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Three new species of *Incrucipulum* (Lachnaceae, Helotiales, Ascomycota) from Japan

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Abstract

Three new species of *Incrucipulum* were described from Japan: *I. foliicola* and *I. pseudosulphurellum* on *Myrica gale* subsp. *tomentosa* and *I. hakonechloae-macrae* on *Hakonechloa macra*. Disposition to *Incrucipulum* was justified by molecular phylogenetic analysis based on ITS-5.8S, LSU and RPB2 regions, and monophyly of *Incrucipulum* was also confirmed. Some apomorphic characters of *Incrucipulum* were identified. By addition of three new species, the genus *Incrucipulum* now contains 13 species.

Key words: *Incrucipulum foliicola, Incrucipulum hakonechloae-macrae, Incrucipulum pseudosulphurellum,* mycobiota, taxonomy

Introduction

The genus *Incrucipulum* Baral (1985: 71) belongs to the family Lachnaceae Raitviir (Helotiales, Leotiomycetes, Ascomycota). Members of *Incrucipulum* were separated from wider genus *Dasyscyphus* Nees ex Gray (1821: 670) (=currently recognized congeneric with *Lachnum* Retzius (1769: 255) (Hosoya *et al.* 2010)) based on their thick-walled (0.5–1.3 µm), cubic ectal excipular cells with densely granulated surface (Baral & Krieglsteiner 1985) as distinguishing characters. Thin to thick-walled hairs with crystallized and thick-walled apices were also mentioned as common characters (Baral & Krieglsteiner 1985). The structure of ectal excipulum was noted also by Le Gal (1939) for *I. ciliare* (Schrader 1799: 63) Baral (1985: 72) and *I. capitatum* (Peck 1878: 60) Baral (1985: 72) but had not been adopted as a genus-level taxonomic character at that time. Now the generic concept of *Incrucipulum* is widely accepted.

Four species (*I. capitatum*, *I. ciliare* [type species], *I. sulphurellum* (Peck 1878: 59) Baral (1985: 72) and *I. virtembergense* (Matheis 1977: 240) Baral (1985: 73)) were originally included (Baral & Krieglsteiner 1985). Six species were later added: *I. densiseptatum* (Raitviir & R. Galán (in Galán & Raitviir 1994: 464)) Raitviir (2006a: 45), *I. longispineum* (Hosoya & Issh. Tanaka (in Tanaka & Hosoya 2001: 598)) Sasagawa & Hosoya (in Hosoya *et al.* 2010: 175), *I. radiatum* (Issh. Tanaka & Hosoya 2001: 606) Sasagawa & Hosoya (in Hosoya *et al.* 2010: 175), *I. saccardoi* (Raitviir & Sacconi (in Raitviir 1991: 166)) Raitviir (2006a: 47), *I. sinegoricum* (Raitviir 1985: 3) Raitviir (2006b: 140) and *I. uralense* (Chlebicki 2002: 84) Chlebicki & Suková (in Chlebicki 2005: 109) (Baral & Krieglsteiner 1985, Index Fungorum 2018, Raitviir 2006b). Species of *Incrucipulum* occur on various substrates such as fallen twigs, culms and leaves and have strong selectivity to host species and their parts.

In the process of elucidating Japanese lachnaceous mycobiota, four undocumented species of *Incrucipulum*, including three undescribed species and one new record from Japan, were collected. Molecular phylogenetic analysis was conducted to confirm the validity of inclusion of the three new species into *Incrucipulum* and identify apomorphic characters in *Incrucipulum*.

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Material and methods

Collection and Isolation

Five *Incrucipulum* specimens of three new species and *I. capitatum* were collected from four localities in Japan. Specimens were air-dried naturally for one week in 20 °C and deposited in the mycological herbarium of the National Museum of Nature and Science (TNS). Multi-spored isolates were obtained by collecting discharged ascospores on potato dextrose agar (PDA, Nissui, Tokyo) and kept on PDA slants at 4 °C. Isolates were numbered beginning with 'FC-' and will be deposited to NITE National Bioresource Center (NBRC).

Morphological examination

External appearance of apothecia was examined using SZ61 stereoscopic microscope (Olympus, Tokyo, Japan) and microphotographs were taken using DS-L4 (Nikon, Tokyo, Japan). To examine microscopic characters, dried apothecia were detached from the substrate and rinsed in 70% ethanol followed by rehydration in ion-exchanged water for six hours, and then observed in cotton blue dissolved in lactic acid (CB/LA) in squash mount using BX51 microscope equipped with Nomarski interference contrast device (Olympus). Microphotographs were taken using DS-L3 with the camera head DS-Fi2 (Nikon). Line drawings were prepared using drawing device U-DA (Olympus) equipped with BX51. Ascal apex iodine reaction was checked by Melzer's reagent.

DNA extraction

Two mL of 2% malt extract broth was inoculated with isolates and cultivated at room temperature for two weeks. The cultivated mycelia were frozen at −80 °C for two hours, dried by a Bulk Tray Dryer (Labconco, Kansas, USA) and then crushed using TissueLyser (Quiagen, Hilden, Germany). Powdered fungal samples were incubated in cetyltrimethylammonium bromide buffer (CTAB buffer, 2% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA) at 65 °C for one hour, and protein was removed using the mixture of chloroform/isoamylalcohol (24:1). The solution was purified by 6M sodium iodine buffer (6 M NaI, 50 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.1 M Na_2SO_3) (Hosaka & Castellano 2008) with GLASSMILK (Funakoshi, Tokyo, Japan) and washed by ethanol/buffer solution (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl, 50% EtOH). Purified DNA was eluted and dissolved in Tris-EDTA buffer (TE buffer, 10 mM Tris-HCl, 1 mM EDTA).

When isolates were not available, DNA was extracted directly from a fresh apothecium using DNA extraction buffer (SDS 0.35 mM, Proteinase K 0.1mg/ml, Tris-HCl 10 mM, $MgCl_2$ 1.5 mM, KCl 50 mM, dH_2O to adjust to total volume 50 mL). Apothecium was put in a 1.5 mL Eppendorf tube and smashed manually using 1.5 mL pellet pestle (Fisher Scientific, Hampton, USA). 50 µl DNA extraction buffer was added and incubated for 90 min. at 40 °C followed by 10 min. incubation at 90 °C. After centrifuged, the supernatant was preserved as extracted DNA.

Extracted DNA samples were deposited to Molecular Biodiversity Research Center in the National Museum of Nature and Science (Tsukuba, Ibaraki, Japan) and available for molecular phylogenetic researches.

