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Study on the phylogeny of *Nephroma helveticum* and allied species

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Abstract — *Nephroma subhelveticum* sp. nov. is described, supported by both morphological and molecular data as a species new to science. The nrDNA ITS sequence analysis indicates that *N. subhelveticum* and *N. flavorhizinatum* are allied species of *N. helveticum*, and that *N. isidiosum* derives from *N. helveticum*.

Key words — Asia, China, evolution, lichen, taxonomy

Introduction

The species of *Nephroma* Ach. (*Nephromataceae*) have middle-sized foliose thalli, cyanobacteria (sometimes green algae) as their photobionts, apothecia produced on the lower surface, 8-spored asci, commonly 3-septate ascospores, and often abundant terpenoids (James & White 1987, Burgaz & Martínez 1999, Brodo et al. 2001, Wetmore & Nash 2002, Louwhoff 2009). This genus includes 37 species worldwide (Tian et al. 2011).

Both *Nephroma helveticum* and *N. isidiosum* are cosmopolitan species with terpenoids, and only these two species in the genus have isidia (Brodo et al. 2001). *Nephroma helveticum* has lobules and flat isidia, while *N. isidiosum* has true cylindrical or branched isidia (Brodo et al. 2001). *Nephroma flavorhizinatum* also has lobules and terpenoids (Tian et al. 2011). The similar morphological and chemical characters indicate that these three species may be closely related. Recently some morphologically similar specimens that also have terpenoids, lobules, and very crisp thalli, were found from China. The nrDNA ITS sequences generated from these *Nephroma* representatives have been analysed so as to establish their phylogenetic relationships.

Table 1. GenBank sequences of *Nephroma* and *Lobaria* included in the molecular analyses.

Species	Origin	GenBank no.
N. helveticum	China, Yunnan	DQ001292
	Macaronesia	HQ455071
	USA, southwest	AY124119
	China, Tibet	AY124120
	USA, northwest	AY124124
N. subhelveticum (recorded as *N. helveticum*)	South Korea	DQ066705
	South Korea	DQ066706
	China, central	AY124118
	China, central	AY124122
	China, northeast	AY124127
	Russia, northwest	AY124128
N. parile	Canada, east	AY124148
N. resupinatum	South Korea	DQ066710
L. macaronesica	Outgroup	GU072745
L. pulmonaria		GU072753
L. retigera		EU626996

Materials & methods

Morphology & chemistry

The specimens studied are preserved in SDNU (Lichen Section of Botanical Herbarium, Shandong Normal University). The morphology and anatomy of the specimens were examined using an Olympus SZX16 stereomicroscope and Olympus BX61 compound microscope; morphological characters were photographed under Olympus with DP72. Lichen substances were identified using standardized thin layer chromatography techniques (TLC) with C system (Orange et al. 2010).

Molecular analyses

Taxon sampling — nrDNA ITS sequences were obtained from 41 Chinese *Nephroma* specimens; 13 additional *Nephroma* sequences and outgroup sequences from three *Lobaria* species were downloaded from GenBank (Table 1).

PCR amplification and sequencing — Total DNA was extracted by the modified CTAB method (Rogers & Bendich 1988). E9 (ttgtacacaccgcccgt), NF1R (atccgaggtcaatcgtgt), NF2R (tgatccgaggtcaatcgt), and CL2R (tttctttcctccgcttattga) were used for PCR amplification of the nrDNA ITS as primers. PCR reaction program: initial denaturation at 95°C for 3 min, followed by 35 cycles of 30 s denaturation at 94°C, 45 s annealing at 58°C, 1 min extension at 72°C, and completed with a final 8 min extension at 72°C. Products were purified with Gel Extraction Mini Kit (SABC). Shanghai Genecore Corp. carried out the sequencing reactions.

Data analysis — The sequences were aligned using MAFFT version 7 (Katoh & Standley 2013). The aligned ITS matrix was edited manually and phylogenetic analyses were conducted using MEGA 5 (Tamura et al. 2011). The phylogenetic tree was inferred using the Minimum Evolution method (Rzhetsky & Nei 1992) and tested by 1000 bootstrap replications (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). All positions containing

alignment gaps and missing data were eliminated in pairwise sequence comparisons (Pairwise deletion option). A total of 606 positions comprised the final dataset.

