EU057912 | NORDIN <i>et al.</i> 2007 |
| <i>Oxneriaria rivulicola</i> | Sweden | Nordin 5957 | EU057922 | NORDIN <i>et al.</i> 2007 |
| <i>Oxneriaria verruculosa</i> | Norway | Owe-Larsson 9007 | EU057940 | NORDIN <i>et al.</i> 2007 |
| <i>Oxneriaria verruculosa</i> | Sweden | Nordin 5942 | EU057942 | NORDIN <i>et al.</i> 2007 |

Table 2. Specimens used in the nrLSU phylogenetic analyses of the *Aspicilia* species. New sequences are in bold

| Name of species | Country/Origin | Collection number | Genbank no. (ITS) | Reference |
|---------------------------------------|---------------------------|------------------------|-------------------|---------------------------|
| <i>Aspicilia cinerea</i> | Sweden, Östergötland | Nordin 5542 (UPS) | HM060734 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia cinerea</i> | Sweden, Dalarna | Hermansson 13275 (UPS) | HM060733 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia dudinensis</i> | Sweden, Torne Lappmark | Nordin 6036 (UPS) | HM060748 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia dudinensis</i> | Sweden, Torne Lappmark | Nordin 5971 (UPS) | HM060757 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia epiglypta</i> | Sweden, Västergötland | Nordin 6303 (UPS) | HM060756 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia epiglypta</i> | Sweden | Nordin 7037 (UPS) | MH255589 | ZAKERI 2018 |
| <i>Aspicilia laevata</i> | Sweden, Uppland | Tibell 23659 (UPS) | HM060730 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia laevata</i> | Finland, Alandia | Nordin 5846 (UPS) | HM060735 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia nigromaculata</i> | Pakistan | LAH36941 | MW969630 | This paper |
| <i>Aspicilia nikrapensis</i> | Svalbard | Ebbestad SVL2:1 (UPS) | HM060759 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia nikrapensis</i> | Svalbard | Ebbestad SVL2:1 (UPS) | HM060759 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia supertegens</i> | Sweden, Torne Lappmark | Nordin 6023 (UPS) | HM060751 | NORDIN <i>et al.</i> 2010 |
| <i>Aspicilia supertegens</i> | Norway, Troms | Owe-Larsson 9002 (UPS) | HM060742 | NORDIN <i>et al.</i> 2010 |
| <i>Circinaria caesiocinerea</i> | Sweden, Uppland | Tibell 22612 (UPS) | HM060731 | NORDIN <i>et al.</i> 2010 |
| <i>Circinaria emiliae</i> | Kazakhstan, Atyrau | Kulakov 3702 (UPS) | HM060728 | NORDIN <i>et al.</i> 2010 |
| <i>Circinaria emiliae</i> | Kazakhstan, Atyrau | Kulakov 3798 (UPS) | HM060729 | NORDIN <i>et al.</i> 2010 |
| <i>Circinaria gibbosa</i> | Sweden, Uppland | Nordin 5878 (UPS) | HM060740 | NORDIN <i>et al.</i> 2010 |
| <i>Circinaria hispida</i> | Turkey, Malatya | Candan 11 (ANES) | HM060760 | NORDIN <i>et al.</i> 2010 |
| <i>Circinaria leproscens</i> | Sweden, Uppland | Nordin 5906 (UPS) | HM060749 | NORDIN <i>et al.</i> 2010 |
| <i>Oxneriaria dendroplaca</i> | Sweden, Torne Lappmark | Nordin 5952 (UPS) | HM060744 | NORDIN <i>et al.</i> 2010 |
| <i>Oxneriaria dendroplaca</i> | Finland, Enontekiön Lappi | Nordin 6366 (UPS) | HM060758 | NORDIN <i>et al.</i> 2010 |
| <i>Oxneriaria haeyrenii</i> | Sweden, Torne Lappmark | Nordin 5997 (UPS) | HM060755 | NORDIN <i>et al.</i> 2010 |
| <i>Oxneriaria mashiginensis</i> | Norway, Troms | Nordin 5708 (UPS) | HM060732 | NORDIN <i>et al.</i> 2010 |
| <i>Oxneriaria permutata</i> | Sweden, Torne Lappmark | Nordin 6027 (UPS) | HM060747 | NORDIN <i>et al.</i> 2010 |
| <i>Oxneriaria verruculosa</i> | Norway, Troms | Owe-Larsson 9007 (UPS) | HM060741 | NORDIN <i>et al.</i> 2010 |