Polymerase chain reaction (PCR) and sequencing

For phylogenetic analysis, the internal transcribed spacer region of nuclear ribosomal DNA containing partial ITS1-5.8S-ITS2 (ITS-5.8S), the partial large subunits nuclear ribosomal RNA gene (LSU) and the section '6–7' of the second largest subunit of the nuclear RNA polymerase II gene (RPB2) were used, because analysis using only ITS-5.8S did not make robust trees in inter- and intra-genus level (Hosoya *et al.* 2010). Extracted DNA was amplified by PCR using EmeraldAmp PCR Master Mix (Takara, Kusatsu, Japan). For ITS-5.8S, the forward primer ITS1F (CTT GGT CAT TTA GAG GAA GTA A) (Gardes & Bruns 1993) and the reverse primer ITS4 (TCC TCC GCT TAT TGA TAT GC) (White *et al*. 1990) were used. For LSU, the forward primer LR0R (ACC CGC TGA ACT TAA GC) and the reverse primer LR5 (TCC TGA GGG AAA CTT CG) (Vilgalys & Hester 1990) were used. For RPB2, the forward primer RPB2-P6F (TGG GGW YTS GTM TGY CCT GC) and the reverse primer RPB2-P7R (CCC ATS GCY TGY TTA CCC AT) (Liu *et al*. 1999) were used.

For ITS-5.8S and LSU, 10 µl PCR reaction were performed in a following protocol: initial denaturation for 3 min at 95 °C, 30 cycles of 94 °C for 35 s, 51 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were purified using ExoProStar Sequencing Clean-up kit (Illumina, San Diego, USA).

For RPB2, 20 µl PCR reaction solution was used in a following protocol: initial denaturation for 3 min at 95 °C, followed by 25 cycles of 95 °C for 45 s, 52 °C for 40 s and 74 °C for 2 min, then another 25 cycles of 95 °C for 50 s, 52 °C for 45 s and 74 °C for 2 min 5 s, and a final extension at 10 min for 72 °C. Appropriate length of amplified DNA in

the agarose gel were checked using the LED transilluminator (Fujifilm Wako, Tokyo, Japan) and cut out using a spatula and then purified using MonoFas DNA Purification Kit I (GL Sciences, Tokyo, Japan) following the manufacturer's instruction.

Cycle sequence reaction was conducted with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) using the same PCR primers mentioned above. Sequencing was carried out using Applied Biosystems 3500xL Genetic Analyzer. Sequences obtained in the present study were assembled using ATGC Ver. 7 (Genetyx, Tokyo, Japan), and then registered to DNA Data Bank of Japan (DDBJ) database synchronized with the GenBank.

For TNS-F-81441, DNA was also extracted directly from an apothecium in addition to DNA extraction from an isolate (FC-6879), because the isolate turned out to be contaminated by bacteria. Sequences derived from the isolate and the apothecia were examined separately.

Phylogenetic analysis

For species of *Incrucipulum*, all available sequences obtained by Hosoya *et al.* (2010) and newly obtained sequences were assorted, and 12 OTUs composed of seven species were obtained (Table 1).

For other lachnaceous members, one or two species were selected from five major lachnaceous genera (*Albotricha* Raitviir (1970: 40)*, Brunnipila* Baral (1985: 49)*, Erioscyphella* Kirschstein (1938: 384)*, Lachnellula* P. Karsten (1884: 138)*, Lachnum* (sensu stricto)), since monophyly of these genera were already suggested (Hosoya *et al.* 2010, Perić & Baral 2014). Species of *Capitotricha* (Raitviir 1970: 88) Baral (1985: 60), *Neodasyscypha* Suková & Spooner (in Suková 2005: 163) and *Proliferodiscus* J.H. Haines & Dumont (in Haines 1983: 536) not included in Hosoya *et al.* (2010) were also added. Since *Lachnum varians* (Rehm 1900: 94) M.P. Sharma (1986: 411) was presumed as a separate lineage from *Lachnum* (sensu stricto) (Hosoya *et al.* 2010, Guatimosim *et al.* 2016), *L. varians* was also included. Two species of *Dasyscyphella* Tranzschel (1899: 11), whose generic affiliation is still unresolved, were incorporated. Species of *Lasiobelonium* Ellis & Everhart (1897: 136), *Trichopeziza* Fuckel (1870: 295) and *Trichopezizella* Dennis ex Raitviir (in Raitviir 1969: 68) were not included because they were indicated to be phylogenetically apart from other lachnaceous genera (Hosoya *et al*. 2010). *Hyaloscypha spiralis* (Velenovský 1934) J.G. Han, Hosoya & H.D. Shin (in Han *et al.* 2014: 161), *Urceolella carestiana* (Rabenhorst 1866: 189) Dennis (1963: 335) and *Hymenoscyphus varicosporoides* Tubaki (1966: 346) were selected as outgroup taxa based on molecular phylogenetic assessment of Hyaloscyphaceae (Han *et al*. 2014). In total, 27 lachnaceous OTUs of 22 species and three outgroup OTUs were gathered (Table 1).

Sequences of 30 OTUs were initially aligned by MAFFT 7 (Katoh 2013) and adjusted manually for each region. Molecular phylogenetic analysis of 30 OTUs was executed based on Maximum likelihood (ML; Felsenstein 1981) and Bayesian inference (BI) analyses. The combined sequence datasets were divided into five data partitions (ITS-5.8S, LSU and each codon position of RPB2). The optimal model was estimated using Kakusan 4 (Tanabe 2011) with default settings. In the ML analysis, the separate model was selected based on Akaike's information criterion (AIC). ML tree search and ML bootstrap analysis (Felsenstein 1985) with 1,000 bootstrap replications were performed using RAxML v.8.2.X (Stamatakis 2016). In the BI analysis, proportional model was selected based on Bayesian information criterion (BIC). Four chains of Metropolis Coupled Markov chain Monte Carlo (MCMCMC) analysis resulted in 5 million generations and sampled every 100 generations using MrBayes v.3.2 (Ronquist *et al*. 2012). The average standard deviation of split frequencies (ASDSF) was verified to become <0.01 and each run was checked to become convergent using Tracer v1.7.1 (Rambaut 2018a). Bayesian Posterior probabilities (BI PPs) were used to evaluate reliability and 50% majority rule consensus tree was adopted.

Trees were illustrated using FigTree v1.4.4 (Rambaut 2018b) based on ML analysis and ML bootstrap value (BS) and BI PPs were plotted on each branch. The data matrix was deposited in TreeBASE with accession ID S24265 (available from http://purl.org/phylo/treebase/phylows/study/TB2:S24265).

