Original Article

Microbial Biosystems 6(2) (2021) 2022.1050
10.21608/mb.2022.121262.1050

Contents lists available at Egyptian Knowledge Bank
Microbial Biosystems
Journal homepage: http://mb.journals.ekb.eg/

MICROBIAL BIOSYSTEMS
INTERNATIONAL SCIENTIFIC JOURNAL OF MICROBIAL BIOLOGY

Deciphering the symbiosis of endemic *Usnea ghattensis* and their photobiont *Trebouxia* sp. through molecular tools from the northern Western Ghats, India

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ARTICLE INFO

Article history
Received 11 February 2022
Received revised 28 March 2022
Accepted 10 April 2022
Available online 22 April 2022

Corresponding Editor:
Degola F
Sridhar KR

Keywords
β tubulin
ITS
LSU
Lichen
Taxonomy

ABSTRACT

Usnea ghattensis is an endemic fruticose lichen species of northern Western Ghats, India. With the changing climate, pollution, land use management and growing population in these terrains, the diversity of endemic lichens of these pristine habitats are under threat. This study attempts to unravel the symbiotic association of flagship fruticose lichen species, *Usnea ghattensis* and their photobiont *Trebouxia.* Based on combined ITS and LSU data, β tubulin data, morphology and chemical evidence, *Usnea ghattensis* is placed in the *Usnea* clades 3 and 4, representing Asian *Usnea* species. The RPB1 data and analyses were also in congruence to that of β tubulin, however the phylogeny is not interpreted due to data deficiency. The ITS sequence data and phylogeny of photobiont of *U. ghattensis* were also established as an undescribed *Trebouxia* species belonging to Clade I within the genus *Trebouxia*. This is the first holistic attempt to study the lichen symbiosis of *Usnea ghattensis* from the Western Ghats of India.

Published by Arab Society for Fungal Conservation

Introduction

Usnea is the largest genus of fruticose lichens worldwide and the second largest genus in the *Parmeliaceae* consisting of about 400 species worldwide, distributed in polar, temperate and tropical regions (Kirk et al. 2008; Clerc 2016). On the basis of inner cartilaginous tissue, Motyka (1936-38) classified fruticose lichens and recognised six subgenera: *Euusnea*, *ProtoUsnea*, *Leithariella*, *Chlorea*, *Neuropogon* and *Eumitria. Usnea* was previously placed in *Usneaceae* together with other similar fruticose lichens (Eschweiler 1824). However, due to similarity in the fruiting body, it was brought under *Parmeliaceae* (Henssen & Jahns 1974; Ericksson and Hawksworth 1986). Ohmura (2002) revealed a new subgenus *Dolichousnea* based on ITS sequence data and analyses and concluded that *Usnea* contains three taxa at subgeneric level *Usnea*, *Eumitria* and *Dolichousnea*.

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Based on the current concepts in *Usnea* phylogeny, Asian *Usnea* species are classified under the complex *Usnea clade* 3 and 4 (Truong et al. 2013).

Secondary metabolites of *Usnea* species have many biological activities such as antimicrobial, antiviral, antiprotozoal, antiproliferative, antioxidant, anti-inflammatory and analgesic activity (Yamamoto et al. 1998; Muller 2001; Ingolfsdottir 2002; Behera et al. 2005a, b). *Usnea* species are reported as bioindicators as their growth is vulnerable to air pollution and environmental changes. Secondary metabolite usnic acid has an antibiotic effect and in Asia, *Usnea* is harvested on a large scale for commercial production of medicines for skin diseases (Brodo et al. 2001; Articus 2004). Higher altitudes of Western Ghats of India are considered to be "lichen hotspots", however climate change and forest fragmentation are causing a significant threat to these pollution indicators in the wild. Even though the morpho-taxonomic studies, biomonitoring, biodeterioration studies on *Usnea ghattensis* are reported (Behera et al. 2005b; Upreti et al. 2012), the phylogeny of this flagship fruticose lichen species is yet to be resolved. Hence, this study attempts to resolve the phylogenetic placement of *Usnea ghattensis* using molecular markers ITS, LSU and protein-coding gene β tubulin and RPB1 from the northern Western Ghats of Maharashtra. This study also focuses on identifying photobiont Trebouxia species through ITS sequence data and phylogenetic analysis to envision the holistic mycobiont and phycobiont authentication and understand the co-evolution and species diversity existing among Indian lichens.

Materials and Methods

Sample collection

Field surveys were conducted during the 2018 monsoon season in the Western Ghats of Maharashtra viz. Mahabaleshwar, Thoseghar area. A total of 10 to 15 fresh specimens were collected from different habitats of semi evergreen forests. Non-destructive minimal quantity of samples was collected to conserve the in-situ diversity. The collected samples were transported to the laboratory, air-dried under shade and stored for detailed morphological and molecular study. Fresh filamentous thalli were collected and stored (4 ℃) specifically for molecular studies to evade cross contamination from fast-growing saprophytic fungi. After preliminary morphological studies, 10 specimens were selected that are subjected to molecular studies.

Morphology and chemical analyses

Thallus morphology of all the samples were studied using a Nikon binocular stereomicroscope (Model SMZ-1500 with Digi-CAM, Japan). Thallus colour, branching and other features of the cortex such as apothecia were observed and noted. For microscopy, thallus sections were taken using a razor blade and mounted in lactic acid cotton blue (with gentle heating over the flame). Morphological characteristics were elaborated and compared with standard taxonomic references (Awasthi G 1986; Troung et al. 2013). Chemical profiles were studied by Thin Layer Chromatography (TLC) following standard protocols (Culberson 1972; White and James 1985; Orange et al. 2001).