Morphology & chemistry

Nephroma helveticum Ach., Lich. univ.: 523 (1810) Figs 1, 2

Thallus foliose, leathery. Lobes with stout lobules (0.5 × 1 mm) at margin. Upper surface brown; isidia and soredia absent; tomentum and pruina often present; lobules marginal and laminal, often branched. Upper cortex pale

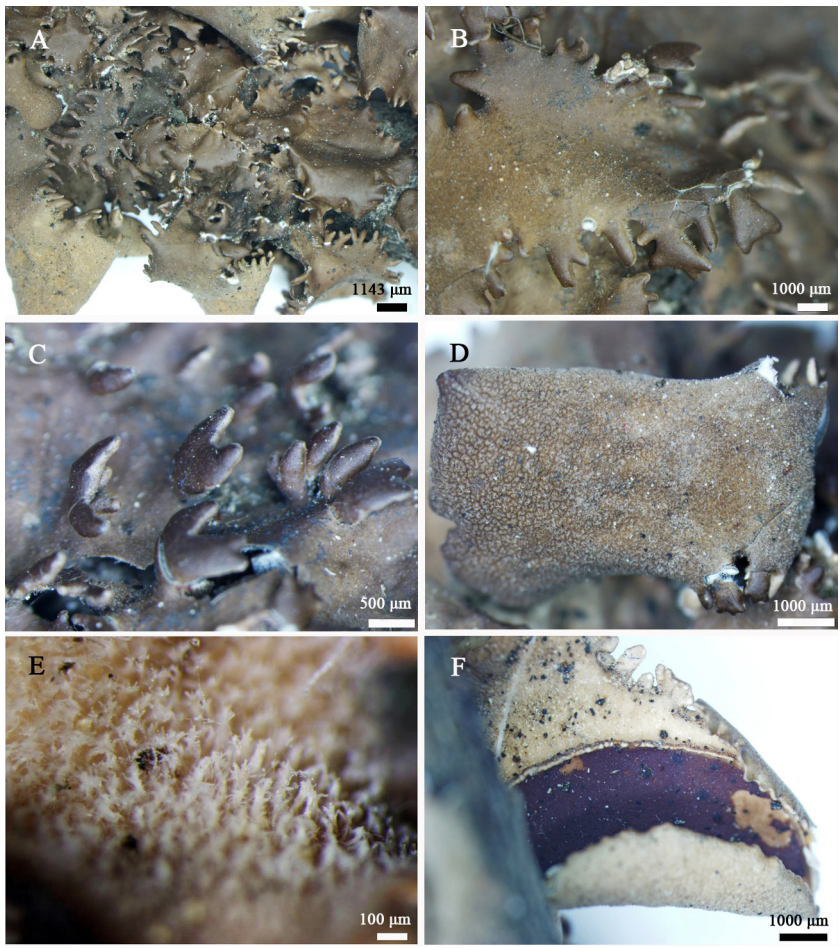

Figure 1. *Nephroma helveticum* (20080653). A: Thallus; B: Lobe with marginal lobules; C: Laminal lobules; D: Rough and tomentose dorsal surface of apothecium; E: Tomentum on lower surface; F: Apothecium immersed on lower surface.

brown, ca. 35 µm thick; photobiont *Nostoc*, ca. 65 µm thick; medulla white, with abundant crystals soluble in water, ca. 80 µm thick; lower cortex pale, ca. 30 µm thick. Lower surface brown, tomentose, paler and smooth at the margins; rhizines stubby, sparse. Apothecia common, immersed on lower surface at tips of lobes, kidney-shaped; disc dark brown, ≤7 mm in diameter; dorsal surface rough, with abundant clustered tomentum; margin usually crenulate. Epihymenium pale brown, without crystals, ca. 15 µm tall; hymenium hyaline, without crystals, ca. 40 µm tall; hypothecium pale brown, without crystals, ca. 35 µm tall. Paraphyses simple. Asci clavate, I+ blue, 8-spored; ascospores pale brown, 3-septate, 15–20 × 3–4 µm. Pycnidia not seen.

Spot tests — Thallus upper surface: K–, C–, KC–, PD–; medulla: K–, C–, KC–, PD–.

Secondary metabolites — N1, N2, ± other lichen substances (Fig. 3).

Ecology & distribution in China— on bark or moss in Guizhou, Jilin, Shanxi, Shaanxi, Sichuan, Tibet, and Yunnan.

