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Usnea jingdongensis sp. nov. from Southwest China

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Abstract—*Usnea jingdongensis* from the Ailaoshan Mountain of Southwest China, is described as a new species of *Parmeliaceae*. It is characterized by the uninflated branches, fistulose axis with pale brown to dark brown loose hyphae, and the absence of pseudocyphellae and soralia. The phylogenetic analysis of the nrDNA ITS sequence data supported the recognition of the species. A key to the eumitrioid *Usnea* species in China is also provided.

Key words—Asia, evolutionary tree, *Lecanorales*, protocetraric acid, taxonomy

Introduction

Usnea Dill. ex Adans. is a hyperdiverse lichen genus, with more than 350 species (Clerc 1998; Kirk & al. 2008; Lücking & al. 2017) distributed from polar zones to tropical areas. It forms a strongly supported monophyletic lineage (Crespo & al. 2007) in the family *Parmeliaceae* (*Lecanorales*, *Lecanoromycetes*, *Ascomycota*). Traditional diagnostic characters of the genus include a fruticose thallus, branches with a cartilaginous central axis, and the presence of usnic acid (Clerc 1998; Ohmura 2001). In taxonomic studies, these characters were proved to be feasible for genus delimitation (Clerc 1998; Ohmura 2001). However, at the species level, the plasticity of morphological characteristics to environmental changes poses a great challenge for the delimitation of species (Truong & Clerc 2013). One of the DNA barcoding markers, the nuclear ribosomal internal transcribed spacer (nrDNA ITS), had been proposed for

fungi, including lichenized fungi (Schoch & al. 2012). A series of phylogenetic studies supported the delimitation of species in *Usnea* described from morphological and chemical features (Ohmura 2008; Liu & Guo 2009; Saag & al. 2011; Gerlach & al. 2019; Temu & al. 2019; Lücking & al. 2020). Eumitrioid *Usnea* (= *Eumitria*), comprising the species with a tubular central axis ± filled with loose hyphae throughout the branches of the thallus, has consistently been recovered as a monophyletic group in all phylogenetic studies (Articus 2004; Temu & al. 2019; Lücking & al. 2020).

Eumitria was described as a genus by Stirton (1882), but has usually been considered to be a subgenus of *Usnea* (Motyka 1936; Ohmura 2001, 2002). Based on the phylogenetic analysis of limited molecular data, Articus (2004) resurrected it at generic level, which was also accepted by Divakar & al. (2017), Kraichak & al. (2017), and Lücking & al. (2020).

Since only a small proportion of species (3/24) with a hollow, often fistulate central axis were included in the phylogeny, and these limited phenotypic features are not always easy to discern and are not completely consistent with the recognized genera, we defend the recognition of a single genus *Usnea* that is morphologically well characterized (Ohmura & Kanda 2004; Wirtz & al. 2006; Thell & al. 2018; Temu & al. 2019). Here, we prefer to use the term 'eumitrioid' for the *Usnea* species with a tubular central axis throughout the whole thallus (Truong & Clerc 2013).

Eumitrioid *Usnea* has been studied in Africa (Motyka 1936; Dodge 1956; Swinscow & Krog 1974, 1986; Krog 1994; Nadel 2016; Temu & al. 2019), Australia (Rogers & Stevens 1988; Stevens 1999), East Asia (Zhao & al. 1982; Ohmura 2001, 2012), and South America including the Galapagos (Truong & Clerc 2013).

Wei (1991) enumerated about 80 species of *Usnea* reported in the literature for lichen collections from China before 1989. Since then, a series of studies on the diversity and taxonomy of *Usnea* from China have been carried out (Aptroot & al. 1999, 2002; Ohmura 2001, 2012; Seaward & Aptroot 2005; Zhang & al. 2006; Ohmura & al. 2010; Han & al. 2020). Approximately 90 species of *Usnea* have now been reported from China, among which seven species are eumitrioid (Zhao & al. 1982; Ohmura 2001, 2012; Han & al. 2020; Wei 2020).

During the study of *Usnea* in China, we discovered a new eumitrioid species based on morphological characteristics and nrDNA ITS sequence data. It is here therefore formally described and its morphological and structural characteristics are compared with those of closely related species.