TABLE 1. Taxa investigated in the molecular phylogenetic analysis. '-' appears when DNA was extracted not from an isolate but directly from an apothecium. Specimens and sequences of *Incrucipulum* newly obtained in this study were shown in bold.

Taxon	Specimen no.	Locality	Substrate	Collecting date	Isolate / Culture no.	GenBank/DDBJ accession no.		
						ITS	LSU	RPB2
Albotricha acutipila (P. Karst.) Raitv.	TNS-F-16740	Japan, Nagano, Sugadaira	Culm of bamboo	6/17/2006	NBRC 104380	AB481234	LC438571	AB481354
Albotricha albotestacea (Desm.) Raitv.	TNS-F-16497	Japan, Nagano, Sugadaira	Culm of *Miscanthus sinensis*	5/18/2005	NBRC 101346	AB481235	LC424943	AB481340
Brunnipila fuscescens (Pers.) Baral	TNS-F-16635	Japan, Gunma, Agatsuma	Unidentified leaf	4/27/2006	NBRC 104365	AB481255	LC424945	AB481348
Capitotricha bicolor (Bull.) Baral	TNS-F-65670	Switzerland, Graubünden, Filisur	Twig of *Prunus spinosa*	6/6/2016	FC-6101	LC424834	LC424942	LC425011
Capitotricha rubi (Bres.) Baral	TNS-F-65752	Switzerland, Verwaltungskreis, Saicourt	Twig of *Rubus idaeus*	6/4/2016	FC-6075	LC438560	LC438573	LC440395
Dasyscyphella longistipitata Hosoya	TNS-F-16439	Japan, Kanagawa, Yamakita	Cupule of *Fagus crenata*	4/17/2005	NBRC 101335	AB481239	LC424947	AB481331
Dasyscyphella montana Raitv.	TNS-F-16527	Japan, Gunma, Agatsuma	Unidentified wood	5/21/2005	NBRC 102336	AB481242	LC438577	AB481336
Erioscyphella abnormis (Mont.) Baral	TNS-F-80478	Japan, Shizuoka, Oyama	Unidentified wood	6/26/2017	NBRC 113934	LC424837	LC424949	LC425009
***Incrucipulum capitatum* (Peck) Baral**	**TNS-F-81420**	Japan, Hokkaido, Sapporo	Leaf of *Quercus crispula*	6/17/2018	NBRC 113938	**LC424838**	**LC424954**	**LC438592**
Incrucipulum ciliare (Schrad.) Baral	TNS-F-81514	Japan, Saitama, Ogano	Leaf of *Quercus crispula*	8/12/2018	-	LC438568	LC438585	LC438598
Incrucipulum ciliare (Schrad.) Baral	TNS-F-81516	Japan, Saitama, Ogano	Leaf of *Castanea crenata*	8/12/2018	NBRC 113940	LC438565	LC438582	LC438595
Incrucipulum ciliare (Schrad.) Baral	TNS-F-81520	Japan, Shizuoka, Shizuoka, Umegashima	Leaf of *Quercus crispula*	8/18/2018	NBRC 113941	LC438566	LC438583	LC438596
***Incrucipulum foliicola* Tochihara**	**TNS-F-81508**	Japan, Hokkaido, Nemuro	Leaf of *Myrica gale* var. *tomentosa*	7/20/2018	NBRC 113942	**LC438567**	**LC438584**	**LC438597**
***Incrucipulum foliicola* Tochihara**	**TNS-F-81526**	Japan, Hokkaido, Oshamambe	Leaf of *Myrica gale* var. *tomentosa*	7/26/2018	-	**LC438569**	**LC438586**	**LC438599**
***Incrucipulum hakonechloae-macrae* Tochihara**	**TNS-F-81512**	Japan, Saitama, Ogano	Leaf and culm of *Hakonechloa macra*	8/12/2018	-	**LC438564**	**LC438581**	**LC438594**
Incrucipulum longispineum (Hosoya & Issh. Tanaka) Sasagawa & Hosoya	TNS-F-17632	Japan, Miyagi, Sendai	Leaf of *Lyonia ovalifolia*	7/29/2006	NBRC 102347	AB481256	LC438579	AB481362
***Incrucipulum pseudosulphurellum* Tochihara**	**TNS-F-81441**	Japan, Hokkaido, Oshamambe	Twig of *Myrica gale* var. *tomentosa*	7/26/2018	FC-6822	**LC438563**	**LC438588**	**LC438601**

...*Continued on next page*

TABLE 1. (Continued)

Taxon	Specimen no.	Locality	Substrate	Collecting date	Isolate / Culture no.	GenBank/DDBJ accession no.		
						ITS	LSU	RPB2
Incrucipulum pseudosulphurellum **Tochihara**	**TNS-F-81441**	Japan, Hokkaido, Oshamambe	Twig of *Myrica gale* var. *tomentosa*	7/26/2018	-	**LC438570**	**LC438587**	**LC438600**
Incrucipulum radiatum (Issh. Tanaka & Hosoya) Sasagawa	TNS-F-16769	Japan, Nagano, Sugadaira	Leaf of *Fagus crenata*	9/24/2006	NBRC 104385	AB481261	LC438572	AB481359
Incrucipulum radiatum (Issh. Tanaka & Hosoya) Sasagawa & Hosoya	TNS-F-36248	Japan, Niigata, Tokamachi	Leaf of *Fagus crenata*	9/20/2010	NBRC 113933	LC438559	LC438580	LC438593
Lachnellula calyciformis (Batsch) Dharne	TNS-F-81248	Japan, Hokkaido, Engaru	Twig of *Abies sachalinensis*	7/12/2017	NBRC 113935	LC438561	LC438574	LC438590
Lachnellula suecica (de Bary ex Fuckel) Nannf.	TNS-F-16529	Japan, Nagano, Sugadaira	Twig of *Larix kaempfer*	5/21/2005	NBRC 101348	AB481248	LC424944	AB481341
Lachnum soppittii (Massee) Raitv.	TNS-F-16551	Japan, Ibaraki, Mt. Tsukuba	Unidentified leaf	5/28/2005	NBRC 104361	AB481266	LC438578	AB481344
Lachnum varians (Rehm) Spooner	TNS-F-17631	Japan, Kagoshima, Yakushima	Stem of unidentified fern	10/23/2005	NBRC 102332	AB481267	LC438576	AB481330
Lachnum virgineum (Batsch) P. Karst.	TNS-F-16583	Japan, Kanagawa, Yamakita	Unidentified wood	7/2/2005	NBRC 104358	AB481268[a)]	AB926119	AB481343
Neodasyscypha cerina (Pers.) Spooner	TNS-F-65625	Switzerland, Verwaltungskreis, Saicourt	Twig of *Crataegus* sp.	6/8/2016	FC-6068	LC424836	LC424948	LC425013
Proliferodiscus alboviridis (Sacc.) Spooner	TNS-F-17436	Japan, Ibaraki, Tsukuba Botanical Garden	Unidentified wood	7/8/2006	NBRC 108594	LC438558	LC424950	LC438589
Hyaloscypha spiralis (Velen.) J.G. Han, Hosoya & H.D. Shin (outgroup)	TNS-F-17909	Japan, Kumamoto, Kikuchi	Unidentified wood	10/10/2005	NBRC 108585	LC438602	LC438604	LC438606
Hymenoscyphus varicosporoides Tubaki (outgroup)	TNS-F-16472	Japan, Ibaraki, Kasumigaura	Unidentified wood	5/5/2005	NBRC 104355	AB926052	LC424952	AB481329
Urceolella carestiana (Rabenh.) Dennis (outgroup)	TNS-F-18014	Japan, Iwate, Hanamaki	Stem of *Thelypteris nipponica*	5/23/2006	NBRC 108588	LC438603	LC438605	LC438607