Specimens examined — **CHINA**. **Guizhou**, Kaili, Mt. Leigong, alt. 1027 m, on bark, 2 Nov 2009, H.Y. Wang 20102726. **Jilin**, Wangqing, Mt. Tulaopoding, alt. 1100 m, on moss, 22 Jul 2012, H.Y. Wang 20129292. **Shanxi**, Ningwu, Mt. Luya, alt. 2500 m, on bark, 26 Aug 2011, H.Y. Wang 20121819A. **Shaanxi**, Meixian, Mt. Taibai, alt. 2100 m, on bark, 2 Aug 2005, W. Fu 20083001; 17 Jun 2011, Z.L. Huang 20114881(GenBank JX867681); alt. 2150 m, on bark, 17 Jun 2011, Z.L. Huang 20114339 (GenBank JX867682); alt. 2200 m, on bark, 2 Aug 2005, W. Fu 20080332, 20100571; alt. 2700 m, on bark, 17 Jun 2011, Z.L Huang 20114639K; alt. 2950 m, on bark, 17 Jun 2011, Z.L. Huang 20114717 (GenBank JX867703). **Sichuan**, Litang, alt. 4200 m, on bark, 5 Nov 2008, H.Y. Wang 20080388; Litang, Mt. Kazila, alt. 4700 m, on bark, 7 Nov 2008, H.Y. Wang 20080323, 20080354. **Tibet**, Nyingchi, Dongjiuxiang, alt. 2550 m, on bark, 17 Jul 2011, Y.L. Cheng 20114928, 20117991 (GenBank JX867707), 20117991A (GenBank JX867710), 20117991C (GenBank JX867713), 20118005, 20118005A, 20118005D, 20118005E, 20118005F (GenBank JX867712), 20118006B (GenBank JX867704), 20118006C (GenBank JX867683), 20118011, 20118044, 20118255A (GenBank JX867709), 20118300 (GenBank JX867705), 20118545; Mt Sejila, alt. 4100 m, on bark, 20 Oct 2007, G.Y. Han 20072907; alt. 4500 m, on bark, 20 Jul 2011, Y.L. Cheng 20118118A, 20118461, 20118729A (GenBank JX867706), 20118729E; Lulangzhen, observation station, alt. 2550 m, on bark, 14 Jul 2011, D.F. Jiang 20119099. **Yunnan**, Shangri-La, Tianshengqiao, alt. 3500 m, on bark, 3 Nov 2008, H.Y. Wang 20080653 (GenBank JX867708), 20081370, 20081391, 20081515, 20081715, 20081885, 20082058, 20082065, 20082181, 20082185, 20082213, 20082264, 20082270, 20082291, 20082355, 20102930, 20083063, 20083097, 20083312, 20083385, 20083861, 20086001, 20086011; Mt. Shika, alt. 4300 m, on bark, 2 Nov 2008, H.Y. Wang 20081991, 20082033, 20082218, 20083336, 20086017; Yulong, Mt. Laojun, alt. 4000 m, on bark, 6 Nov 2009, H.Y. Wang 20100564 (GenBank JX867711); 7 Nov 2009, H.Y. Wang 20090001, 20100562; Jianchuan, Mt. Shibao, alt. 2600 m, on bark, 31 Oct 2008, H.Y. Wang 20083409; Lijiang, Mt. Yulong, alt. 3200 m, on bark, 7 Nov 2009, M. Li 20102729A.

Comments — *Nephroma helveticum* is considered to produce flat isidia (Brodo et al. 2001). However, we did not find true isidia on our specimens. The lobules of *N. helveticum* from China are varied (Fig. 2 I–N), sometimes very narrow and