Materials & Methods

Specimens, morphology and chemistry

All specimens examined were collected from Mt. Ailaoshan, Yunnan Province, Southwest China and were deposited in the Herbarium Mycologicum Academiae Sinicae-Lichenes, Beijing, China (HMAS-L) and the Herbarium of Hebei Normal University, Shijiazhuang, China (HBNU). Morphological characters of the specimens were examined using stereomicroscopes (Motic SMZ-140 and Zeiss-SteREO Discovery-V12) and compound microscope (Leica DM500). The micromorphological structure of the branches was analyzed according to Ohmura (2001). The sizes of cortex, medulla, and central axis were measured in longitudinal sections of branches at 40× magnification. The relative thickness of cortex/medulla/axis of the total branch diameter (CMA) and the ratio of axis/medulla (A/M) of all the studied specimens were calculated according to Clerc (1984, 1987) and were ascribed to the categories defined by Clerc (2011). Percentages of the tubular part of the axis (TBA) were calculated according to Truong & Clerc (2013). The apothecia were cut by hand with a razor and mounted in water. The slices were stained with 0.2% toluidine blue for about 15 min. The asci and ascospores of apothecia were observed and photographed at 400× magnification with Leica DM500 microscope. Thin layer chromatography (TLC) was performed on all specimens examined using solvent systems C and G (Orange & al. 2010).

DNA extraction, PCR amplification and sequencing

The samples of branch tips were cut for DNA extraction from the specimens examined. DNA was extracted using the DNAsecure Plant DNA Kit (DN-14, Aidlab Biotechnologies Co., Ltd) following the manufacturer's protocol. The nrDNA ITS region (ITS1+5.8S+ITS2) was amplified according to Han & al. (2020). The primers ITS1F-forward (5'–CTT GGT CAT TTA GAG GAA GTAA–3', 22 nt) (Gardes & Bruns 1993) and ITS4-reverse (5'–TCC TCC GCT TAT TGA TAT GC–3', 20 nt) (White & al. 1990) were used for the Polymerase Chain Reaction (PCR). The amplification reaction was performed in a 25 μL volume containing 0.75 units of TransStart Taq Polymerase (Tiangen, China), 2.5 μL of buffer, 0.5 μL of a 5 μM solution of the primers, 2 μL of 2.5 mM for each dNTP solution, and 1 μL of genomic DNA. PCR cycling was performed with the following protocol: initial denaturation for 3 min at 95°C; 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min; with final elongation at 72°C for 10 min. PCR products were screened on 1% agarose gels stained with ethidium bromide and the sequences were generated by Genewiz Inc. (Suzhou, Jiangsu, China).

Phylogenetic analysis and sequence comparing

Seven newly obtained sequences (two from the holotype; one each from five other specimens) were submitted to GenBank (Table 1).

The Blastn (NCBI) searches were carried out by using the entire nrDNA ITS (ITS1+5.8S+ITS2) sequences and the searches were limited to records that excluded uncultured/environmental sample sequences, and the results were filtered to match

Table 1. Specimens and sequences of *Usnea* and *Eumitria* spp. used in the ITS phylogenetic analyses. Newly produced sequences are in bold, with the holotype annotated with [T].

Species	Country	Voucher	GenBank #	Reference
U. baileyi	Japan	Ohmura 4488A	AB051050	Ohmura 2002
	USA	Ohmura 4516	AB051051	Ohmura 2002
	China	TNM L00004731	FJ494924	—
	Tanzania	Temu SGT156	MN080245	Temu & al. 2019
	Tanzania	Temu SGT119	MN080248	Temu & al. 2019
	Tanzania	Temu SGT112	MN080249	Temu & al. 2019
	Tanzania	Temu SGT110	MN080250	Temu & al. 2019
	Tanzania	Temu SGT63	MN080251	Temu & al. 2019
E. cf. *baileyi*	São Tomé & Principe	MN0417	MW267140	Nadel 2016
	São Tomé & Principe	MN0600a	MW267144	Nadel 2016
	São Tomé & Principe	MN0179	MW267145	Nadel 2016
E. firmula	São Tomé & Principe	MN0117	MW267133	Nadel 2016
	São Tomé & Principe	MN0581	MW267134	Nadel 2016
	São Tomé & Principe	MN0550b	MW267135	Nadel 2016
	São Tomé & Principe	MN0550a	MW267136	Nadel 2016
	São Tomé & Principe	MN0084	MW267137	Nadel 2016
U. glabrescens	Estonia	glabrescens_02	JN086304	Saag & al. 2011
	UK	glabrescens_15	JN086307	Saag & al. 2011
	Finland	glabrescens_16	JN086308	Saag & al. 2011
U. jingdongensis	**China**	**2018120543 [T]**	**MN080697**	**This study**
	China	**2018120543 [T]**	**MT261829**	**This study**
	China	**2018120562b**	**MN080698**	**This study**
	China	**2018120582**	**MN080700**	**This study**
	China	**2018120532**	**MN080702**	**This study**
	China	**2018120566**	**MN080704**	**This study**
	China	**2018082322a**	**MN080706**	**This study**
U. pectinata	China	TNM L00004729	FJ494946	—
	Tanzania	Temu SGT109	MN080236	Temu & al. 2019
	Tanzania	Temu SGT115	MN080237	Temu & al. 2019
E. pectinata	Japan	Ohmura 2989	AB051655	Ohmura 2002
	Indonesia	Ohmura 4373	AB051656	Ohmura 2002
E. cf. *pectinata*	São Tomé & Principe	MN0068a	MW267147	Nadel 2016
	São Tomé & Principe	MN0125	MW267148	Nadel 2016
	São Tomé & Principe	MN0597	MW267149	Nadel 2016

