

Phlegmacium kausarii (Cortinariaceae), a new species of Phlegmacium, subgenus Phlegmacium, sect. Phlegmacioida, from coniferous forests of Kashmir Himalaya, India

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Abstract

Phlegmacium kausarii is a new species in the genus *Phlegmacium* reported from the Kashmir Himalaya, India, based on distinct morphological characters and molecular phylogenetic analysis of nrITS sequences. The species is characterized by pileus that is glutinous, glabrous with brownish universal veil remnants scattered over the surface, with incurved and entire thick margin. Lamellae are pale yellow, moderately crowded, adnate with crenulate margins. Stipe clavate (pear-shaped), greyish and tapering towards the end. Basidiospores $8.7\text{--}10.3 \times 5.5\text{--}6.4 \mu\text{m}$, amygdaliform. The odor is strong, pleasant like a banana. Molecularly, the ITS sequences differ from other species by at least 11 substitutions and 9 indel positions.

Introduction

Earlier, *Cortinarius* was considered as the largest genus in the order Agaricales, with reportedly 2250 species distributed throughout the world (Zhao et al. 2017, He et al. 2019). The *Cortinarius* genus has been thoroughly studied in Europe and North America, (Frøslev et al. 2005, Garnica et al. 2005, Frøslev et al. 2007, Teasdale et al. 2013, Liimatainen et al. 2014, 2020, 2022; Garnica et al. 2016, Soop et al. 2019), while only a few species have been reported from other regions, particularly Asia (Peintner et al. 2004). Despite recent advances in molecular techniques, the diversity of this genus from the South Asian region, particularly from the Indian subcontinent remains poorly understood. Considerable phenotypic variability and convergence in morphological traits are displayed by the species of the genus *Cortinarius* (Liimatainen et al. 2014). This poses a significant difficulty in the correct delimitation of different species based on the conventional morphological approach alone (Garnica et al. 2016, Badotti et al. 2017).

Recent studies have used several DNA barcodes that include ITS, LSU, Tef1- α , RPB1, and RPB2 for molecular phylogenetics and delimitation of various fungal taxa up to species level. Several studies have found that ITS barcode to be significantly effective in distinguishing *Cortinarius* species (Garnica et al. 2016, Brandrud et al. 2018). The detailed studies of type specimens based on ITS sequence data in *Cortinarius* include subgenus *Phlegmacium* (Fr.) Trog (Frøslev et al. 2007, Liimatainen et al. 2014) and *Telamonia* (Fr.) Trog (Liimatainen et al. 2020). Similarly, several relatively large phylogenetic studies on the genus *Cortinarius* were published that delimited the species into several distinct sections and clades (Garnica et al. 2005, 2016, Harrower et al. 2011, Peintner et al. 2004, Soop et al. 2019, Stensrud et al. 2014, Liimatainen et al. 2017).

Recently, the genus *Cortinarius* (Pers.) Gray was revised based on molecular phylogenomic study and was segregated into 10 genera (each having several subgenera and sections) (Liimatainen et al. 2022). Based

on the revised phylogenetic analysis, the subgenera *Cortinarius*, *Phlegmacium* and *Thaxterogaster* were reintroduced as genera while seven new genera were described as new to science (Liimatainen et al. 2022).

Phlegmacium (Fr.) Wünsche, em. Niskanen & Liimat. (2022) includes taxa with relatively fleshy agaricoid basidiomes with vivid colors, a viscid to glutinous pileus surface and a dry stipe. The development type is stipitocarpic or pileocarpic. Surface dry, viscid or glutinous with varying colors. The surface in some species is innately fibrillose or radially wrinkled, rarely scaly or with patches of veil. Lamellae in the vast majority of species are crowded, emarginate. Stipe is wide at the apex, clavate to bulbous with a rounded or a marginate bulb, or cylindrical to rooting, dry. Universal veil sparse to abundant, forming incomplete and complete girdles on the stipe. Basidiospores amygdaloid, ellipsoid or citriform, in some species subglobose, finely to strongly verrucose.

Subgenus *Phlegmacium* includes species having medium- to large-sized basidiomata that are predominantly stipitocarpic, agaricoid (phlegmacioid). The stipe is dry, and the pileus is viscid to glutinous, or if dry then the KOH reaction in the context of the pileus is usually yellow. The pileipellis is either duplex or simplex.