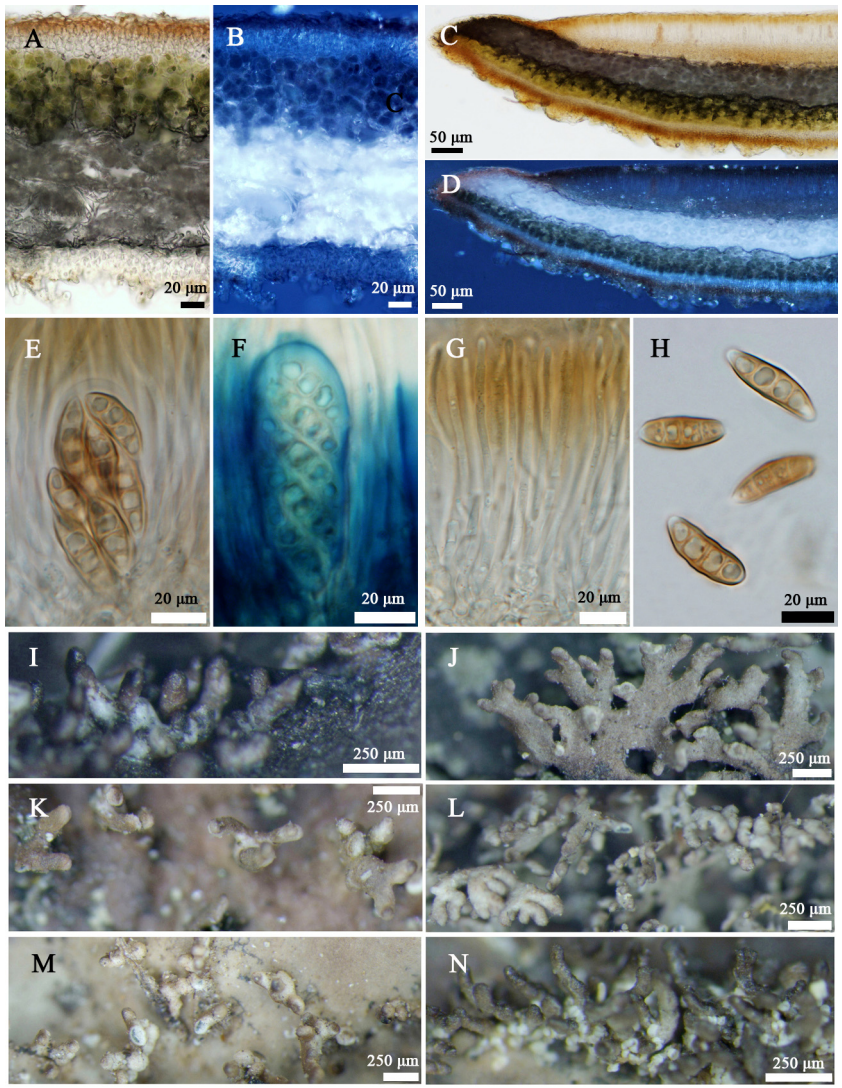

Figure 2. *Nephroma helveticum* (A–H, 20080653; I, 20080323; J, 20118011; K, 20118005D; L, 20118729E; M, 20080388; N, 20100564). A: Section of thallus; B: Crystals in thallus; C: Section of apothecium; D: Crystals in apothecium; E: Ascus and ascospores; F: Iodine reaction of ascus and paraphyses; G: Paraphyses; H: Ascospores; I–N: Various laminal lobules.

isidium-like but always dorsiventral. Although *N. helveticum* and *N. isidiosum* have similar secondary substances (TLC, Fig. 3), *N. isidiosum* can be clearly separated by its typical cylindrical to coralloid isidia and lack of apothecia.

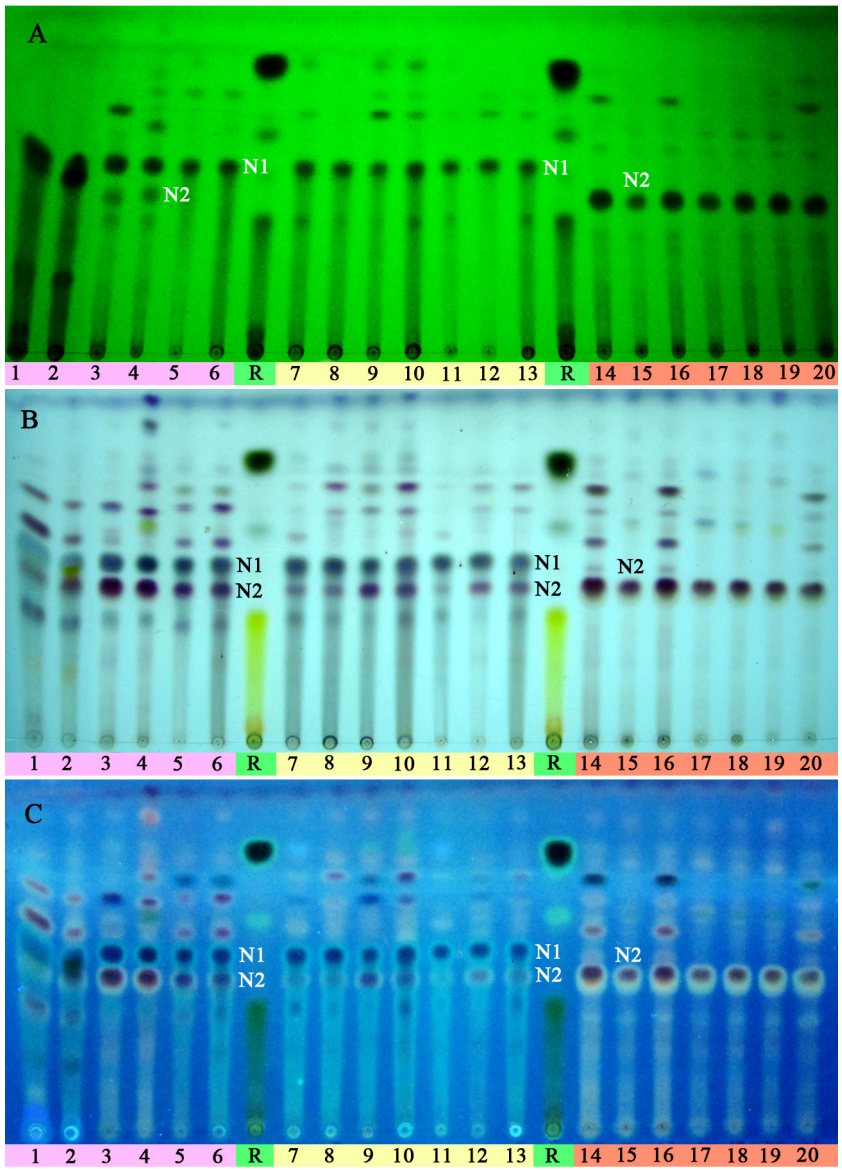

Figure 3. TLC results: A: UV254 nm before acid; B: Sunlight after acid and heating; C: UV365 nm after acid and heating. *Nephroma helveticum* (1, 20100564; 2, 20114717; 3, 20118255A; 4, 20117991C; 5, 20118300; 6, 20080653); *N. isidiosum* (7, 20080192; 8, 20081753; 9, 20084006; 10, 20083227; 11, 20084002; 12, 20114639D; 13, 20081280); *N. subhelveticum* (14, 20108511; 15, 20106583; 16, 20108510; 17, 20106494; 18, 20106581; 19, 20106595; 20, 20105971).