Species	Country	Voucher	GenBank #	Reference
	São Tomé & Principe	MN0060	MW267150	Nadel 2016
	São Tomé & Principe	MN0070b	MW267151	Nadel 2016
	São Tomé & Principe	MN0583	MW267162	Nadel 2016
	São Tomé & Principe	MN0063	MW267163	Nadel 2016
	São Tomé & Principe	MN0556	MW267164	Nadel 2016
	São Tomé & Principe	MN0567	MW267165	Nadel 2016
	São Tomé & Principe	MN0585	MW267166	Nadel 2016
E. aff. *pectinata*	São Tomé & Principe	MN0481	MW267153	Nadel 2016
	São Tomé & Principe	MN0065	MW267156	Nadel 2016
	São Tomé & Principe	MN0163	MW267157	Nadel 2016
U. wasmuthii	Norway	O-L-198061	MK812232	Marthinsen & al. 2019
	Colombia	41018b	MW241067	Moncada & al. 2020
Outgroup:				
U. diffracta	Japan	Ohmura 1124	AB051057	Ohmura 2002
	China	TNM L00004750	FJ494931	—
U. longissima	Japan	Ohmura 3664	AB051645	Ohmura 2002
	Japan	Ohmura 3816B	AB051647	Ohmura 2002
	China	TNM L00004685	FJ494936	—
U. trichodeoides	Japan	Ohmura 3809	AB051670	Ohmura 2002
	China	TNM L00004677	FJ494954	—

records with query coverage more than 95%, and percent identity between 88% and 100%. The representative taxa were selected mainly according to the Blastn search results in GenBank, morphological characters, and some references (Ohmura 2001, 2002; Articus 2004; Ohmura & Kanda 2004; Wirtz & al. 2006; Truong & Clerc 2013; Thell & al. 2018; Temu & al. 2019; Lücking & al. 2020).

The entire ITS sequences of seven samples of our examined specimens and the 46 representatives selected were aligned both by ClustalW and Muscle implemented in MEGA 6 (Tamura & al. 2013), then adjusted manually. The final data matrix can be obtained from the corresponding authors and was deposited in TreeBase (S29861).

Two model selection strategies Akaike (corrected) and Bayesian information criteria (AICc and BIC) implemented in MEGA 6 were used to determine the substitution models. The models K2+G with the lowest BIC score and GTR+G+I with the lowest AICc value were chosen to infer the phylogenetic trees by using the Maximum Likelihood (ML) in MEGA 6. For ML analysis, initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analyses performed a non-parametric bootstrap analysis (1000 replicates) to assess branch support.

Result

nrDNA ITS sequences and the phylogenetic analysis

The ITS region was successfully sequenced for six specimens (two sequences for the holotype; Table 1; Fig. 1, in bold). According to the NCBI Blastn identity, the ITS sequence data indicate that all of our specimens of *Usnea* belong to the subgenus *Eumitria*, and are close to reference sequences of *Eumitria firmula* (MW267133, 92.59%), *Usnea baileyi* (MN080242, 92.78%), and *Eumitria* cf. *pectinata* (MW267149, 91.98%).