Aspicilia, five sequences of *Oxneriaria* S.Y. Kondr. & Lököš and two sequences of *Circinaria* Link were available from the literature (see Table 1 for voucher details). The aligned ITS1-5.8S-ITS2 region comprised 574 sites, of which 359 were conserved and 195 variable; 134 sites were parsimony informative. The tree resulting from the analyses of the ITS region is shown in Fig. 1. *Aspicilia nigromaculata* forms a well-supported clade with *A. subradians* (Nyl.) Hue (HQ259268, HQ259267), *A. fluvialis* A. Nordin & Owe-Larss. (HQ259264), *A. granulosa* A. Nordin (HQ259265) and *A. epiglypta* (Norrl. ex Nyl.) Hue (HQ259261, EU057907). *Ochrolechia parella* (L.) A. Massal. (KJ607905) was selected for rooting the tree (PAUKOV & DAVYDOV 2020).

LSU sequences were newly prepared for one specimen (see Table 2 for voucher details). The tree resulting from the analyses of the LSU region is shown in Fig. 2. The Pakistan sequence is recovered as sister to *Aspicilia epiglypta* (GenBank no. MH255589, HM060756) (NORDIN *et al.* 2011).

Taxonomic treatment

Aspicilia nigromaculata Fayyaz, Afshan, Niazi & Khalid sp. nov. (Figs. 1-3)

Diagnosis. A well-developed, grey to greenish grey frequently black spotted thick thallus, well-developed thicker peripheral and flat to slightly concave areoles, and small conidia 10–15 × 0.5–1 µm; differing from related species in the ITS and nrLSU region.

Mycobank No: MB839363

Holotype. PAKISTAN: Azad Jammu and Kashmir, Jhelum Valley (N 33°23', E 73°32'), 2500 m alt., on rock, 24 September 2020, Najam-ul-Sehar Afshan and Abdul Rehman Niazi (JV-07) (LAH36940-holotype), ITS GenBank accession number MW969628 LSU, Genbank accession number MW969630.

Thallus: crustose, areolate to rimose areolate, immarginate, frequently black spotted, thick, 8 to 10 cm wide,