DNA isolation, Polymerase Chain Reaction and Sequencing

Prior to DNA isolation, manual cleaning of specimens was done using a brush to avoid plant parts and bryophytic remnants and further washed in distilled water to facilitate the recovery of high-quality DNA.

Total genomic DNA from lichen thallus was isolated using the modified CTAB method (Porebski et al. 1997; Cubero et al. 1999). Additionally, the sorbitol wash method (Inglis et al. 2018) was also used to achieve quality DNA from samples with dark pigmentation. DNA isolation kit (FavoPrep™ Plant Genomic DNA Extraction Mini Kit, Taiwan) was also used as an alternate for achieving high-quality DNA. DNA was quantified using NanoDrop ND-1000 spectrophotometer V3.8.1 (Thermo scientific, USA) and quality was ensured for further PCR studies. For amplifying Internal Transcribed Spacer regions (ITS) from photobiont, primer pair ITS1T and ITS4T (Kroken and Taylor 2000) were used. For amplifying Internal Transcribed Spacer regions (ITS) of mycobiont, primer pair ITS5 and ITS4 (White et al. 1990) and ITS1F (Gardes and Bruns 1993) were used. The partial 28S nrDNA (LSU) was amplified using the primer pair; LROR and LR5 (Vilgalys and Hester 1990). Protein coding RPB1 gene was amplified using gRPB1-A (Stiller and Hall 1997) and fRPB1 -C (Matheny et al. 2002) primers and BT_2a and BT_2b (Glass and Donaldson 1995) primers were used to amplify β tubulin (BenA) gene. The PCR reactions (25 µl) contained 10× buffer (containing 100mM Trizma–HCL, pH 8.3 at 25ºC, 500 mM KCL, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 0.2 mM each dNTP, 0.5 µM each primer, 1unit *Taq* DNA polymerase (Sigma–Aldrich) and 1–10 ng genomic DNA extract. DNA with dark pigments was also subjected to a ready-to-use reaction mixture, EmeraldAmp® GT PCR Master Mix, 2X Premix (containing DNA polymerase, optimized reaction buffer, dNTPs, and a density reagent) (TaKaRa, Japan) for PCR. The amplifications for ITS, LSU rDNA, Ben A and RPB1 were carried out in an automatic thermocycler ProFlex™ PCR system (Applied Biosystems, USA). Thermal cycling parameters used for amplification were:

initial denaturation at 95ºC for 5 min, and 30 cycles of 94ºC for 1 min, 45 ºC–50ºC (ITS5–ITS4) for 30 sec, 54ºC–56ºC (ITS1F–ITS4) for 1 min, 54ºC–56ºC (LSU rDNA) for 1 min, 55ºC (BenA) for 30 sec and 56ºC (RPB1 nrDNA) for 50 sec, 72 ºC for 90 sec and a final extension at 72 ºC for 10 min. The PCR products were purified with StrataPrep PCR Purification Kit (Agilent Technologies, TX, USA) and sequenced with the same primers using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA).

Phylogenetic analyses

Sequences with high similarity percentages were determined from a BLASTn search to find the closest matches with taxa and from recently published data in GenBank sequence database. Sequences generated from different primers of the four genes (ITS, LSU, Ben A and RPB1) were analysed with other sequences (following Truong et al. 2013) retrieved from GenBank (Table 1). The multiple sequence datasets were aligned with MAFFT v. 7 at the webserver (http://mafft.cbrc.jp/alignment/server; Katoh et al. 2017), and manually edited where necessary in BioEdit v.7.0.9.0 (Hall 1999). The phylogeny website tool "ALTER" (Glez-Peña et al. 2010) was used to transfer the alignment file in to PHYLIP format for RAxML analysis. Phylogenetic analyses of both individual and combined aligned data were performed with the maximum likelihood (ML) method and Bayesian analysis (PP). Phylogeny was inferred using the program RAxML v8.1.11 (Stamatakis 2006; Stamatakis et al. 2008). Based on J- Model test, best fit model of nucleotide substitution 'GTRGAMMA+I' model was implemented, with locus-specific model partitions treating all loci as separate partitions, and evaluated nodal support using 1000 bootstrap pseudo-replicates. Exploratory analyses using alternative partitioning schemes resulted in identical topologies and highly similar bootstrap support values. For the Bayesian tree sampling, the concatenated ITS & LSU data set was partitioned as described in the ML analysis in siMBa (Mishra and Thines 2014), specifying the best fitting model, and allowing unlinked parameter estimation and independent rate variation. Posterior probabilities (PP) were estimated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. Every 1000th tree was sampled to avoid sample auto-correlation. Based on the likelihood profile, the first 25% trees were discarded as burn in. Only clades with bootstrap support equal or above 50% under ML and PP equal to or above 0.95 in a Bayesian framework were considered as supported. Phylogenetic trees were visualized using the program FigTree 1.4.0. (Rambaut 2014). Trees were edited using Microsoft Power Point. DNA sequences that were newly generated in this study were deposited in GenBank.

Results

Phylogenetic analyses

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits of the *Usnea ghattensis* AMH 18.10 (SF3) (GenBank MN892640) & AMH 18.25(SF31) (GenBank MN893227) using the ITS sequence had highest similarity to *Usnea* sp. isolate KAR30 as (*Usnea articulata*, GenBank AJ457140; identities= 499/511 (98%), no gaps), *Usnea malmei* (GenBank AJ457140; identities= 513/534 (96%), no gaps). Closest hits using the LSU sequences AMH 18.10 (SF3) (GenBank MN892714) & AMH 18.25 (SF31) (GenBank MN893229) are *Usnea ceratina* (GenBank KY033353; identities= 716/726 (99%), no gaps), *Usnea subdasaea* (GenBank JQ837409; identities= 715/726 (98%), no gaps), *Usnea rubicunda* (GenBank JQ837400; identities= 715/726 (98%), no gaps).