Newly described species as well as specimen number and sequences are shown in bold.

a) The duplicate sequence is registered to DDBJ as 'AB705235'.

Taxonomy

Incrucipulum foliicola Tochihara, *sp. nov.* (Figs. 1, 2)
MycoBank no.:—MB829358

Ecology:—Saprotrophic on dead leaves of *Myrica gale* subsp. *tomentosa* (C.DC.) E. Murray. Abundant on the fallen leaves accumulated on the wetlands.

Holotype:—JAPAN. Hokkaido: Yamakoshi-gun, Oshamanbe-cho, Shizukari wetland, 5 m, 42.573644° N, 140.430907° E, 26 June 2018, on dead leaves of *Myrica gale* subsp. *tomentosa*, Y. Tochihara (TNS-F-81526!).

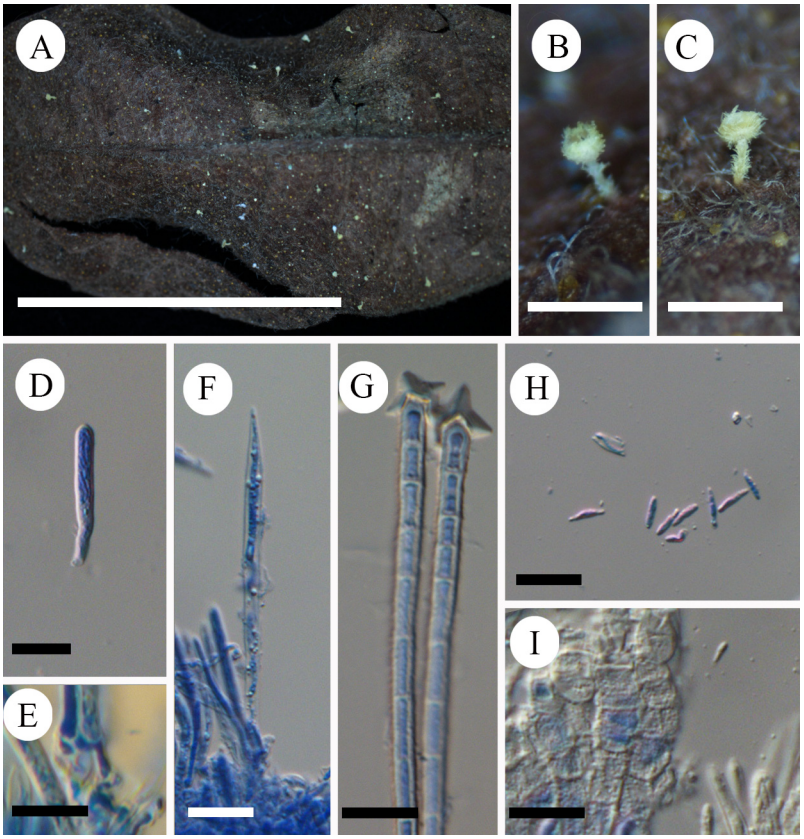

FIGURE 1. *Incrucipulum foliicola*. **A:** Apothecia scattered on the substrate. **B:** Fresh apothecium. **C:** Dried apothecium. **D:** Ascus. **E:** Ascal bases with croziers. **F:** Paraphysis. **G:** Hairs with cruciate crystals like 'cross shuriken'. **H:** Ascospores. **I:** Ectal excipular cells with granulate surface. TNS-F-81508 (**A, C–I**), TNS-F-81526 (**B**). Scale bars = 1 cm (**A**), 0.5 mm (**B, C**), 10 µm (**D–I**).

Paratype:—JAPAN. Hokkaido: Nemuro-city, Cape Ochiishi wetland, 47 m, 43.166311° N, 145.512608° E, 20 July 2018, on dead leaves of *Myrica gale* subsp. *tomentosa*, Y. Tochihara (TNS-F-81508!).

Etymology:—Referring to the part of substrates.

Description:—**Apothecia** scattered on the substrate, superficial, cup-shaped, 0.1–0.4 mm in diameter when fresh and dry, having long and slender stipes, up to 0.6 mm high, externally covered with hairs, lemon yellow throughout when fresh and dry. **Disc** concave, concolourous with external receptacle. **Ectal excipulum** *textura prismatica* composed of hyaline cubic cells with granulated surface arranged in parallel rows, thick-walled; wall up to 2 µm thick. **Medullary excipulum** *textura intricata* of hyaline hyphae up to 2 µm wide. **Hairs** straight, cylindrical, up to 90 × 3–5 µm, densely septate, hyaline, granulated throughout, thick-walled; wall up to 2 µm thick; individual cells 2.5–10 µm long; apex blunt or a little bit swollen, equipped with one cross-shaped crystal like "cross shuriken" ca. 10 µm across or one tetrahedral crystal detached easily in squash mounts. **Asci** 27–37 × 2.5–4 µm, 8-spored, cylindrical-clavate with slightly protruding apices; apical pore blue in Melzer's reagent without 3% KOH pretreatment; croziers present at the basal septa. **Ascospores** 5–8 × 1.2–1.5 µm, long ellipsoid to narrowly fusiform, aseptate, containing some small lipid bodies. **Paraphyses** straight, lanceolate, septate, up to 4 µm wide, exceeding the asci up to 25 µm.