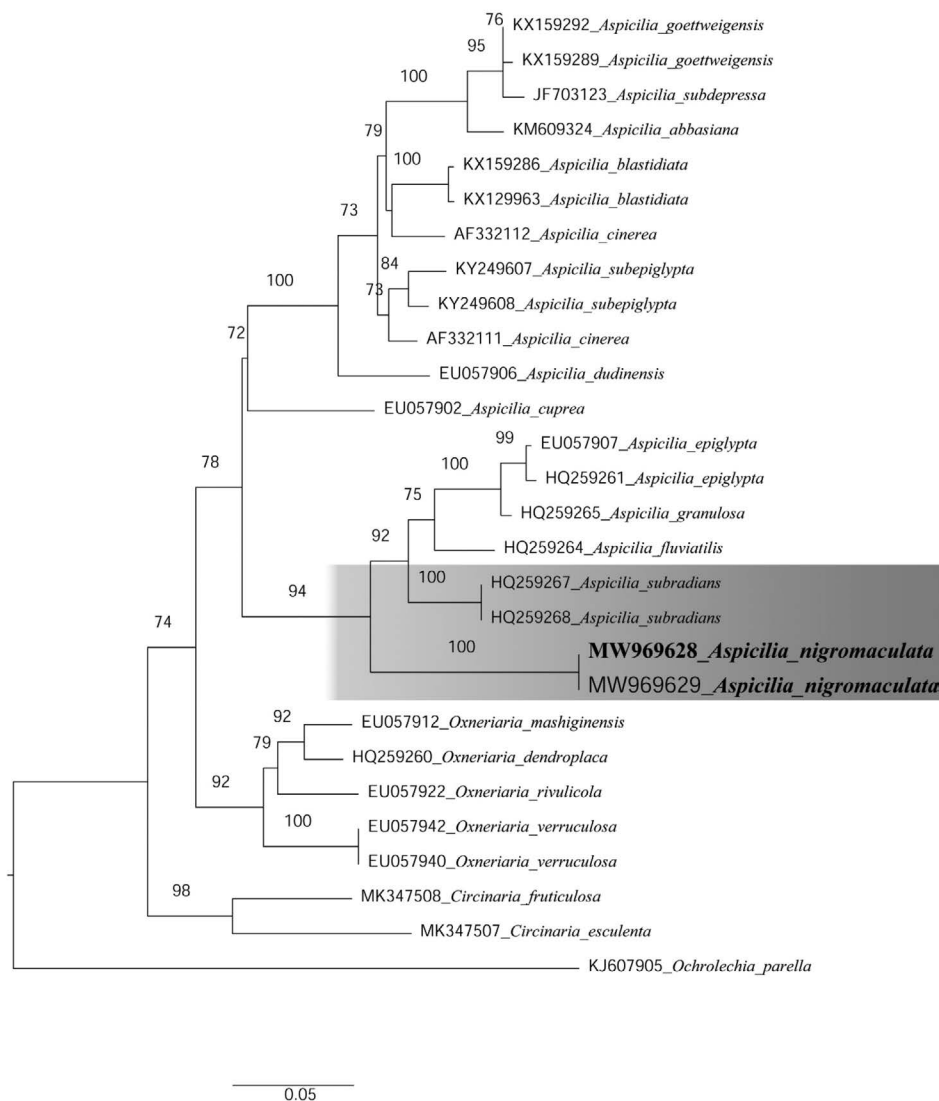


Fig. 1. Most likely phylogenetic relationship of *Aspicilia nigromaculata* with their associated taxa inferred from the nrITS region on rooting with *Ochrolechia parella* (L.) A. Massal. (KJ607905) as an outgroup.

epruinose. **Areoles:** 0.5 to 2 mm wide and 1 mm thick, thinner centrally but thicker towards the periphery, broadly attached to the stipitate, separated by distinct cracks, discrete to rarely contiguous, smooth to thinly wrinkled, angular to irregular, replicating by divisions, flat to slightly concave. **Upper surface:** grey to greenish grey. **Lower surface:** brown to black. **Prothallus:** not seen. **Upper cortex:** 20–30 μm thick, smooth, upper layer dark brown, hyaline adjacent to algal layer, paraplectenchymatous, textura globularis, cells 8–12 μm in diam. **Algal layer:** continuous, 40 to 60 μm thick. **Photobiont cells:** globose to sub-globose, 13–24 μm . **Medulla:** 60–110 μm thick, dark brown, hyphae prosoplectenchymatous 1–3 μm wide. **Pycnidia:** immersed, rare, indistinct, often black at the ostiole, 0.1–0.4 mm. **Conidiophores:** 13–15 \times 2–2.5 μm . **Conidia:** filiform, acrogenous, straight, hyaline, 10–15 \times 0.5–1 μm .

Chemistry– Cortex; K+ (yellowish–green), C–, KC–. Medulla; K–, C–, KC–, no substance detected by TLC.

Habitat and distribution. The known collections of the new species are from the Himalayan moist temperate forest of Azad Jammu and Kashmir, Pakistan. The specimens were found on siliceous rocks. The coniferous forest is dominated by species of *Pinus roxburghii* Sarg., *Quercus oblongata* D. Don, *Quercus glauca* Thumb and *Pyrus pashia* L. etc. The maximum daily temperature of the region varies from 20 to 30°C during the summer with an average temperature of 4°C during the winter, and there is moderate rainfall.

Etymology. The species epithet “*nigromaculata*” refers to the black-spotted thallus of the type.