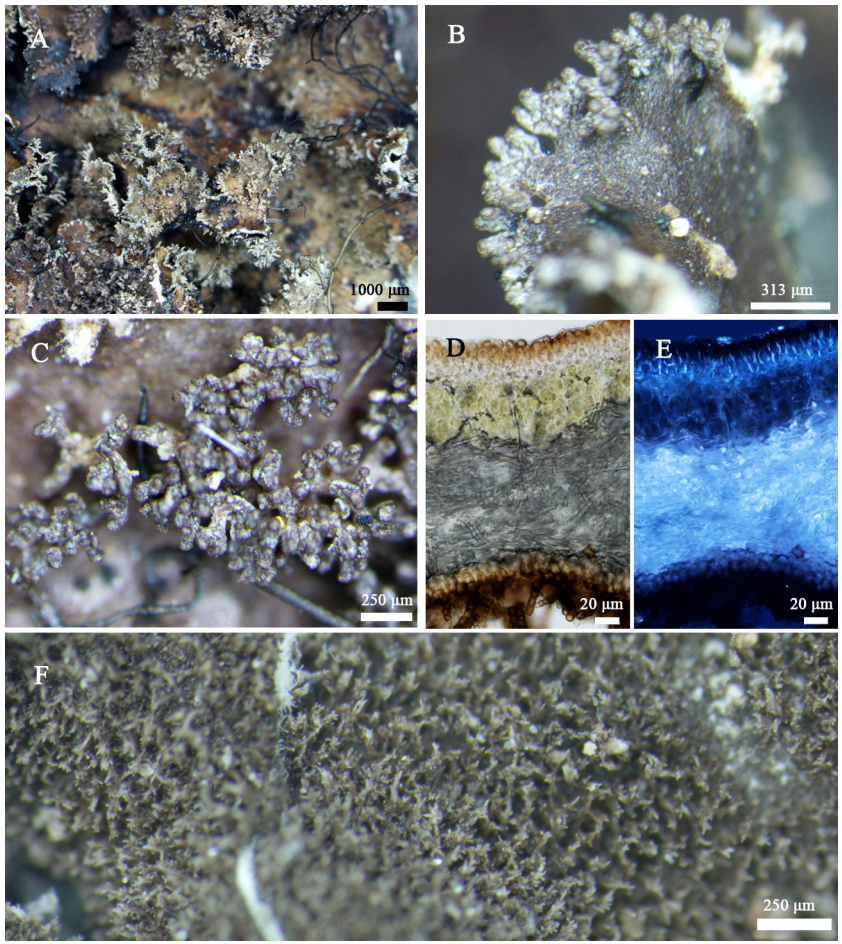

Figure 4. *Nephroma isidiosum* (20080192). A: Thallus; B: Lobe with marginal isidia; C: Laminal isidia; D: Section of thallus; E: Crystals in thallus; F: Tomentum on lower surface.

Nephroma isidiosum (Nyl.) Gyeln., Annals Cryptog. Exot. 4: 126 (1931) Fig. 4

Thallus foliose, leathery. Lobes with cylindrical isidia at margin. Upper surface brown; lobules and soredia absent; tomentum and pruina often present; isidia marginal and laminal, cylindrical to coralloid. Upper cortex pale brown, ca. 30 µm thick; photobiont *Nostoc*, ca. 50 µm thick; medulla white, with abundant crystals soluble in water, ca. 90 µm thick; lower cortex brown, ca. 12 µm thick. Lower surface brown, tomentose, paler and smooth at the margins; rhizines stubby, sparse. Apothecia and pycnidia not seen.

Spot tests—Thallus upper surface: K–, C–, KC–, PD–; medulla: K–, C–, KC–, PD–.

Secondary metabolites — N1, N2, ± other lichen substances (Fig. 3)

Ecology & distribution in China — on bark or ground with moss in Shaanxi, Sichuan, Tibet, and Yunnan.

Specimens examined — **CHINA**. **Shaanxi**, Meixian, Mt. Taibai, alt. 2700 m, on bark, 17 Jun 2011, Z.L. Huang 20114639D (GenBank JX867700). **Sichuan**, Litang, alt. 4200 m, terricolous, 5 Nov 2008, Z.S. Sun 20080225, 20080345, 20080753, 20080843, 20081280 (GenBank JX867701), 20084000, 20084006 (GenBank JX867689), 20084008 (GenBank JX867698); on bark, 5 Nov 2008, Z.S. Sun 20080661 (GenBank JX867694), 20083995, 20084004 (GenBank JX867693); 7 Nov 2008, H.Y. Wang 20084003 (GenBank JX867690); Mt. Kazila, alt. 4700 m, 7 Nov 2008, on bark, H.Y. Wang 20080238 (GenBank JX867695), 20080286, 20080769, 20080871, 20080865, 20081923 (GenBank JX867697), 20082001 (GenBank JX867687), 20084002 (GenBank JX867696), 20084005, 20084009, 20084010, Z.S. Sun 20080192 (GenBank JX867688), 20080846 (GenBank JX867691), 20080855 (GenBank JX867684), 20081753 (GenBank JX867699), 20083018 (GenBank JX867686). **Tibet**, Nyingchi, Lulangzhen, Dongjiuxiang, alt. 2550 m, on bark, 17 Jul 2011, Y.L. Cheng 20118005C; Lulangzhen, Observation station, alt. 3400 m, on bark, 12 Jul 2011, Y.L. Cheng 20118228. **Yunnan**, Lijiang, Jiuhexiang, alt. 2700 m, on bark, 30 Oct 2008, Z.S. Sun 20081681; Shangri-La, Tianshengqiao, alt. 3500 m, on bark, 3 Nov 2008, H.Y. Wang 20082176, 20082177, 20083800, 20086000, Y.D. Du 20082092 (GenBank JX867702), Z.J. Ren 20082225 (GenBank JX867685), Z.S. Sun 20083227 (GenBank JX867692).