Similarly, for β–tubulin sequences AMH 18.10 (SF3) (GenBank MN895929) & AMH 18.25 (SF31) (GenBank MN895930), *Usnea articulata* (GenBank JN086237; identities= 359/569 (97%), no gaps) is the closest match. Closest hits using the RPB1 sequence AMH 18.25 (SF31) (GenBank MN895931) are *Usnea articulata* (GenBank JN992558; identities= 747/758 (99%), no gaps), *Usnea cornuta* (GenBank JN992604; identities= 743/765 (97%), no gaps), *Usnea hirta* (GenBank JN992575; identities= 730/749 (97%), no gaps).

The combined ITS and LSU sequence data of the genus *Usnea* to determine the species placement was given in Table 1. The tree is rooted with *Plastimatia glauca* (AY756342). The analysed dataset comprised LSU (818 bp) and ITS (506 bp) sequence data (a total of 1327 characters including gaps) for 54 taxa. Analyses of each single gene were generated and the topology of each tree had clade stability. The best RAxML tree (Fig. 1) with a final likelihood value of -5887.814099 is presented. The matrix had 357 distinct alignment patterns, with 16.21% undetermined characters or gaps. Estimated base frequencies were: A = 0.238752, C = 0.237929, G = 0.290254, T = 0.233065; substitution rates AC = 2.050325, AG = 2.643418, AT = 2.508831, CG = 0.757930, CT = 12.973441, GT = 1.000000; gamma distribution shape parameter α = 0.623263. Phylogenetic trees were sampled every 1000th generation (resulting in 4000 total trees) in 4,000,000 generations from the running of six simultaneous Markov chains. The first 1000 trees, which contained the burn-in phase of the

analyses were discarded. The remaining 3000 trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. Maximum parsimony, maximum likelihood and Bayesian posterior probability analyses resulted in similar topologies. Analyses based on β tubulin (Fig. 2) and RPB1 data were also performed for accurate phylogenetic placement of the *U. ghattensis*. The evolutionary relationships were estimated from a concatenated, two locus (ITS, LSU) data matrix for *Usnea* species based on Truong's concept (Truong et al. 2013).

Table 1 Species, specimens and GenBank accession numbers used in the phylogenetic study (newly generated sequences are indicated in bold).

Taxa	Specimen no.	GenBank Accession no.		
		ITS	**LSU**	***BenA***
Plastimatia glauca	-	AF451752	AY756342	AF502271
Usnea acanthella	NW289	DQ235482	-	-
U. angulate	85	JQ837291	JQ837376	
U. antarctica	NW138	DQ235517	-	-
U. aranea	120	KP668964	KP668969	-
U. articulata	19	JN943545	JN939696	-
U. articulata	59	JN943508	JN939728	-
U. articulata	02	-	-	JN086237
U. articulata	05	-	-	JN086238
U. articulata	01	-	-	JN086236
U. aurantiacoatra	NW211	DQ767954	-	-
U. baileyi	SGT63	MN080251	MN080252	-
U. barbata	KA7	-	-	AF502257
U. brasiliensis	44	JQ837294	JQ837379	-
U. ceratina	14	JN943550	JN939694	-
U. ciliate	NW287	DQ235476	-	-
U. clerciana	126	KP668967	KP668972	-
U. cornuta	1	JN943562	JN939688	-
U. cornuta	19	JQ837299	JQ837384	-
U. crocata	35	JQ837303	JQ837388	-
U. dasaea	41	JQ837305	JQ837390	-
U. dasaea	81	JQ837306	JQ837391	-
U. dasaea	01	-	-	JN086239
U. dasaea	02	-	-	JN086240
U. diffracta	KDIF 87	-	-	AJ748093
U. diplotypa	07	-	-	JN086244
U. erinacea	104	JQ837307	JQ837392	-
U. esperantiana	13	JN943551	JN939693	-
U. filipendula	7	JN943556	JN939692	-
U. filipendula	6	JN943557	JN939691	-
U. filipendula	KFP18	-	-	AF502269
U. flavocardia	03	-	-	JN086246
U. flavocardia	01	-	-	JN086245
U. florida	26	JN943538	JN939703	-
U. florida	03	-	-	JN086248
U. florida	02	-	-	JN086247
U. fragilescens	96	JQ837310	JQ837394	-
U. fragilescens	119	JQ837309	JQ837393	-
U. fulvoreagens	43	JN943522	-	-
U. fulvoreagens	01	-	-	JN086249
U. fulvoreagens	05	-	-	JN086250
U. ghattensis	**AMH 18.10/SF3**	**MN892640**	**MN892714**	**MN895929**
U. ghattensis	**AMH 18.25/SF31**	**MN893227**	**MN893229**	**MN895930**
U. glabrescens	21	JN943543	JN939698	-
U. glabrescens	61	JN943506	JN939730	-