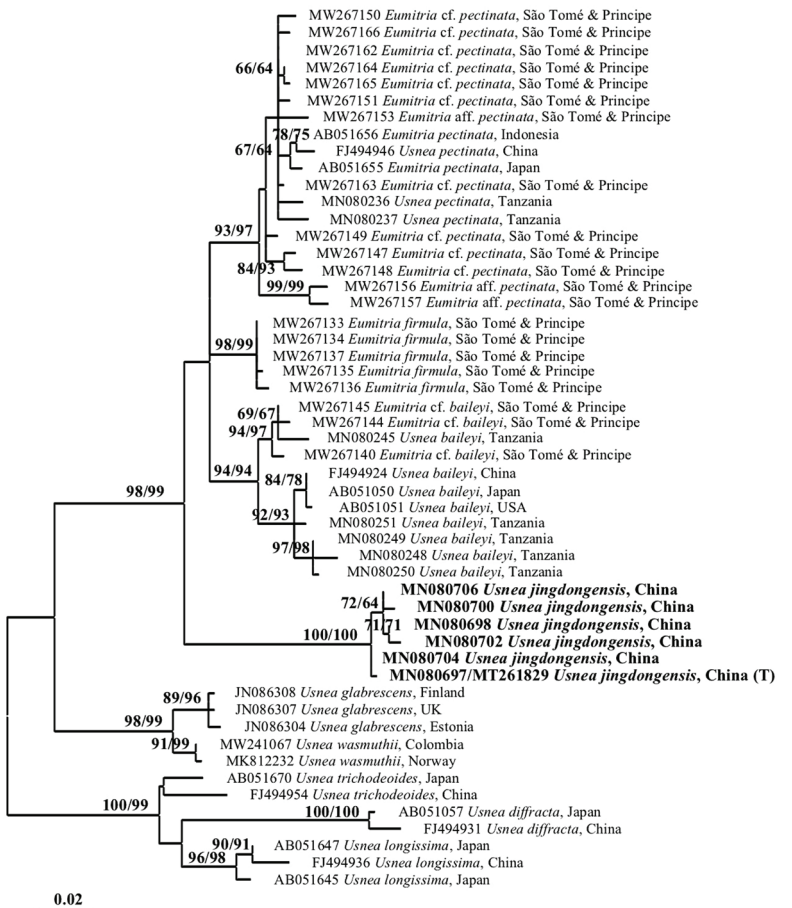

Fig. 1. Phylogenetic relationships of *Usnea jingdongensis* and similar species in *Usnea* subg. *Eumitria*, inferred from nrDNA ITS (ITS1+5.8S+ITS2) sequence data. Species in *U.* subg. *Dolichousnea* were selected as outgroup. Support is indicated for branches characterized by bootstrap frequencies ≥50% with K2+G / GTR+G+I models. Our new sequences are in bold.

The Maximum Likelihood (ML) tree inferred from the nrDNA ITS with the highest log likelihood (–2351.33) is presented in Fig. 1. The sequence data matrix comprised 52 samples (from nine taxa) and 508 characters, of which 355 (69.88%) were constant and 116 (22.83%) were parsimony-informative. All samples of *Usnea jingdongensis* were well defined as a distinct monophyletic lineage with 100% bootstrap support frequencies (BS) by both the Kimura 2+G and GTR+G+I models (BS: 100%/100%). The ML tree of our aligned matrix also recovered our new samples in a strongly supported sister relationship (BS: 98%/99%) with the samples of related species including *U. baileyi* (BS: 94%/94%), *U. firmula* (BS: 98%/99%), and *U. pectinata* (BS: 93%/97%), which all belong to subgenus *Eumitria*.

The results indicated that sequences of nrDNA ITS were sufficient to distinguish all species in the current data set of *Usnea*.

Our proposal of *Usnea jingdongensis* as a new species is justified by its demonstration as a distinct monophyletic clade.

Taxonomy

Usnea jingdongensis S.Y. Guo & L.F. Han, **sp. nov.** Fig. 2

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Differs from *Usnea flaveola* by its thallus trunk jet black at the base, its white or pale brown to brown medulla, its white to pale brown fistulose axis, its tubular axis with loose, pale yellow or pale brown to brown hyphae inside, and its production of protocetraric acid.

Type: China. Yunnan Province, Jingdong county, Ailaoshan Mountain, 24.53°N, 101.02°E, alt. 2450 m, on bark, 5 December 2018, L.F Han & H.D. Wen 2018120543 (**Holotype**, HMAS-L; GenBank MN080697, MT261829).