Comments — See *N. helveticum*.

Nephroma subhelveticum H.Y. Wang, **sp. nov.** Figs 5, 6

MycoBank MB 801765

Differs from *Nephroma helveticum* by its crisp thin thallus, its finer marginal lobules, its smooth ridged non-tomentose dorsal apothecial surface, and its lack of lichen substance N1.

Type — China. Jiangxi, Ji-An, Qianmocun, Mt. Nanfengmian, alt. 1300 m, 1 Nov 2010, on bark, H. Y. Wang 20106583 (**Holotype**, SDNU; GenBank, JX867680).

Etymology — Referring to the very close genetic relationship and morphological similarity with *Nephroma helveticum*.

Thallus foliose, very crisp, about 4 cm in diameter. Lobes long and narrow, usually 5 × 10 mm, with fine lobules at margin (0.1 × 0.2 mm). Upper surface gray, smooth; tomentum, isidia, soredia and pruina absent; lobules marginal and laminal, long or round, branched or not. Upper cortex pale, ca. 20 μm thick; photobiont *Nostoc*, ca. 55 μm thick; medulla white, with abundant crystals soluble in water, ca. 55 μm thick; lower cortex pale, ca. 15 μm thick. Lower surface pale or pale brown, tomentose, paler and smooth at the margins; rhizines stubby, sparse. Apothecia common, immersed on lower surface at tips of lobes, kidney-shaped; disc dark brown, 1–4 mm in diameter; dorsal surface with obvious ridges and lobules, without tomentum; margins usually with

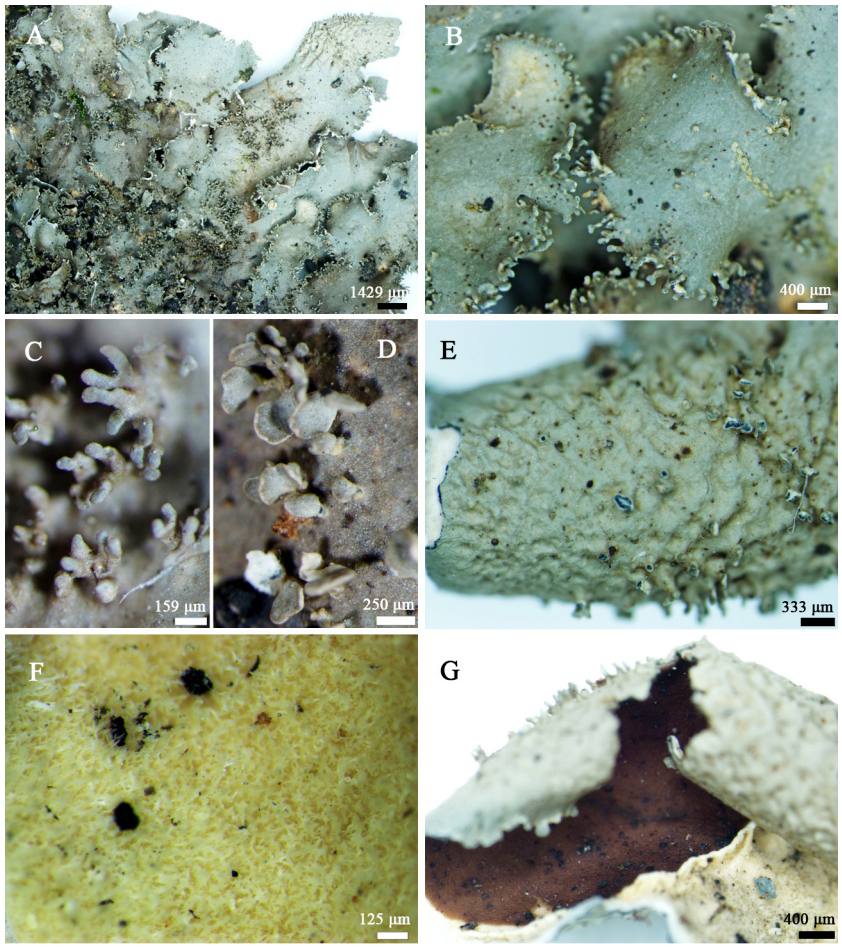

Figure 5. *Nephroma subhelveticum* (Holotype, 20106583). A: Thallus; B: Lobe with marginal lobules; C-D: Laminal lobules; E: Smooth and ridged dorsal surface of apothecium; F: Tomentum on lower surface; G: Apothecium immersed on lower surface.