U. glabrescens	17	-	-	JN086253
U. glabrescens	01	-	-	JN086251
U. hirta	35	JN943529	JN939711	-
U. hirta	01	-	-	JN086254
U. hirta	02	-	-	JN086255
U. lambii	NW251	EF492207	-	-
U. lapponica	08	-	-	JN086259
U. lapponica	09	-	-	KU352373
U. longissimi	U0737	JX978205	-	-
U. longissimi	KL68	-	-	AJ748094
U. mutabilis	03	-	-	JN086262
U. patagonica	NW63	DQ235487	-	-
U. pectinate	YO4373	AB051656	AB720729	-
U. perhispidella	137	JQ837290	JQ837375	-
U. perpusilla	NW2183	EF492216	-	-
U. rigida	KRI47	-	-	AF502272
U. rubicunda	47	JN943518	JN939719	-
U. rubicunda	49	JN943516	JN939721	-
U. rubicunda	04	-	-	JN086263
U. rubricornuta	34	JQ837323	JQ837404	-
U. rubrotincta	YO4405	AB368489	-	-
U. silesiaca	88	JQ837331	JQ837412	-
U. silesiaca	SIL03	-	-	KU352382
U. steineri	65	JQ837334	JQ837415	-
U. subaranea	122	KP668963	-	-
U. subcornuta	130	JQ837325	JQ837406	-
U. subcornuta	01	-	-	JN086264
U. subdasaea	8	JQ837328	JQ837409	-
U. subfloridana	27	JN943537	JN939704	-
U. subfloridana	10	-	-	JN086266
U. subfloridana	05	-	-	JN086265
U. subglabrata	25	JQ837312	JQ837396	-
U. subrubicunda	76	JQ837332	JQ837413	-
U. substerilis	01	-	-	JN086267
U. substerilis	02	-	-	JN086268
U. substerilis	06	-	-	JN086269
U. trachycarpa	NW173	DQ235496	EF116570	-
U. trachycarpus	KN91	-	-	AJ748089
U. trichodeoides	YO5316	AB720727	AB720728	-
U. ushuaiensis	NW235	EF492146	-	-
U. wasmuthii	34	JN943530	JN939710	-
U. wasmuthii	23	JN943541	JN939700	-
U. wasmuthii	08	-	-	JN086275
U. wasmuthii	09	-	-	JN086276

****Usnea ghattensis*: RPB1 GenBank Number = MN895931**

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits of the for the photobiont, *Trebouxia* species from *Usnea ghattensis* (GenBank MN907405) using the ITS sequence had highest similarity to *Trebouxia* sp. isolate L1177 GenBank KJ754204; identities= 632/677 (93%), 1% gap), *Trebouxia* sp. isolate L1177 GenBank KJ754203; identities= 632/677 (93%), 1% gap), *Trebouxia impressa* isolate L2239p GenBank KX181276; identities= 599/649 (92%), 1% gap), The sequence data of the *Trebouxia* species was analyzed to determine the species placement (Table 2). The tree is rooted with *Asterochloris mediterranea* (AF345435). The analysed dataset comprised ITS sequence data of 703 bp characters including gaps for 31taxa. The best RAxML tree with a final likelihood value of -4534.479276 is presented. The matrix had 328 distinct alignment patterns, with 19.14% undetermined characters or gaps. Estimated base frequencies were: A = 0.216610, C = 0.240395, G = 0.275360, T = 0.267635; substitution rates AC = 1.294466, AG = 4.306267, AT = 3.051192, CG = 0.726654, CT = 5.149581, GT = 1.000000; gamma

distribution shape parameter α = 0.439693. Phylogenetic trees were sampled every 1000th generation (resulting in 4000 total trees) in 4,000,000 generations from the running of six simultaneous Markov chains. The first 1000 trees, which contained the burn-in phase of the analyses were discarded. The remaining 3000 trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. Maximum parsimony, maximum likelihood and Bayesian posterior probability analyses resulted in similar topologies (Fig. 1).

Table 2 Species, specimen accession and sequences database accession numbers used in the phylogenetic study (newly generated sequences are indicated in bold).

Taxa	**Specimen No.**	**GenBank Accession no. ITS (Photobiont)**
Asterochloris mediterranea	IO3	AF345435
Trebouxia arboricola	92.011A1	AJ249481
T. asymmetrica	SAG 48.88	AJ249565
T. corticola	UTEX 909	AJ249566
T. corticola	98	AB630328
T. decolorans	SW 44b	FJ705191
T. decolorans	P 320 IIc	AJ969601
T. flava	UTEX 181	AF242467
T. galapagensis	UTEX 2230	AJ249567
T. gelatinosa	L1780	KT768205
T. gelatinosa	AO33	JQ004571
T. gelatinosa	AO32	JQ004570
T. gelatinosa	AO31	JQ004569
T. gigantea	UTEX 2231	AJ249577
T. higginsiae	UTEX 2232	AJ249574
T. impressa	IB345	KY559117
T. impressa	IB327	KY559112
T. impressa	IB344	KY559116
T. impressa	L2238p	KX181275
T. impressa	L2239p	KX181276
T. incrustata	LEC 1	AM920666
T. jamesii	1457 Sv4 20	GQ375353
T. jamesii	1146 Sp1 7	GQ375342
T. potteri	UTEX 900	AF242469
Trebouxia sp.	A000965	MH258947
Trebouxia sp.	L1178	KJ754204
Trebouxia sp.	L1177	KJ754203
Trebouxia sp.	17775	MN397127
Trebouxia sp.	A012539	MH258954
***Trebouxia sp.* (*Usnea ghattensis*)**	**AMH18.25/SF31**	**MN907405**
T. usneae	UTEX 2235	AJ249573