Etymology. The epithet refers to Jingdong County, where the species was collected.

Thallus corticolous, fruticose, erect or subpendent, up to 11 cm long, greyish-green to yellowish-green when fresh, greyish-green when dry, with anisotomic-dichotomous, sparse to dense ramification (Fig. 2A). Trunk always thinner at the contact point and thickening upwards, with jet black pigmentation extending to the base of the branches, not annulated, up to 1.0 cm long. Main branches 1.0–1.8 mm in diameter, slowly tapering in longitudinal section, terete in cross-section, uninflated. Lateral branches not constricted at the ramification point; foveolae and maculae absent; fibrils slender (up to 5 mm long), abundantly distributed on the branches (Fig. 2B-D); fibercles rare; papillae present on main and lateral branches, many to numerous, short verrucose to cylindrical (Fig. 2B–D); tubercles (containing medulla), numerous, cylindrical (Fig. 2B–D); soralia and isidiomorphs absent.

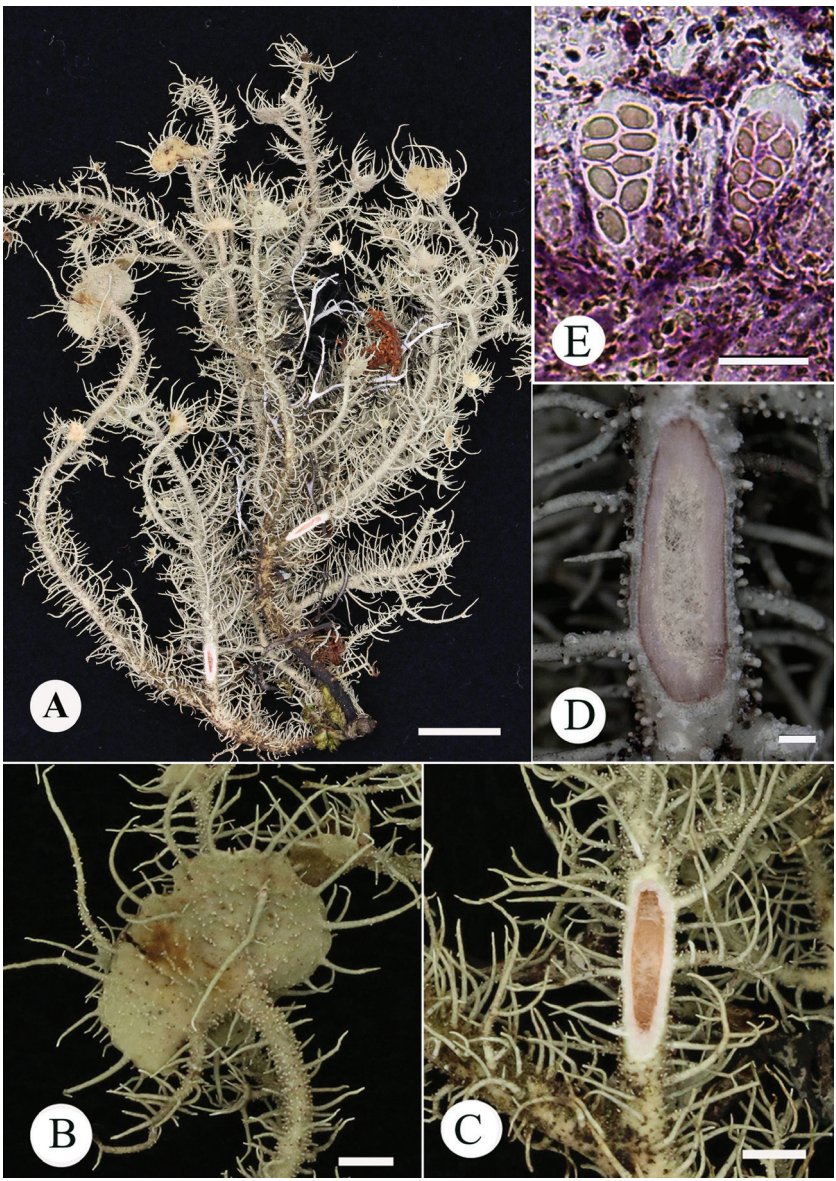

Fig. 2. *Usnea jingdongensis* (A–C, E = holotype, Han & Wen 2018120543; D = Han & Wen 2018120566). A. thallus; B. the reverse side of apothecia; C. fistulose axis with brown loose hyphae inside; D. medulla with brown pigment; E. asci and ascospores. Scale bars: A = 10 mm; B, C = 2 mm; D = 500 μm; E = 20 μm.