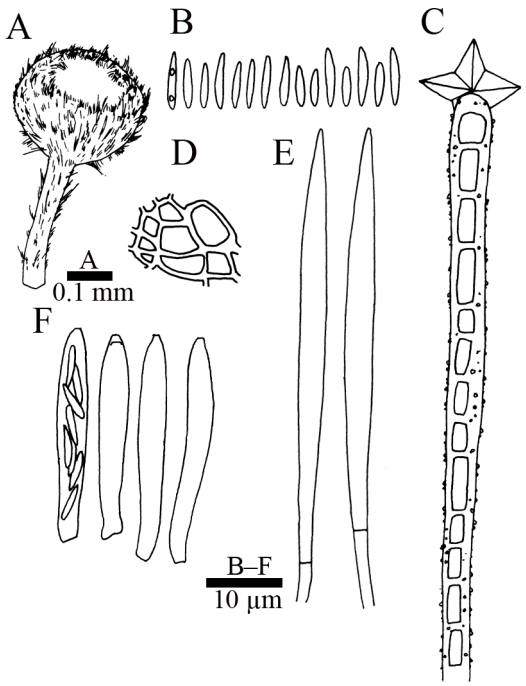

FIGURE 2. *Incrucipulum foliicola* (TNS-F-81508). **A:** Apothecium. **B:** Ascospores. **C:** Hair with a crystal. **D:** Ectal excipular cells. **E:** Paraphyses. **F:** Asci.

Notes:—*Incrucipulum foliicola, I. pseudosulphurellum* and *I. sulphurellum* occur on *Myrica gale* L. (including *M. gale*. subsp. *tomentosa*) and share tetrahedral or cross-shaped crystals of hairs (Figs. 1G, 2C) as a common character. *Incrucipulum foliicola* much resembles *I. sulphurellum* which differs in the absence of croziers and parts of host, based on the original description (Peck 1878) and redescription of Haines (1989). *Incrucipulum foliicola* differs from *I. pseudosulphurellum* in short stipes, the presence of croziers, hyaline ectal excipular cells and parts of host.

Considering that all *Incrucipulum* species and other lachnaceous species have strong selectivity to the parts of host and that foliicolous species never occur on twigs and other parts, *I. foliicola* should be treated as a new species.

Incrucipulum foliicola is currently known from two wetlands in Hokkaido.

Incrucipulum hakonechloae-macrae Tochihara, *sp. nov.* (Figs. 3, 4)

MycoBank no.:—MB829308

Ecology:—Saprotrophic on fallen and damp leaves, culms and other leaf-like parts of *Hakonechloa macra* (Munro) Honda stuck to the ground.

Holotype:—JAPAN. Saitama Pref.: Chichibu-gun, Ogano-machi, Mt. Futagoyama, 1008 m, 36.068342° N, 138.867364° E, 12 August 2018, on dead leaves and culms of *Hakonechloa macra*, Y. Tochihara (TNS-F-81512!).

Etymology:—Referring to the host plant *Hakonechloa macra.*

Description:—**Apothecia** scattered on the substrate, superficial, cup-shaped, 0.2–0.4(–0.5) mm in diameter, externally covered with short, white, capitate hairs bearing drops of dew when moist, stipitate, up to 0.2–0.5 mm high when fresh and dry; stipe white, sometimes brown in the lower part. **Disc** concave, lemon yellow to orange when fresh and dry. **Ectal excipulum** *textura prismatica* composed of hyaline cubic cells like stone pavings, 2.5–15 × 5–13 µm, containing yellow oil drops, thick-walled; wall up to 3 µm thick with granulate surface; cells arranged in parallel rows. **Medullary excipulum** *textura intricata* of hyaline hyphae up to 2 µm wide. **Hairs** straight, cylindrical, sometimes with rounded apices, 38–90 × 3–8 µm, 2–5-septate, hyaline, completely covered by granules, thin to relatively thick-walled; wall up to 1.5 µm thick; apices blunt or a little bit swelled, bearing amber crystal caps detached easily in squash mounts. **Asci** 46–63(–75) × 6–10 µm, 8-spored, cylindrical-clavate; apical pore blue in Melzer's reagent without 3% KOH pretreatment; croziers present at the basal septa. **Ascospores** 8–14 × 2.5–5 µm, fusiform, aseptate or rarely 1-septate, usually packed by gelatinous sheath, containing some large lipid bodies conspicuous in CB/LA mount. **Paraphyses** straight, cylindrical to narrowly lanceolate, rarely swollen at the apices, sometimes branched near the bases, septate, up to 3 µm wide, containing hyaline to orange oil drops, exceeding the asci up to 7.5 µm.