lobules. Epihymenium pale brown, without crystals, ca. 10 µm tall; hymenium hyaline, without crystals, ca. 38 µm tall; hypothecium hyaline, without crystals, ca. 25 µm tall. Paraphyses simple. Asci clavate, I+ blue, 8-spored; ascospores pale brown, 3-septate, 15–20 × 3–4 µm. Pycnidia not seen.

Spot tests—Thallus upper surface: K–, C–, KC–, PD–; medulla: K–, C–, KC–, PD–.

Secondary metabolites— N2 (Fig. 3)

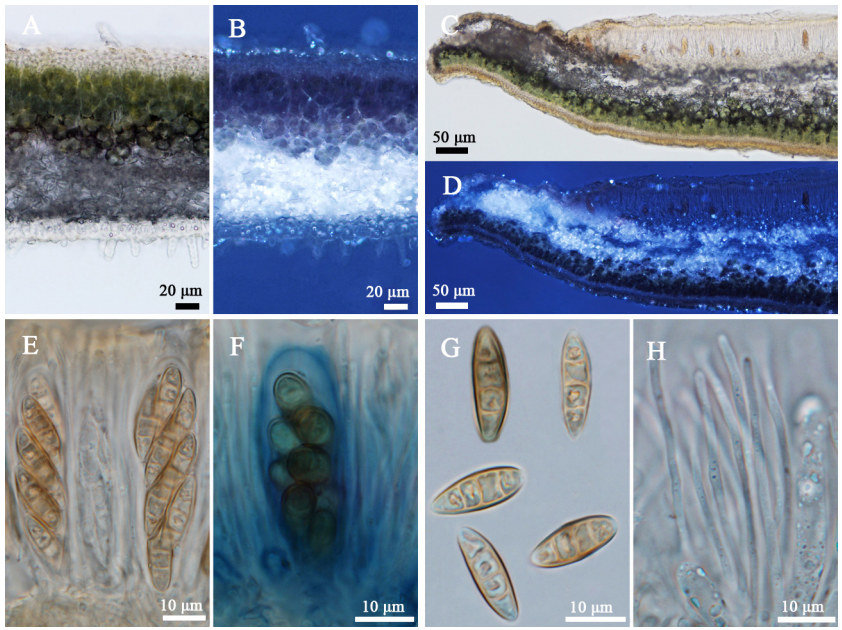

Figure 6. *Nephroma subhelveticum* (Holotype, 20106583). A: Section of thallus; B: Crystals in thallus; C: Section of apothecium; D: Crystals in apothecium; E: Asci and ascospores; F: Iodine reaction of ascus and paraphyses; G: Ascospores; H: Paraphyses.

Ecology & distribution in China — on bark or moss in Jiangxi and Fujian.

Additional specimens examined — **CHINA**. **Jiangxi**, Ji-An, Qianmocun, Mt. Nanfengmian, alt. 1300 m, on bark, 1 Nov 2010, H.Y. Wang 20106581, 20106595; 2 Nov 2010, H.Y. Wang 20106494 (GenBank JX867679). **Fujian**, Wuyishan, Tongmucun, alt. 1200 m, on moss, 25 Oct 2010, M. Li 20105971, 20108510 (GenBank JX867677), 20108511 (GenBank JX867678).

Comments—The crisp and thin (rather than leathery and thick) thallus, the fine (not stout) marginal lobules, the smooth ridged apothecial dorsal surface of apothecia lacking in tomentum (rather than rough and tomentose), and the absence of N1 lichen substances distinguish the new species from *N. helveticum*.

Phylogeny **Fig. 7**

The ITS sequence analysis indicates that *Nephroma flavorhizinatum, N. subhelveticum, N. helveticum,* and *N. isidiosum* are very closely related. *Nephroma flavorhizinatum* is a sister group (92% bootstrap support) of the clade including *N. subhelveticum, N. helveticum,* and *N. isidiosum*, with a 0.066–0.095