Section *Phlegmacioida* (Fr.) Niskanen & Liimat. (2022), from subgenus *Phlegmacium*, has also been revised recently and includes species having viscid to glutinous pileus, not fibrillose, that is yellow to brownish yellow. Lamellae are emarginate, almost crowded, greyish white, and later very pale greyish brown. The stipe is white, thick at apex, wide at base, and almost cylindrical with clavate to sub-bulbous base. Universal veil yellow, forming girdles on the stipe. Spores narrowly amygdaloid, with rounded apex, moderate to fairly strongly verrucose, moderately dextrinoid, often with dark red-brown angular granules in the spore. Lamellar trama hyphae with moderate to very large sand brown to red-brown granules. Pileipellis: epicutis strongly gelatinous, uppermost hyphae ochraceous yellow to ochraceous brown, mostly not granulose.

Cortinarius species were also reported in some earlier studies from Kashmir based on morphological observations (Abraham 1993). However, only a few reports of *Cortinarius* species associated with coniferous forests of Kashmir Himalaya are based on molecular phylogeny (Ito et al. 2014, 2015). Recently, two new species *Cortinarius himalayensis* and *Thaxterogaster carneus* were reported from Kashmir Himalaya, India (Ahmed et al. 2023a, b).

In the present study, an integrated morphological and molecular phylogenetic approach was employed for correct taxonomic identification of the specimens belonging to the genus *Phlegmacium*.

Materials and methods

Study area

The study area included the coniferous forests of Kashmir Himalaya (34°20'33"N and 74°05'10"E) that extend along the elevational gradient of 1600–3800 meters asl. The sporocarps were collected from the study sites dominated mainly by *Cedrus deodara* (Roxb. ex D. Don) G. Don (1830:388), *Pinus wallichiana* A.B. Jacks. (1938:85), *Abies pindrow* (Royle ex D. Don) Royle (1836:86) and *Picea smithiana* (Wall.) Boiss. (1844:700) along with *Betula utilis* D. Don (1825:58) – the deciduous treeline species in the region. The samples were labelled and the specimens were stored under deep freeze conditions until molecular investigations. The dried voucher specimens have been deposited in Kashmir University Botanical Garden Herbarium (KASH).

Morphological observation

The sporocarps were photographed in the field using a digital camera and later dug out carefully using a knife and collected in separate paper bags. Fresh sporocarps were used for describing macroscopic characters, such as color, size, shape and detailed anatomical features of various parts. The color of the specimens was ascribed based on color codes from the Methuen Handbook of Color (Kornerup and Wanscher 1978). The microscopic characters were examined from thin sections of fresh and dried tissue mounted in water, 3% KOH and Melzer's reagent using a light microscope (AmScope MLX) with 100× resolution and fitted with an AmScope camera (Garnica et al. 2003; Niskanen et al. 2012). The basidiospore measurements and morphology were based on several mature spores obtained from lamella that were observed under a light

microscope and photos were taken using 100× resolution. The pileipellis structure was studied from sections cut halfway through the pileus. In the description, ‘Q’ refers to the length/width ratio of basidiospores.

DNA extraction, PCR and Sequence amplification

DNA was extracted from fresh sporocarp specimens using the standard cetyltrimethylammonium bromide (CTAB)-chloroform method with few modifications (Rogers & Bendich 1994; Porebski *et al.* 1997). The CTAB mix along with sample lysate was transferred to the tubes of the QIAGEN Plant mini DNA purification kit and the steps for purification of DNA were followed as mentioned in the manufacturer’s protocol. The isolated DNA was used as a template and complete sequences of ITS1, 5.8S rRNA gene and ITS2 with partial sequences of 18S were amplified by polymerase chain reaction (PCR) in Applied Biosystems 2720 Thermal Cycler using two previously reported and widely used primers ITS1/ITS4, ITS1F/ITS4B (Gardes and Bruns, 1993). The purified PCR products of the nrITS amplified region were directly sequenced in both directions using the ITS1F/ITS4B pair of amplification primers in Sci-Genome and Biobench solutions labs). Chromatograms were analyzed using Chromas version 2.33 (Technelysium Pty Ltd).

Data analysis

A BLAST (Basic Local Alignment Search Tool) search was carried out for the sequences using the National Center for Biotechnology Information (NCBI), USA (<https://www.ncbi.nlm.nih.gov/>) and UNITE fungal database (<https://unite.ut.ee/>). The final data set comprised 28 ITS sequences available from section *Phlegmacioida*, including 3 newly generated sequences of nrITS region. The closely matched sequences of neighbouring sections retrieved from the NCBI database were used as outgroups in the phylogenetic analysis (TABLE 1) (Xie *et al.* 2019).