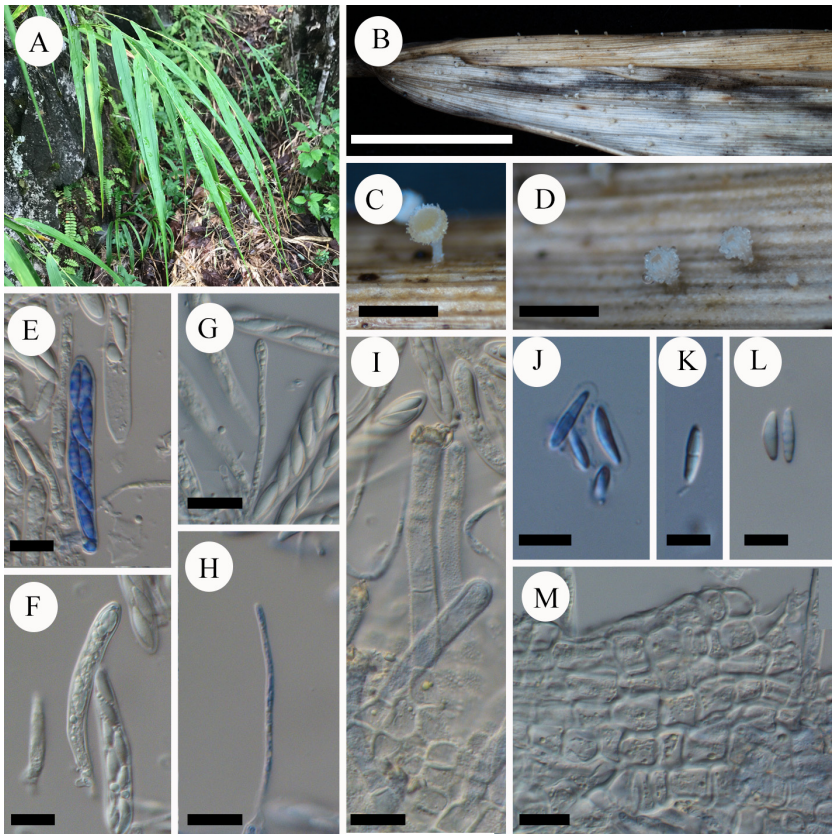

FIGURE 3. *Incrucipulum hakonechloae-macrae* (TNS-F-81512). **A:** The host *Hakonechloa macra* and its litter in the collection site of *I. hakonechloae-macrae.* **B:** Scattered apothecia of *I. hakonechloae-macrae* occurring on the host leaf. **C, D:** Fresh apothecia. **E:** Ascus. **F:** Ascus arising from a crozier. **G, H:** Paraphysis. **I:** Hair arising from ectal excipular cells with granulate surface. **J:** Ascospores packed by gelatinous sheath. **K:** Ascospore with a septum. **L:** Ascospores containing large lipid bodies. **M:** Thick-walled ectal excipular cells containing orange oil drops. Scale bars = 1 cm (**B**), 0.5 mm (**C, D**), 10 µm (**E–M**).

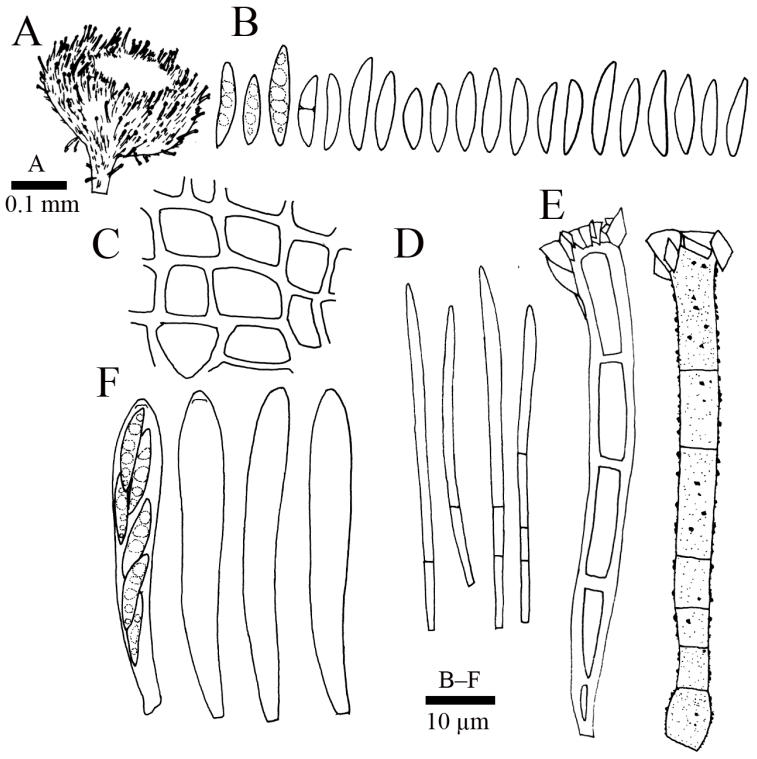

FIGURE 4. *Incrucipulum hakonechloae-macrae* (TNS-F-81512). **A:** Apothecium. **B:** Ascospores. **C:** Ectal excipular cells. **D:** Paraphyses. **E:** Hairs with crystals. **F:** Asci.

Notes:—*Incrucipulum hakonechloae-macrae* is distinguishable from other species of *Incrucipulum* in yellow to orange disc (Fig. 3C) and ascospores containing very large oil drops and packed by gel sheath (Figs. 3J–3L). Ascospores did not germinate in PDA at all. Some spores germinated in water agar or corn meal agar, but no further extension of hyphae was observed.

Incrucipulum hakonechloae-macrae was collected only from *Hakonechloa macra*, an endemic grass in the central Honshu, Japan (Katoh & Ebihara 2011). *Incrucipulum hakonechloae-macrae* is possibly also endemic to the Honshu Island corresponding to the host distribution, because most of lachnaceous species have strong host selectivity.

Incrucipulum pseudosulphurellum Tochihara, *sp. nov.* (Figs. 5, 6)

MycoBank no.:—MB829362

Ecology:—Saprotrophic on fallen twigs of *Myrica gale* subsp. *tomentosa*.

Holotype:—JAPAN. Hokkaido: Yamakoshi-gun, Oshamanbe-cho, Shizukari wetland, 5 m, 42.573644° N, 140.430907° E, 26 June 2018, on dead twigs of *Myrica gale* subsp. *tomentosa*, Y. Tochihara (TNS-F-81441!).

Etymology:—Referring to the similarity to *I. sulphurellum*.