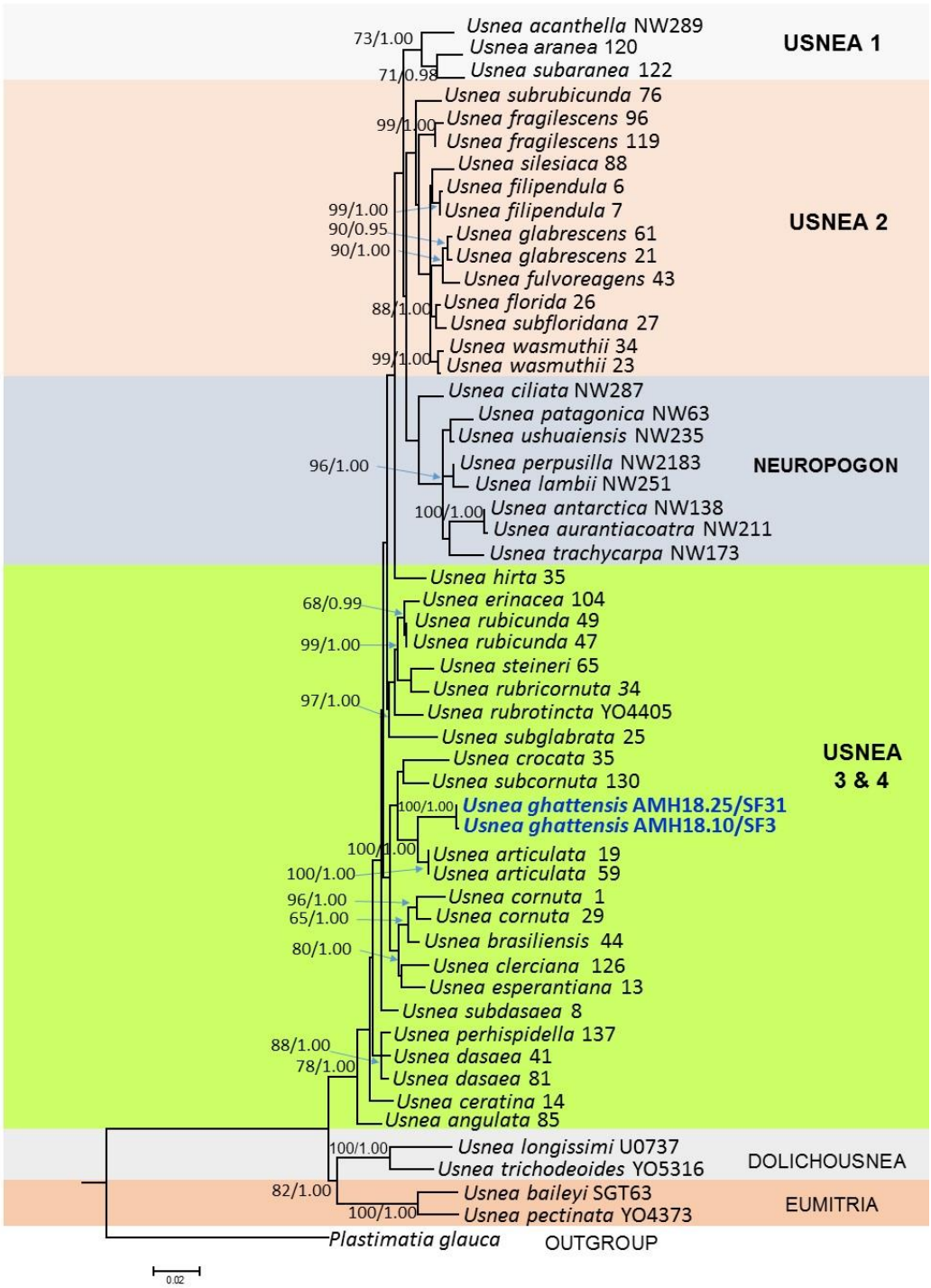

Fig 1. Phylogram generated from RAxML analyses based on analyses of combined ITS and LSU sequence data for the genera *Usnea* (*Parmeliaceae*, Lecanorales). Bootstrap support values for ML greater than or equal to 50% are given above the nodes and PP greater than or equal to 0.95 are given. The tree is rooted to *Plastimacia glauca* (AY756342, AY756342). The new sequences generated are shown in bold.