The initial sequence alignment was performed for all the sequences using the MAFFT program (<https://mafft.cbrc.jp/alignment/server/>) and the alignment was further refined with BioEdit 7.0.9 (Hall 1999, 2004). Maximum likelihood (ML) analysis was performed using phyML and Branch support was assessed with Bootstrap analysis run with 1000 replicates (Felsenstein 1985). The best-fit model for the dataset was chosen as GTR+I using the jModel Test program. The newly generated sequences were deposited in the NCBI database with accession numbers listed in TABLE 1.

Table 1. ITS sequences of *Phlegmacium* species used in the phylogenetic analysis. The new species is shown in bold.

Sequence name	Voucher	Accession No.	Geog
Sec. <i>Phlegmacioida</i> (<i>Percomes</i>)	Sec. <i>Phlegmacioida</i> (<i>Percomes</i>)	Sec. <i>Phlegmacioida</i> (<i>Percomes</i>)	Sec. <i>Phlegmacioida</i> (<i>Percomes</i>)
<i>P. acystidiosus</i>	CLO4681	KF732419	USA.
<i>P. albofragrans</i>	IB19950595	NR130187	USA.
<i>P. aurantiopallidus</i>	AB05-11-404	NR132061	France
<i>P. castaneicolor</i>	MICH.10331	NR130203	USA.
<i>P. cephalixoides</i>	IB19870188	NR130204	USA.
<i>P. cf. rhizophorus</i>	IK98-2451	KF732570	USA.
<i>P. citrinifolius</i>	MICH.10334	NR130206	USA.
<i>P. coelopus</i>	MEL2350382	NR173459	USA
<i>P. delaporteii</i>	RH8673	NR130215	France
<i>P. glaucopus</i>	SF44760	KF732315	Sweden
<i>P. luteiaureus</i>	H6033617	NR130302	Finland
<i>P. luteobrunnescens</i>	MICH.10371	NR130227	USA.
<i>P. neotriumphans</i>	G2970631	NR157947	France
<i>P. norrandicus</i>	CFP526	DQ117928	Sweden
<i>P. ochraceobrunneus</i>	G00126800	NR130235	France
<i>P. pallidifolius</i>	MICH.10392	NR130240	USA.
<i>P. pansa</i>	IK901826	KF732522	Finland

Sequence name	Voucher	Accession No.	Geog
<i>P. papulosus</i>	TN06319	KF732629	Finlan
<i>P. papulosus</i>	IK90.1822	KF732630	Finlan
<i>P. percomis</i>	TN08041	KF732380	Finlan
<i>P. pseudocephalixus</i>	IK981842	KF732634	Swede
<i>P. russus</i>	SF44757	NR130259	Swede
<i>P. serarius</i>	SF44754	NR130263	Swede
<i>P. superbus</i>	MICH.10430	NR130274	USA.
<i>P. vixolivascens</i>	RH89.123	NR130286	France
<i>P. kausarii</i> sp. nov.	K.HIM.1.1	MZ203578	Kashm
<i>P. kausarii</i> sp. nov.	K.HIM.1.2	MZ203579	Kashm
<i>P. kausarii</i> sp. nov.	K.HIM.1.3	MZ203580	Kashm

Results

Phylogenetic analysis

The phylogenetic tree illustrating the phylogenetic position of new species in the section *Phlegmacioida* (*Percomes*) is shown in FIG. 1A–C. The ML tree generated from the ITS sequences of *Phlegmacium* placed the new sequences into a distinct clade along with closely related sequences from members of section *Phlegmacioida* obtained from GenBank, with high statistical support values. Sequences of *Phlegmacium kausarii* clustered on a separate branch sister to *P. percomis* along with other sequences from the section *Phlegmacioida* complex having a statistical support of 94%. The ITS type sequence MZ203580 belonging to *Phlegmacium kausarii* was 684 base pairs long and showed a similarity of 96% with the ITS sequence KJ421068 (*P. olidus*) and 96% with the ITS sequence AY174784 (*P. cephalixus*) that were the closest matched sequences in GenBank having a query cover of 100%.