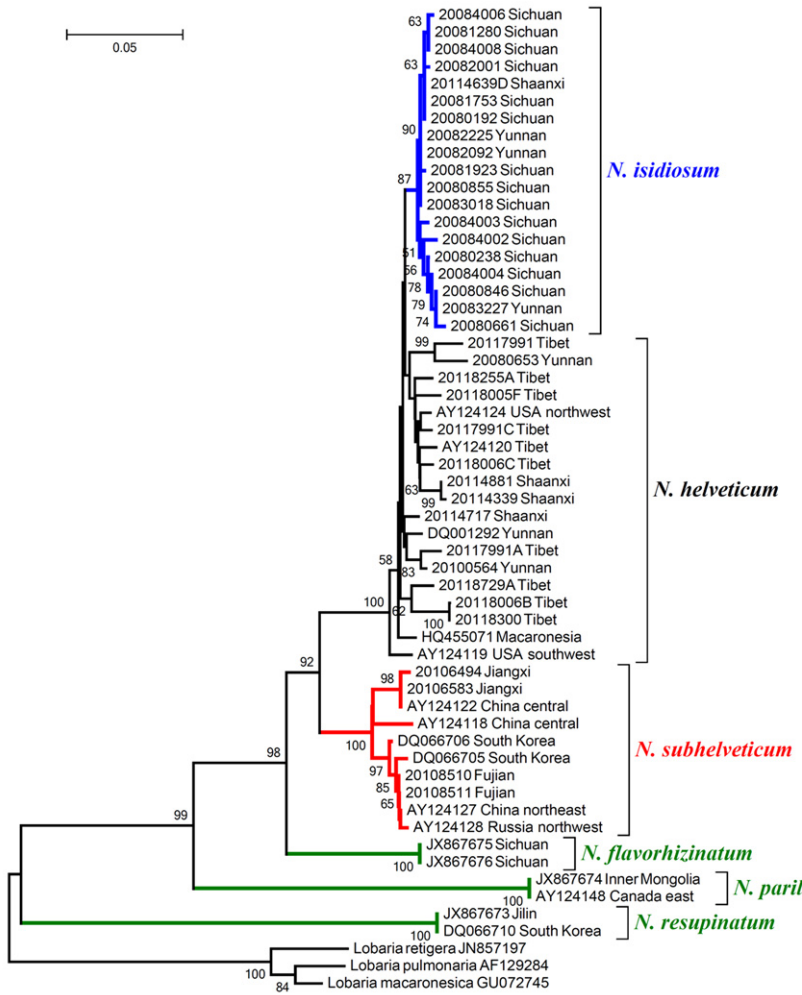

FIGURE 7. The ME tree inferred from ITS data. Bootstrap values >50% (1000 replicates) are shown next to the branches.

evolutionary distance separating sister groups. *Nephroma flavorhizinatum* was first reported from China as a new species differentiated by a golden yellow rhizinal base and distinct pruina at the dorsal surface of apothecia, lobules, and terpenoids (Tian et al. 2011). This morphological differentiation is supported here by the molecular phylogenetic analysis.

Nephroma subhelveticum is sister (100% bootstrap support) to the clade containing *N. helveticum* and *N. isidiosum*, with a 0.046–0.068 evolutionary distance separating sister groups. The *N. subhelveticum* clade (100% bootstrap support) includes the samples from China, South Korea, and northwest Russia; however, the evolutionary distances among these samples are only 0–0.021. The samples within the *N. subhelveticum* sister group are from China, USA, and Macaronesia, among which the evolutionary distances are also very close (0–0.031). These results indicate that the *N. subhelveticum* clade is monophyletic and not conspecific with *N. helveticum*. This result is supported by morphological and chemical characters.

The *N. subhelveticum* clade includes six samples from GenBank (AY1241XX sequences submitted by Lohtander et al. 2002; DQ06670X sequences by Hur et al. 2004). The mtSSU and ITS based research by Lohtander et al. (2002) also included samples of *N. helveticum* and *N. subhelveticum* (identified as '*N. helveticum*') from USA, China, and Russia; their phylogenetic result is identical with our study: two clades, both with >90% bootstrap support. Lohtander et al. (2002) considered that *N. helveticum* might represent closely related taxonomic aggregates. However, they did not compare their specimens morphologically. We have not found detailed information regarding the specimens sequenced by Hur et al. (2004).

The *N. isidiosum* clade is not sister to *N. helveticum*, but rather a child (derived) clade of *N. helveticum;* evolutionary distances between *N. isidiosum* and *N. helveticum* are only 0.009-0.029, suggesting that *N. isidiosum* and *N. helveticum* are conspecific. However, *N. isidiosum* is a monophyletic group with 87% bootstrap support. The genetic diversity level of ITS within *N. isidiosum* is obviously lower than that within *N. helveticum* (Table 2). Moreover, *N. isidiosum* has quite different isidia from *N. helveticum*. These results indicate that all the members of *N. isidiosum* have a common ancestor, and the gene flow between *N. isidiosum* and *N. helveticum* has possibly stopped. In other words, while not conspecific with *N. helveticum*, *N. isidiosum* is a species recently derived from *N. helveticum.*

Table 2. Evolutionary ITS distances within *Nephroma isidiosum* and *N. helveticum*.

Species	Collection locality	No. of Samples	Evolutionary distance (MCL)
N. isidiosum	Shaanxi, Sichuan, Yunnan	19	0–0.011
N. helveticum	Shaanxi	3	0.001–0.015
	Tibet	10	0–0.027
	Yunnan	3	0.009–0.025

Acknowledgements

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