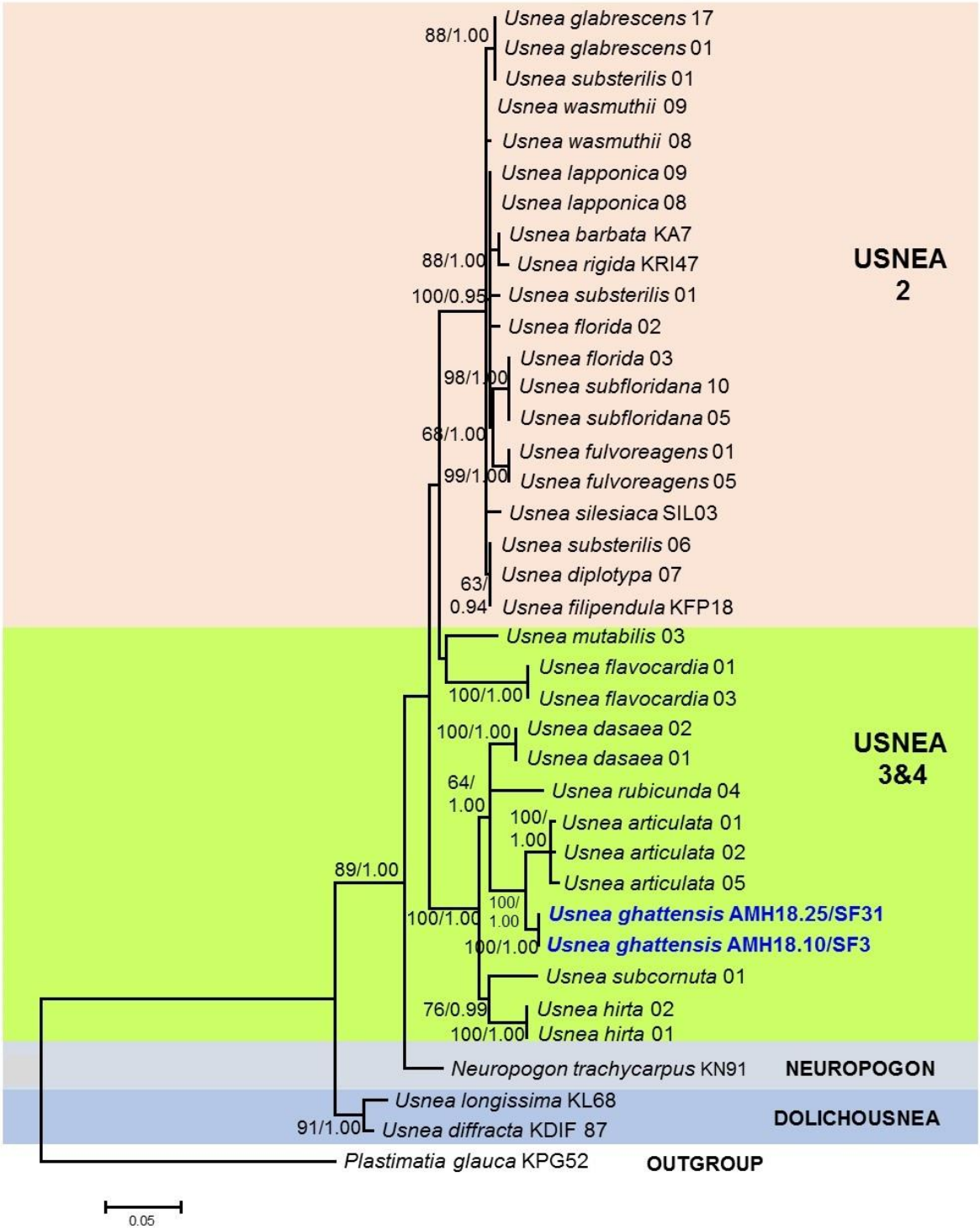

Fig 2. Phylogram generated from RAxML analyses based on analyses of B tubulin (*BenA*) gene sequence data for the genera *Usnea* (*Parmeliaceae*, Lecanorales). Bootstrap support values for ML greater than or equal to 50% are given above the nodes and PP greater than or equal to 0.95 are given. The tree is rooted to *Plastimacia glauca* (AF502271). The new sequences generated are shown in bold and blue.