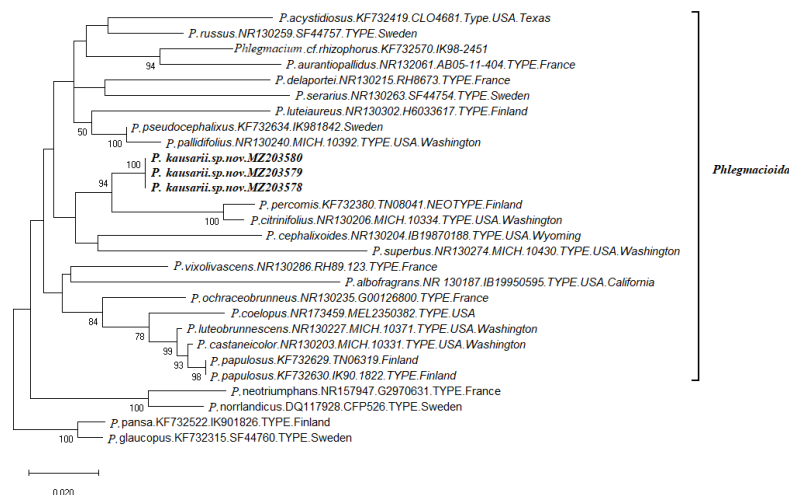


FIG.1A–C. Phylogenetic trees constructed from the ITS sequences illustrating the position of the new species in section *Phlegmacioida*.

TAXONOMY

MycoBank no.

Phlegmacium kausarii S.S. Ahmed and Z.A. Reshi, *sp. nov.* (Figs. 2A–B, 3A).

Diagnosis :— Pileus glutinous, glabrous with brownish universal veil remnants scattered over the surface, with incurved and entire thick margin. Lamellae are moderately crowded, and pale yellow. Stipe clavate, greyish, tapering towards the end. Basidiospores $8.7\text{--}10.3 \times 5.5\text{--}6.4 \mu\text{m}$, amygdaliform. The odor is strong, and pleasant like a banana. The ITS sequences differ from other species by at least 11 substitutions and 9 indel positions.

Holotype. India. Union Territory of Jammu and Kashmir, District Kulgam, Kausar-Nag conifer forests, $33^{\circ}31'29''\text{N}$, $74^{\circ}45'16''\text{E}$, Altitude 2785m, 15 July 2017, coll. S.S. Ahmed, (Holotype KASH K.HIM.1.1); ITS barcode Genbank acc. No. MZ203580; District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, coniferous forest (dominated by *Abies pindrow*), $34^{\circ}33'35''\text{N}$, $74^{\circ}11'10''\text{E}$, Altitude 2635m, 5 July 2017, coll. S.S. Ahmed, (Paratype KASH K.HIM.1.2); ITS barcode Genbank acc. No. MZ203578.

Etymology. The name refers to the famous lake site Kausar-Nag from which the holotype of this species was collected.

Description. Pileus 3.6–5.4 cm in diam., hemispherical to convex when young, becoming broadly convex at maturity, ochraceous yellow (4B7–8), reddish brown (9E6–8) at the centre, pale yellow (4A4) towards margins, glutinous, glabrous, with very small scales in the centre of the pileus, margins smooth and incurved. Universal veil remnants usually abundant, forming brown, loose scales or patches on the upper surface of pileus. Lamellae 6–9 mm broad, adnate, moderately crowded, pale yellow to greyish white (4A2) when young, turning brown (5F8) at maturity, crenulate margins. Stipe 3.5–5.4 long, 0.7–0.9 cm thick at apex, 1.5–2.5 cm thick near base, base cylindrical to clavate, thick girdled to fibrillose of yellow-brown (6C–4) to more rarely whitish (4A3) veil, color greyish white (2A2), ochraceous white (2A1–2) towards the base, often completely brownish in lower part from veil remnants; Universal veil prominent, ochraceous brown (9F6) to rather dark brown (8F6–8), rendering the lower part of stipe distinctly girdled-floccose, sometimes more fibrillose-peronate. Context white, more greyish in stipe apex when young. Odor strong fruit like, and taste distinct mushroom like or yeast-like. Reaction to 3% KOH– negative to light brown to dark brown at pileus, stipe and base. Exsiccata brown (6E5) to dark brown (6F6) in color.



FIG. 2A, 2B (Sporocarp of *P. kausarii*).