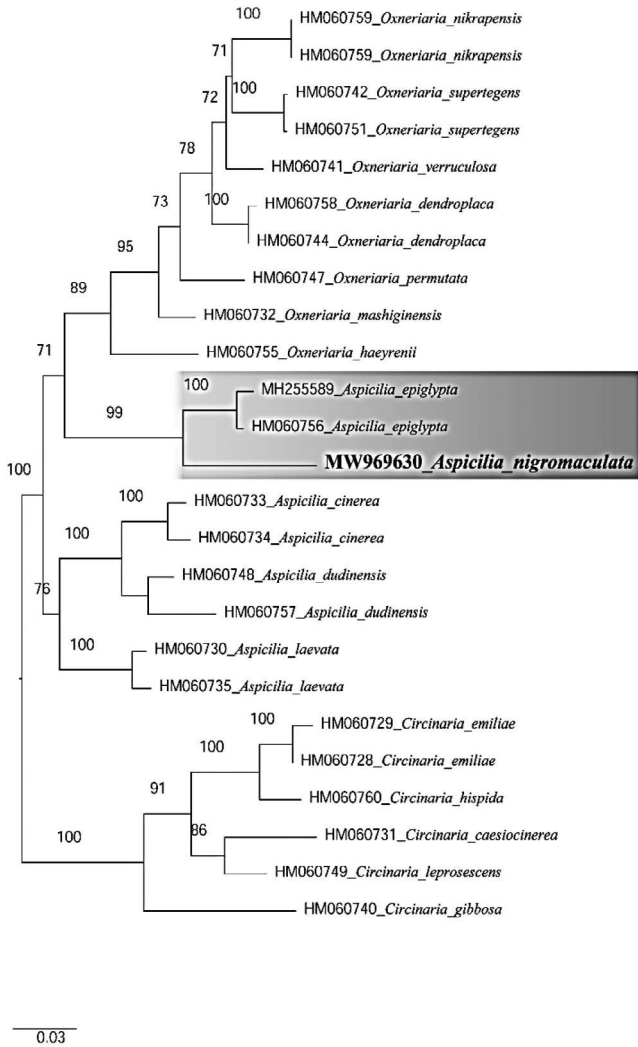


Fig. 2. Most likely phylogenetic relationship of *Aspicilia nigromaculata* with their associated taxa inferred from the nrLSU region.

Additional specimens examined. PAKISTAN. Azad Jammu and Kashmir, Muzaffarbad, Peer Chanasi (N 34°23', E 73°32', 2000 to 2924 m alt.), on rock, 26 September 2020, Najam-ul-Sehar Afshan and Abdul Rehman Niazi, (JV-07H) (LAH36941) (ITS GenBank accession number MW969629).

DISCUSSION

Two collections of this taxon were made from Peer Chanasi and Muzaffarabad (Azad Jammu & Kashmir). The two sites vary significantly in terms of elevation, vegetation and climatic conditions. However, the phylogenetic analyses confirmed that the two collections of *Aspicilia nigromaculata* form a monophyletic clade with good support (Figs. 1 & 2).