Cortex shiny to vitreous in section, very rigid, lacking pigment, thin to moderately thick, 9–16% of the radius. Medulla compact in density, thin, 3–5% of the radius, white or with pale brown to brown pigmentation. Fistulose (Tubular) axis extremely large, axis pale white to pale brown, 79–87% of the diameter; fistulose 36–58%, with loose hyphae inside, pale yellow, pale brown to brown (Fig. 2C, D).

Photobiont *Trebouxia*.

Apothecia common, lateral, up to 10 mm in diameter, disc-shaped, usually flat, with many long cilia and short branches. Disc pale white to pale yellow or brown, not pruinose. Paraphyses simple, septate, ± swollen at the apices; Epihymenium 12.0–18.0 μm thick; Hymenium 48.0–70.0 μm thick; Hypothecium 45.0–75.0 μm thick; Asci clavate; Ascospores, broad ellipsoid, 8.5–12.5 × 4.5–8.5 μm (Fig. 2E).

Pycnidia not seen.

Chemistry—K+ pale yellow, C–, KC+ yellow, Pd+ brick-red. Usnic acid, protocetraric acid, zeorin and three unidentified substances (TLC).

Ecology & distribution—*Usnea jingdongensis* is known only from the type locality where it is abundant with epiphytic lichens in the tropical and subtropical montane forest ecosystems. More than 60 epiphytic lichen species belonging to 26 genera and 17 families were recorded in the primary and secondary forests including six species in *Usnea* (Li & al. 2007; Han & al. 2020).

Variation—The abundance of fibrils and papillae may vary among individuals, but both are conspicuously present, at least on the main branches. The colours of medulla, axis, and tubular axis also vary among individuals or in response to environmental changes (Fig. 3).

Selected additional specimens examined: (All in HMAS-L and HBNU). **China, Yunnan Province, Jingdong County**, Mt. Ailaoshan, 24.53°N 101.02°E, alt. 2450 m, on bark, 23 August 2018, L.F. Han 2018082322a (GenBank MN080706, medulla brown, axis white, tubular axis pale brown, Fig. 3d); 5 December 2018, L.F. Han & H.D. Wen 2018120532 (GenBank MN080702); 5 December 2018, L.F. Han & H.D. Wen 2018120562b (GenBank MN080698, medulla grayish white, axis pale brown, tubular axis grayish brown, Fig. 3c); 5 December 2018, L.F. Han & H.D. Wen 2018120566 (GenBank MN080704; medulla with obviously brown pigment, Fig. 2d); 5 December 2018, L.F. Han & H.D. Wen 2018120582 (GenBank MN080700, medulla and axis white, tubular axis brown, Fig. 3b); 23 September 2020, L.F. Han & H.D. Wen 2020092310 (medulla and axis dark brown, tubular axis brown, Fig. 3f); 23 September 2020, L.F. Han & H.D. Wen 2020092316 (medulla, axis, and tubular axis all white to pale white, Fig. 3a); 18 December 2020, L.F. Han & H.D. Wen 2020121815 (medulla dark brown, axis brown, tubular axis faint yellow, Fig. 3e).

Fig. 3. *Usnea jingdongensis*: colour of medulla, axis, and tubular axis in longitudinal section of branch at ITS widest diameter above first ramification. A. Han & Wen 2020092316, medulla, axis, and tubular axis all white to pale white; B. Han & Wen 2018120582, medulla and axis white, tubular axis brown; C. Han & Wen 2018120562b, medulla grayish white, axis pale brown, tubular axis grayish brown; D. Han 2018082322a, medulla brown, axis white, tubular axis pale brown; E. Han & Wen 2020121815, medulla dark brown, axis brown, tubular axis faint yellow; F. Han & Wen 2020092310, medulla and axis dark brown, tubular axis brown. Scale bars: A, B, D, E = 500 μm; C, F = 750 μm.

Comments—*Usnea jingdongensis* is an eumitrioid *Usnea* species characterized by the fruticose, erect or subpendent thallus with anisotomic-dichotomous branching; jet black trunk base; uninflated branches with numerous fibrils; loose hyphae in fistulose axis with pale yellow to brown pigmentation; and the absence of soralia.