Basidiospores $7.9\text{--}10.3 \times 5.5\text{--}6.4 \mu\text{m}$, $Q = 1.31\text{--}1.67$, $'X = 9.2\text{--}9.8 \times 5.8\text{--}6.2 \mu\text{m}$, $'Q = 1.37\text{--}1.48$ (30 spores, 3 collections), Amygdaliform, distinctly and usually fairly densely verrucose, moderately dextrinoid. Basidia 4-spored, cylindrical to clavate, $24\text{--}46 \times 5\text{--}9 \mu\text{m}$, moderately thin-walled, yellowish-brown to olivaceous brown in 5% KOH. Lamellar edge sterile, sterile cells cylindrical-clavate, $9\text{--}22 \times 3\text{--}8 \mu\text{m}$, thin-walled and slightly hyaline in 5% KOH. Lamellar trama hyphae regular, smooth, olivaceous in 5% KOH. Universal veil having thin-walled hyphae, often with well-developed, yellow-brown, encrusted, parietal and intracellular pigment, hyaline to olivaceous yellow in 5% KOH. Gelatinous layer composed of various hyphal strata; Pileipellis duplex, with a distinctly subcellular hypoderm, elements often irregular, almost isodiametric, and embedded in a brown, amber-like matrix, basal epicutis often with distinct, brown, encrusted pigment.



FIG. 3A (Spore picture of *P. kausarii*).

Ecology and distribution. Cosmopolitan in distribution across Kashmir Himalaya, found across the elevational gradient; Mycorrhizal with conifers (*Pinus*, *Abies*); mostly growing scattered and rarely sighted during the summer season, growing in the mid altitudes of Kashmir Himalaya.

Additional specimens examined (paratypes). India. Union Territory of Jammu and Kashmir, District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, coniferous forest (dominated by *Abies pindrow*), 34°34' 39" N, 74°13' 12" E, Altitude 2622m, 5 July 2017, S.S. Ahmed, (KASH K.HIM. 1.2); Genebank acc. No. MZ203578; 33°28' 22" N, 74°18' 16" E, Altitude 2645m, 6 July 2017, S.S. Ahmed (KASH K.HIM. 1.3); Genebank acc. No. MZ203579; District Kulgam, Kausar-Nag conifer forests, 33°31'29"N, 74°45'16"E, Altitude 2785m, 15 July 2017, S.S. Ahmed (KASH K.HIM. 1.4); Genebank acc. No. MZ203580.

Comments. In the phylogenetic analysis based on the ITS sequences, *Phlegmacium kausarii* clusters on a branch that is sister to *P. percomis* (BS=94) in the section *Phlegmacioida*. The ITS sequence shows a similarity of less than 96% to closely related sequences and differs from other sequences from members of the section *Phlegmacioida* by at least 11 substitutions and 9 indel positions. The interspecific difference between the species was found to be greater than 4% in the ITS region.

Morphologically, *Phlegmacium kausarii* is characterized by the thick glabrous pileus with scattered universal veil remnants covering the surface and incurved margins. Lamellae are thick, moderately crowded, staining deeply in 3% KOH. A short pear-shaped stipe that is cylindrical to clavate and tapering towards the basal end, having universal veil prominent in the lower part, surrounded by distinctly girdled-floccose veil remnants, sometimes more fibrillose-peronate. Basidiospores are slightly large in size and amygdaliform, densely verrucose.

In comparison, *P. kausarii* shares a close morphological affinity with other species in the section *Phlegmacioida*. *Phlegmacium viscidoamarus* and *P. stjernegaardii* of the same section share some morphological similarities with *P. kausarii*. Like in the case of *P. viscidoamarus* , pileus is (hemi) spherical, convex to plano-convex with entire margins, whitish towards the margin, pale yellow (3A3) or brownish orange (6C8) in the disc, cuticle easily peeled, glossy and distinctly viscid, soon almost dried. Glutinous universal veil remnants present, but not abundant. Lamellae are moderately crowded, adnate or emarginate with a decurrent

tooth, whitish, and edges entire or crenulated. Stipe is variable in size, cylindric, clavate to robust-swollen, rigid, clavate or more frequently submarginate, and attenuate at base (Ortega et al. 2009). However, a closer examination of various structures reveals contrasting differences. For example, *Phlegmacium viscidoamarus* is distinguished by the pileus color that is yellowish, yellowish cream or orange towards the disc, cream yellow, cream ochre or orange in mature basidiomes. The yellowish universal veil remnants present on cap and stipe. The pinkish lilac hues present on gills, but absent on stipe and context. The distinct and sub-marginate bulb, the bitterish cap surface, with distinctly viscid fresh, becoming dry. The spores measure $11\text{--}13.8 \times 6.2\text{--}7.2 \mu\text{m}$. This species grows in Mediterranean subhumid sclerophyllous *Quercus rotundifolia* woodlands (Ortega et al. 2009) while *P. kausarii* grows in temperate coniferous forests of Kashmir Himalaya, India.