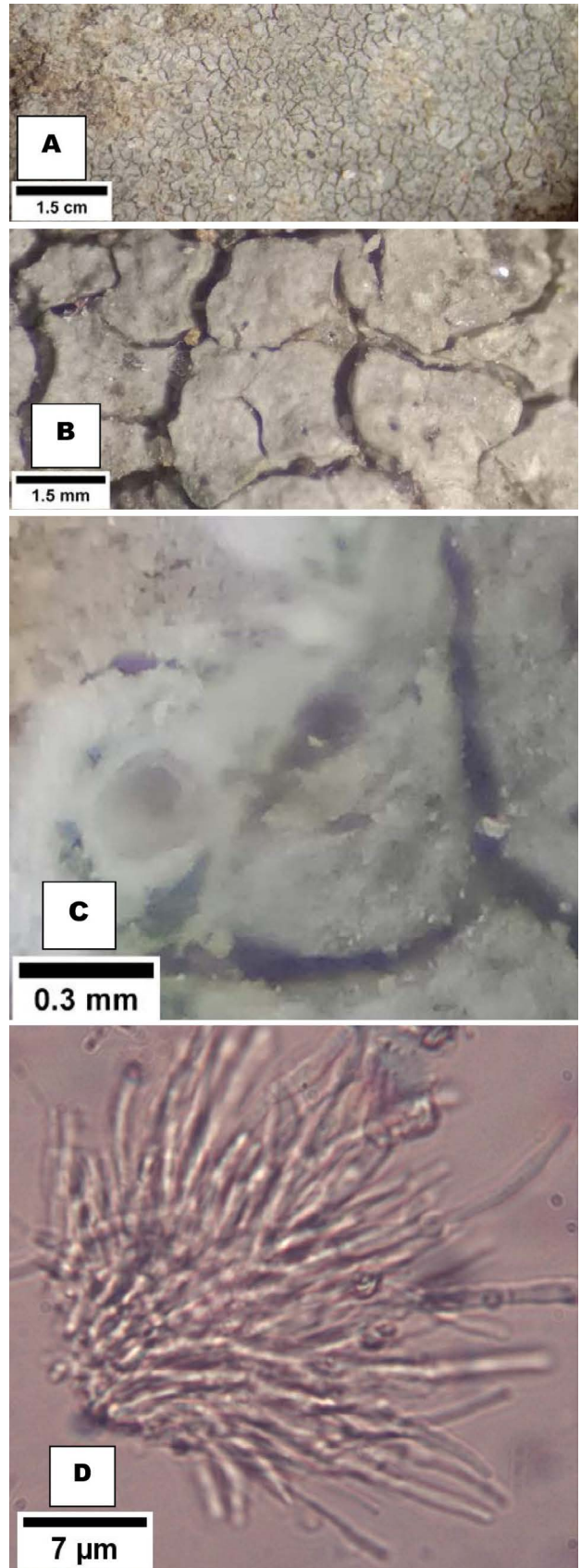


Fig. 3. *Aspicilia nigromaculata* Fayyaz, Afshan, Niazi & Khalid sp. nov. A. Thallus of the specimen, B. Areoles of the specimen, C. Pycnidia, D. Conidiomata

The areoles of *Aspicilia nigromaculata* are flat to slightly concave peripheral areoles and no apothecia were found in the present collections. The other species of *Aspicilia* have mostly convex areoles and apothecia are common. It shows certain morpho-anatomical differences from *A. subradians* i.e. in having flat to slightly convex well developed peripheral areoles, smaller conidia which are 10–15 µm and the prothallus is absent in our specimens (vs. marginal areoles poorly developed, central areoles sometimes convex and a distinct prothallus) (NORDIN *et al.* 2011). The new species differs from *Aspicilia fluviatilis* in having a thick, frequently black spotted, greenish grey thallus, a 20–34 µm thick cortex (vs. thin, often minutely white spotted, grey to ochraceous gray thallus, a 22–43 µm thick cortex). Similarly, the Pakistani collection also differs from *A. granulosa* in having a thick, greenish grey thallus (vs. a thin, green grey, brown grey to brownish thallus, and in lacking vegetative propagules (NORDIN *et al.* 2011). In the phylogenetic analyses the Pakistani collection also forms a separate subclade from the other *Aspicilia* species, suggesting it is not conspecific with any of the above-mentioned species.

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***Aspicilia nigromaculata* sp. nov. (Megasperaceae, Pertusariales, Ascomycota) iz Azad Jammua i Kashmira, Pakistan, dokazi iz morfologije i podataka o sekvencama DNK**

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Nova vrsta u okviru Megasperaceae, *Aspicilia nigromaculata* sp. nov. je opisana iz Pakistana. Usporedne morfo-anatomske i molekularne analize zasnovane na ITS–LSU potvrđuju njegovu poziciju u okviru roda *Aspicilia*. Od drugih vrsta roda razlikuje se po sledećem skupu karakteristika: dobro razvijenim sivim do zelenkasto sivim, često crno pegavim debelim talusom, dobro razvijenim debljim perifernim i ravnim do blago konkavnim areolima i malim konidijama veličine 10–15 × 0.5–1 μm. Različitim od drugih poznatih vrsta roda ga čini i položaj u izdvojenoj grani na filogenetskom stablu.

Ključne reči: ITS nrDNA, nrLSU, filogenija, saksikolozni lišajevi