Usnea flaveola Motyka is similar to *U. jingdongensis* in having fistulose axis

and many fibrils, lacking soralia, but differs by the trunk concolorous with the branches, colorless medulla, and the tubular axis with colorless loose hyphae inside (Truong & Clerc 2013).

Usnea pectinata Taylor resembles *U. jingdongensis* in morphology, but is discriminated by a pendulous thallus, sometimes with soralia, pale punctiform maculae on lateral branches, and dark brown pigmented solid axis with some fistulose areas in the central part of the axis in main branch (Ohmura 2001, 2012; Temu & al. 2019). *Usnea jingdongensis* is somewhat related to *U. baileyi* (Stirt.) Zahlbr., which is characterized by the punctiform soralia on the branches, the red pigment in the medulla and the presence of zeorin (Ohmura 2001, 2012; Truong & Clerc 2013; Temu & al. 2019).

Usnea firmula (Stirt.) Motyka is also similar to *U. jingdongensis*, but can be distinguished by being annularly cracked at the base showing the rose medulla, branches slightly inflated, and sparse cilia on the margin of apothecia (Dodge 1956, 1957), and with fibrils numerous along all branches, 1–2 cm long (Swinscow & Krog 1974).

Usnea jingdongensis perhaps resembles *U. brunnescens* C.W. Dodge, which can be distinguished by the thallus being faint brown to russet in the herbarium and producing single isidia on the verrucae (Dodge 1956, 1957).

Morphologically, the new species is closely similar to *Usnea pulvinulata* C.W. Dodge and *U. trullifera* Nyl. in having esorediate and uninflated branches, but *U. pulvinulata* can distinguished by thallus smaller, up to 5 cm, annularly cracked and subareolate in the lower blackened portion; apothecia larger, up to 15 mm in diameter, disc with white-pruinose when young; containing salazinic acid as constant substance (Dodge 1956, 1957; Swinscow & Krog 1974); and *U. trullifera* can be distinguished by the thallus sparsely branched, branches with strongly tuberculate, medulla pale rose next to the axis, and apothecia with a few fibrils along the margin, disc cinnamon with slightly white-pruinose (Motyka 1936; Dodge 1957; Swinscow & Krog 1986), as well as containing salazinic and norstictic acids (Truong & Clerc 2013).

Our sequences for *Usnea jingdongensis* were mentioned in the study of Lücking & al. (2020) as "yet unnamed species composed of unpublished sequences from China (deposited by S. Guo & al. in 2019 and L. Han & al. in 2020)". They demonstrated that the strongly supported clade *Eumitria* formed four supported subclades based on the ITS data, corresponding to our new species *U. jingdongensis*, as well as *U. firmula*, *U. pectinata* s.lat., and *U. baileyi* s.lat. (Ohmura 2002; Nadel 2016; Jaouen & al. 2019; Temu & al. 2019). Our result is consistent with theirs.

Due to the lack of sequence data, the relationship of *Usnea jingdongensis* with other eumitrioid taxa needs to be further investigated. Here, a key to eumitrioid *Usnea* species from China is provided, for better understanding the new species.

Key to eumitrioid Usnea species from China

1. Soralia absent; apothecia common ..... ***U. jingdongensis***
1. Soralia present; apothecia rare or absent 2
2. Medulla with red, pink or orange pigment 3
2. Medulla without pigment 7
3. Soralia without isidiomorphs ..... ***U. vainioi***
3. Soralia usually with isidiomorphs, abundant, especially on thinner branches 4
4. Medulla extremely thin (<5%); the entire medulla with pink-red pigmentation ..... ***U. baileyi***
4. Medulla thicker (>5%); medulla without pink-red pigmentation 5
5. Medulla pigmentation pink to orange; branch segments terete to ridged ..... ***U. perplectata***
5. Medulla pigmentation pale red; branch segments terete 6
6. Bases of branches almost at right angles to the main branches; the tops of some branches curved in all directions ..... ***U. subrectangulata***
6. Bases of branches at acute angles to the main branch; the tops of branches distinctly curved downward, especially upper branches ..... ***U. recurvata***
7. Branches distinctly ridged; axis fistulose ..... ***U. himantodes***
7. Branches distinctly terete; axis solid or partially fistulose ..... ***U. pectinata***

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