P. stjernaardii is another species with some morphological similarity but is distinguished from *P. kausarii* by its bicolored pileus with a greenish-yellow margin and an olivaceous (red) brown centre, a more or less marginate bulbous stipe base, and a smell of banana or apples. This species differs from the *P. kausarii* in its more distinctly marginate bulb and stronger and more persistently greenish-yellow colors on stipe, pileus margin and in the context. The pileus of *P. stjernaardii* is fairly bright and persistently greenish-yellow towards margin, dark greyish-brown to olive-brown at centre when young, more red-brown with age, and then ochre-brown at margin. Likewise, stipe has a fairly distinctly marginate bulb, greenish-yellow as pileus margin, apex sometimes with a bluish reflex. Basidiospores comparatively large, faintly citriform to amygdaliform, very distinctly and fairly coarsely verrucose. *P. stjernaardii* sometimes has a faint bluish-green tinge in the context and on the lamellae; this is never seen in *P. kausarii*. Furthermore, *P. kausarii* has been reported from the coniferous forests of Kashmir Himalaya, India, with sparse distribution, whereas *P. stjernaardii* seems to be more or less restricted to boreonemoral zone of Fennoscandia (Frøslev et al. 2017).

Phlegmacium majoranae shares some similarity with *P. kausarii* having pileus hemispherical to convex, glutinous, glabrous and glossy, olivaceous to olivaceous brown from centre. Stipe clavate to abruptly bulbous, with pale orange yellow to brownish veil remnants. Taste mild but with components of the smell. Spores ellipsoid to slightly amygdaloid, coarsely verrucose. However, *P. majoranae* is easily distinguished by the peculiar (greenish) yellow basidiocarps and context, the clavate to slightly abruptly bulbous stipe, and a smell similar to that of *P. percomis* (Frøslev and Jeppesen 2008). *P. majoranae* has a greenish to olivaceous pileus and grows with frondose trees. *P. percomis* has an orange yellow pileus and grows exclusively with *Picea*.

Another species *Phlegmacium cephalixus* being morphologically similar to *P. kausarii*, but possesses a different cap and lamellae color (greenish-yellow hues in *P. cephalixus*); bitterish viscosity of the pileus surface; a universal yellow veil with no olive hues; scarcer and less evident universal veil remnants on the stipe, (which form girdles or scales in *P. cephalixus*); and frequently has submarginate bulbs (Bidaud et al., 1999; Consiglio et al., 2005, 2007). The *P. percomis* is distinguished from *P. kausarii* by the pileus that is broadly convex, obtuse, with glabrous surface, viscid and chamois. Lamellae are acuminate-adnate, crowded and narrow. Stipe cespitose to sub-cespitos, clavate-bulbous to rounded-bulbous (Orton PD 1955).

Conclusion

P. kausarii clustered with species of section *Phlegmacioida* during phylogenetic analysis, having several other species recorded from Europe and North America. Further molecular studies are required to validate the accurate phylogenetic position of this taxon. This taxa discovered from the Indian subcontinent is a new species reported for the first time, thus highlighting the knowledge gaps and the need for further taxonomic studies on this diverse mycorrhizal genus. Thus, further studies focusing on the diversity of *Phlegmacium* genus in the Kashmir Himalaya will help us understand the mycorrhizal associations formed by these species with conifers. Further explorative studies can broaden the horizons of the diversity in this genus associated with the coniferous forests of this region.

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Declarations

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Availability of data and material: The ITS data is available in the Genebank public database and the specimens have been deposited in the KASH herbarium.

Code availability: Not applicable.

Authors' contributions

All the authors have contributed to the study and in the preparation of the manuscript.

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Tables

Table 1. ITS sequences of the section used in the phylogenetic analysis.

Figure legends

Figure 1. Maximum likelihood tree inferred from ITS sequences. Newly reported species in the section is shown in Bold. The ML bootstrap values above 50% are shown on each branch.

Figure 2A, B. Sporocarp of *Phlegmacium kausarii*. **3A** . Spore picture of *P. kausarii* .