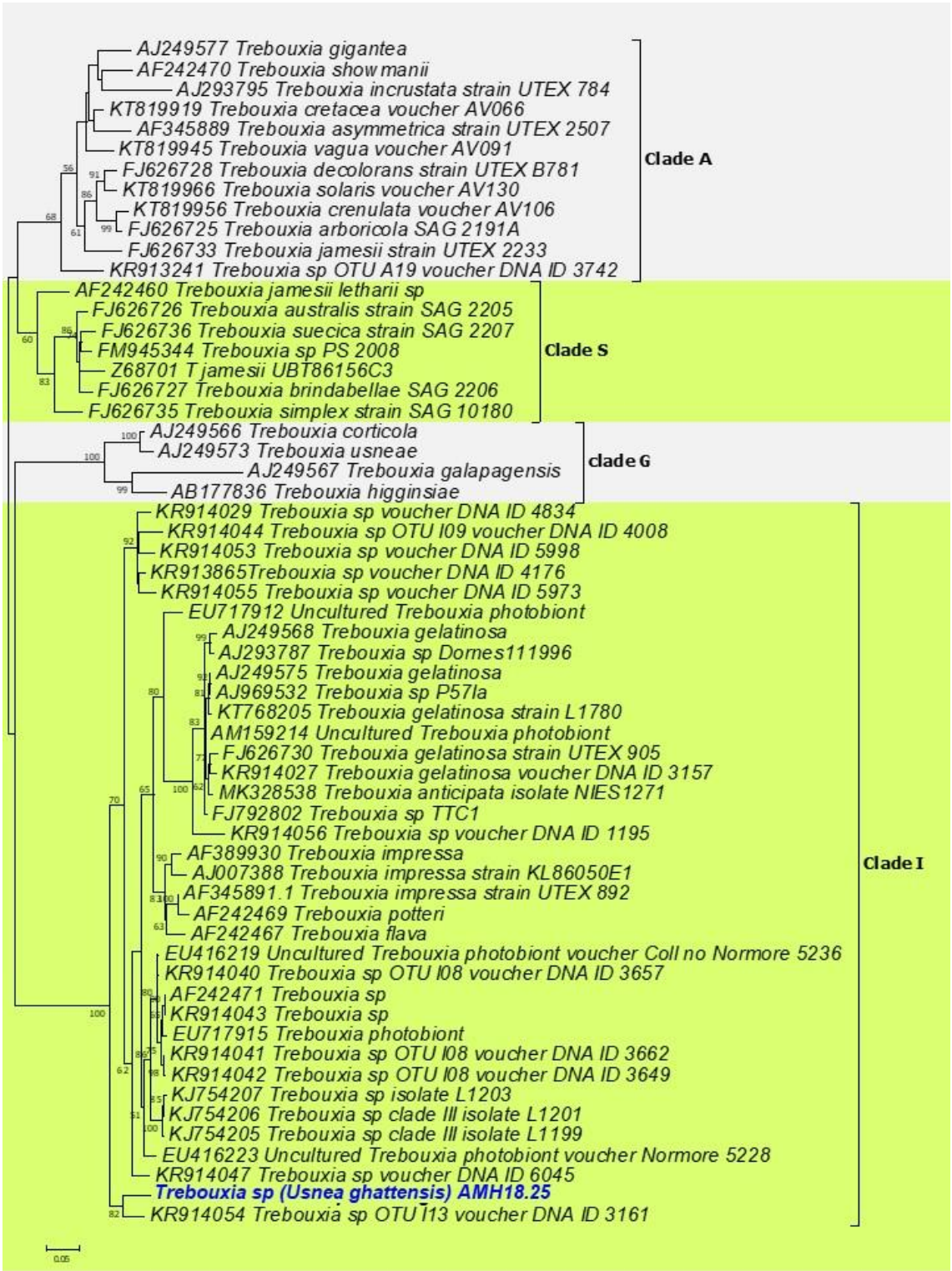

Fig 3. Phylogram generated from RAxML analyses based on analyses of ITS sequence data for the photobiont genera *Trebouxia* (*Trebouxiaceae*). Bootstrap support values for ML greater than or equal to 50% are given above the nodes and PP greater than or equal to 0.95 are given. The new sequence generated are shown in blue and bold.

Phenotypic characterization and TLC

Usnea Dill. ex Adans., Fam. Pl. 2: 7 (1763)

The genus *Usnea* form one of the largest genera within *Parmeliaceae* having ca. 400 species globally and about 60 species are reported from India (Singh and Sinha 2010). Most of the species are corticolous, some are saxicolous or lignicolous, characterized by a fruticose, pendulous or erect thallus, attached to the substrate by a short trunk, with cylindrical branches, holding the structures of sexual (apothecia) or vegetative (soralia) reproduction. Branch section shows a cartilaginous central axis, a medulla formed by loose to compact hyphae, and an outer cortical layer containing a yellow pigment called usnic acid (Truong et al. 2013). *Usnea ghattensis* is a flagship foliose lichen species found in the northern Western Ghats with stiff dense thallus, dense lateral branchlets and brown eupruinose apothecia. The taxon is restricted to Maharashtra and Karnataka states of India.

Usnea ghattensis G. Awasthi J. Hattori Bot. Lab. 61: 373 (1986)

Thallus corticolous, erect, 4–6 cm in height, bright green to light brown when hydrated, becomes yellowish brown when dehydrated, basal disc black, branching sympodial with compact branches, stiff, terete, up to 3 mm in diameter, tapering; lateral branches stiff, dense, simple or branched, 0.5–1 cm long, flexuos, apices blackish; papillate, papillae concolorous to thallus; pseudocyphellae, soredia or isidia absent; cortex palisade like, medulla getting dense near algal stratum, lax inwards, axis solid; Apothecia terminal, geniculate, 5–8 mm in diam., disc concave to plane, brownish, epruinose; margin ciliate, cilia simple, thick up to 3 mm long; receptacle scrobiculate, shortly ciliate; epithecium greenish black; hymenium hyaline, I+ blue, hypothecium yellowish; asci 8–spored; spores simple, hyaline (12) 16–18 × 8–10 µm.

Chemistry: — Usnic acid

Known distribution: —limited to northern Western Ghats regions of Karnataka and Maharashtra

Material examined: — India, Maharashtra, Mahabaleshwar, on tree branch, 16 July 2018, S. Fatima, Rajeshkumar KC & B Sharma, Specimen Accession numbers: AMH18.10/ SF3; INDIA, Maharashtra, Thoseghar, 25 July 2018, S Fatima & B Sharma, Specimen Accession numbers: AMH18.25/ SF31

Discussion

Concatenated analyses based on ITS and LSU data delineated *U. ghattensis* as a sister clade from *U. articulata* (100 BS/ 1.00 PP). Both *U. ghattensis* and *U. articulata* along with *U. crocata* and *U. subcornuta* formed a well-supported stable clade within the Asian *Usnea* species (*Usnea* 3 & 4 Clade). Analysis based on β tubulin data also reinforced the placement of the *U. ghattensis* adjacent to the *U. articulata,* forming a robust clade (100 BS/1.00 PP) (Fig. 2) within *Usnea* Clade 3 & 4. More studies using β tubulin sequencing and analyses may be needed to elaborate the interspecies relationships of Asian *Usnea* as there are limited sequences present to represent all the species that are included in ITS and LSU concatenate analyses. Similarly, RPB1 sequence data analyses also delineated *U. ghattensis* as a sister clade to *U. articulata* forming a well-supported clade (100 BS/ 1.00 PP) (Figure not shown). More RPB1 data may be needed to elaborate the circumscription of *Usnea* Clade 3 and 4.

Based on ITS phylogeny, the photobiont from *Usnea ghattensis* formed a stable clade along with *Trebouxia* spp. (A012539 (MH 25894754) & A000965 (MH 258947) identified from uncultured *Myelochroa irrugans* and *Hypotrachyna neodissecta* respectively) from Korea. The species level identification of *Trebouxia* species from *Usnea ghattensis* cladded with other *Trebouxia* spp. is yet to be resolved as it formed an adjacent clade to *T. gelatinosa* and *T. impressa* major clade.

While describing the species *U. ghattensis*, Awasthi (1986) mentioned the morphology of *U. ghattensis* somewhat close to *U. complanata*; but differs in its very stiff thallus with very dense branchlets and papillae. Sequences of *U. complanata* are not available to support this morphological assessment. Even though the phylogenetic analyses reveal close evolutionary relationship between *U. articulata* and *U. ghattensis*, morphologically both species are highly distinct.