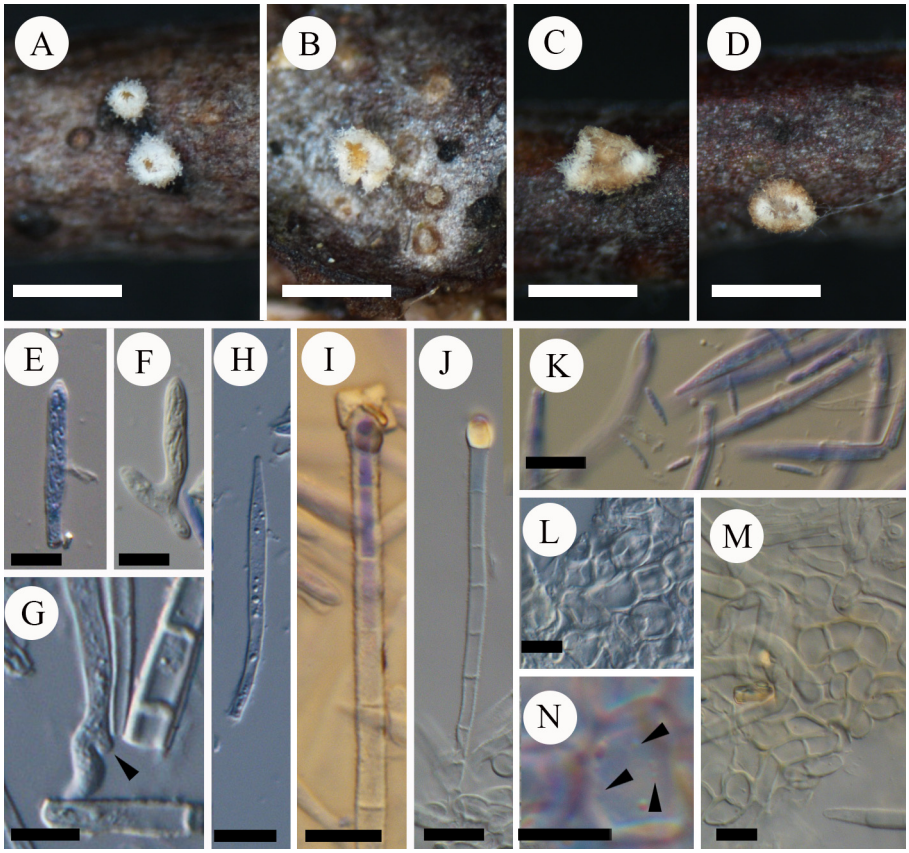

FIGURE 5. *Incrucipulum pseudosulphurellum* (TNS-F-81441). **A, B:** Fresh apothecia. **C, D:** Dried apothecium. **E:** Ascus. **F:** Ascus with an irregularly branched base. **G:** Immature ascus with a crozier-like structure (arrow head). **H:** Paraphysis. **I:** Hair with a tetrahedral crystal. **J:** Hair with an irregular-shaped crystal. **K:** Ascospores. **L:** Hyaline ectal excipular cells. **M:** Subhyaline ectal excipular cells. **N:** Surface of ectal excipular cells with granules (arrow heads). Scale bars = 0.5 cm (**A–D**), 10 µm (**E–N**).

Description:—**Apothecia** scattered, superficial, 0.4–0.8 mm in diameter, having short and dark brown stipes, up to 0.5 mm high, white to somewhat dull yellow on the upper part and thin to dark brown in the lower part, densely covered by white, capitate hairs. **Disc** concave, pure white to orange when fresh and deep orange when dry. **Ectal excipulum** *textura prismatica to textura angularis*, 4–21 × 3–15 µm, hyaline in the upper part and subhyaline to brown in the lower part, slightly thick-walled; wall up to 2 µm thick; surface smooth or rarely equipped with sparse granules. **Medullary excipulum** *textura intricata* of hyaline hyphae up to 2 µm wide. **Hairs** straight, cylindrical with slightly rounded apices, up to 130 × 4.5–7.5 µm, completely granulate, multiseptate, arising from rounded basal cells; hair cells 5–25 µm long, thin to slightly thick-walled; wall up to 1.5 µm thick; apical cells thick-walled; wall up to 2 µm thick; apices bearing various shapes of crystals easily detached by squash mounts, i.e. tetrahedral ones, cross-shaped

ones like ‘cross shuriken’ or amber crystal caps. **Asci** 33–44(–52) × 3.5–7.5 µm, 8-spored, cylindrical-clavate; pore blue in Melzer’s reagent without 3% KOH pretreatment; croziers absent, but base sometimes irregularly branched and forming ‘fake-croziers’. **Ascospores** 6.3–10 × 1.5–2.2 µm, oblong to narrowly fusiform, aseptate. **Paraphyses** straight, lanceolate, up to 5.5 µm wide, exceeding the asci by 25 µm.

Notes:—*Incrucipulum pseudosulphurellum* resembles *I. foliicola* and *I. sulphurellum*, but differs in short and dark brown stipes (Figs. 5A–5D), brownish ectal excipulum rarely equipped with granules (Figs. 5L–5M). Little granules on ectal excipular cells is an atypical character of *Incrucipulum*, but other characters agrees with generic definition. *Incrucipulum pseudosulphurellum* is currently known only from the type locality, but probably widely distributed corresponding to the distribution of host plants.

Wooly appearance and short stipes of this species are similar to *Capitotricha bicolor* (Bulliard 1789: 410) Baral (1985: 60) macroscopically but differs in smaller asci and lacking orange oil drops in paraphyses.

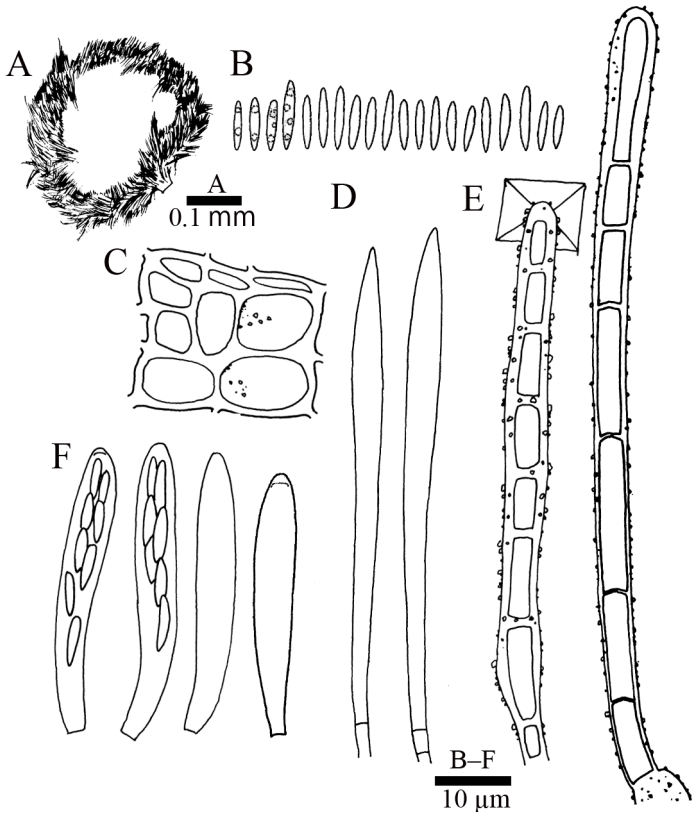

FIGURE 6. *Incrucipulum pseudosulphurellum* (TNS-F-81441). **A:** Apothecium. **B:** Ascospores. **C:** Ectal excipular cells. **D:** Paraphyses. **E:** Hairs with and without crystals. **F:** Asci.

Results and Discussion

In the molecular phylogenetic analysis, we obtained sequence matrices composed of 350 bases of ITS-5.8S, 808 bases of LSU and 665 bases of RPB2. The optimal model was selected as follow; GTR model with a discrete gamma distribution (GTR+G) for ML analysis, and K80+G for ITS, GTR+G for LSU, SYM+G for RPB2 first and third codon position, and HKY85+G for RPB2 second codon position for BI analysis. In the BI analysis, the first 10% of the generated trees in the cold chain were discarded as the burn-in, because ASDSF became <0.01 and model parameters converged when 500,000 sample trees generated. ML tree with BI PPs calculated by the remaining trees was shown (Fig. 7).