Molecular identification of species of *Usena* are important for understanding their evolutionary positions and in-situ conservation as a potential biological resource. Upreti et al. (2012) reported *U. ghattensis* as a potential source of purple dye yielding species for dying silk and other fibres from India. *In vitro* studies of *U. ghattensis* proved its antioxidant potential and antibacterial activities against *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus aureus* (Behera et al. 2005a, 2006). Similarly, Srivastava et al. (2013) also proved the antimicrobial property of extracts of *U. ghattensis* against human pathogenic bacteria, *Bacillus cereus* and *Pseudomonas aeruginosa*. Behera et al. (2009) also optimized the culture conditions for *U. ghattensis* and increased its biomass for antioxidant metabolite production. Verma et al. (2008) evidenced the antioxidant and hepatoprotective activity of a lichen *U. ghattensis* in vitro. They have established that the cultured lichen extract showed hepatoprotection against ethanol-induced toxicity in the mice liver slice culture model by a

significant decrease in the antioxidant enzymes, glutathione peroxidase, catalase, and superoxide dismutase, along with a decrease in lipid peroxidation and lactate dehydrogenase release. Even though more than 60 species are reported from India (Singh and Sinha 2010), most of these species are yet to be subjected to molecular studies to resolve their phylogenetic placement in the fungal tree of life.

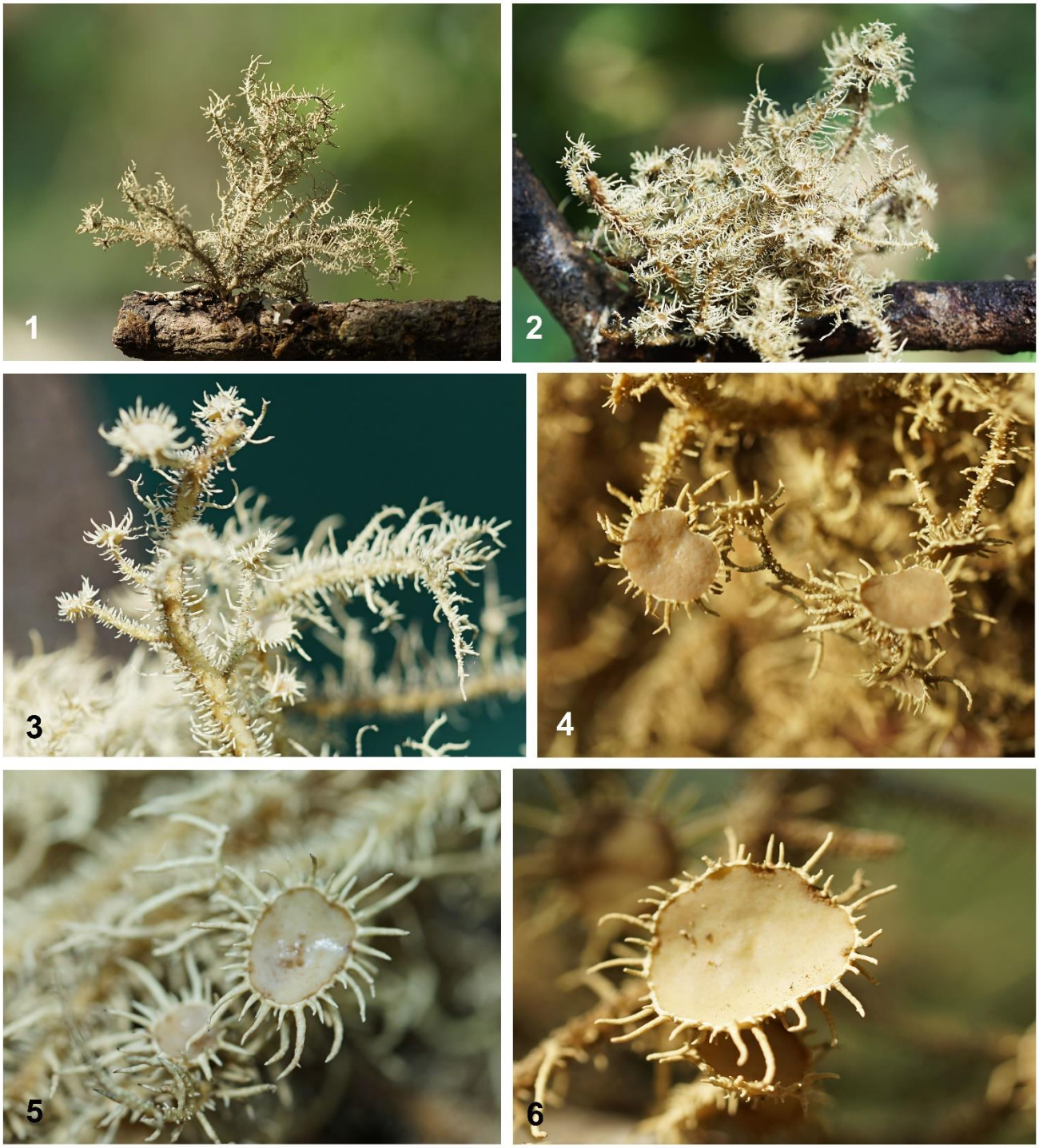

Fig 4. *Usnena ghattensis*. 1–2. Habit. 3. Errect branch and brachlets. 4–6. Epruinose apothecia.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

Rajeshkumar KC and B Sharma thank SERB, Department of Science and Technology, Government of India for financial support under the project CRG/2020/000668 and Director, ARI for providing the facility. Ansil PA thanks CSIR-HRDG, INDIA, for the financial support under the JRF fellowship (09/670(0093)/2021-EMR-I).

Funding

SERB, Department of Science and Technology, Government of India CRG/2020/000668

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