All seven species of *Incrucipulum* formed a strongly supported clade and robust phylogenetic inner relationship of *Incrucipulum* was revealed. Inclusion of the three new species into *Incrucipulum* was justified and monophyly of *Incrucipulum* was also confirmed.

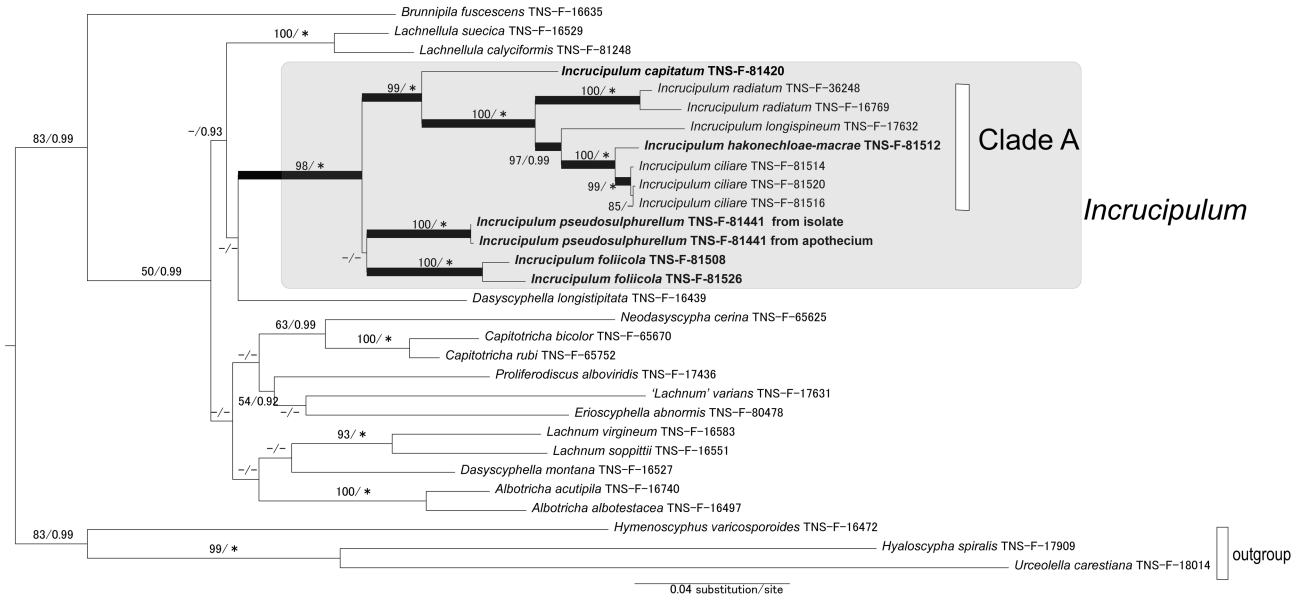

FIGURE 7. Phylogenetic tree of *Incrucipulum* and other lachnaceous species constructed by RAxML based on combined sequences of ITS (350 bases), LSU (808 bases) and RPB2 (665 bases). ML bootstrap values (ML BS; before the slash marks) and BI posterior probabilities (BI PPs; after the slash marks) were shown in each branch. '-' represents ML BS are under 50% or BI PPs are under 0.90, or a branch did not appear in BI analysis. '＊' represents BI PPs are 1.00. Bolded branches show ML BS are 90% or more and BI PPs are 0.95 or more in *Incrucipulum* clade. Sequences of *Incrucipulum* species newly described in this study were shown in bold.

Within *Incrucipulum*, a strongly supported clade excluding *I. foliicola* and *I. pseudosulphurellum* was recognized. Within this clade another strongly supported clade (Clade A), composed of *I. ciliare* (type species) and other three species of *Incrucipulum*, was recognized. The species in Clade A all had cylindrical to narrowly lanceolate paraphyses while others had lanceolate paraphyses. Since lanceolate paraphyses are shared by most of lachnaceous genera and Clade A is terminal in *Incrucipulum*, cylindrical to narrowly lanceolate paraphyses are presumed to be apomorphic and evolved multiple times.

Species of Clade A were also characterized by large (>10 µm long) and conspicuously fusiform ascospores and longer asci (mainly > 40 µm long), while others have small (<10 µm long) and oblong to narrowly fusiform ascospores and smaller asci (mainly < 40 µm long). Fusiform ascospores and larger asci are also presumed to be apomorphic in *Incrucipulum*, as has been suggested in the morphological data of Tanaka & Hosoya (2001).

In the present research, *I. capitatum* was newly collected from Japan. Eight species of *Incrucipulum* are currently known from Japan. Five species, *I. foliicola, I. hakonechloae-macrae, I. longispineum, I. pseudosulphurellum* and *I. radiatum,* have been known only from Japan (Katsumoto 2010, Otani 1967, Raitviir 1977, Tanaka & Hosoya 2001, this study). Considering that lachnaceous species tend to show strong host specificity, it is presumed that species of *Incrucipulum* were diversified in the Far East corresponding to characteristic vascular plant flora, such as tall herb grasslands and bamboo grove (Raitviir 1979).

With the addition of three new species, *Incrucipulum* now contains 13 species. In addition to the currently known members, *Lachnum radovii* Svrček (1984: 201), *L. roseum* (Rehm 1881: 41) Rehm (1893: 882) and *L. soppitii* (Massee 1895: 330) Raitviir (1986: 2) were mentioned as potential members of *Incrucipulum* (Baral & Krieglsteiner 1985). *Lachnum soppitii* is phylogenetically apart from the genus *Incrucipulum*. (Fig. 7). Based on the description and the illustration of Raitviir (1977) *Lachnum lespedezae* (Raitviir 1977: 691) Raitviir (1986: 2) is also a potential member due to cylindrical paraphyses, thick-walled hairs and thick-walled ectal excipulum like stone pavings, but the presence or absence of granules on ectal excipulum of the type was not documented. We hesitate to propose their new combinations in this study because type studies and phylogenetic analyses (excluding *L. soppitii*) have not been done.

Key to species of Incrucipulum

1. Having obvious lanceolate paraphyses 2
- Having cylindrical to narrowly lanceolate paraphyses 6
2. Having hairs with >6 septa 3
- Having hairs with ≤6 septa ..... *I. capitatum*
3. Occurring on *Myrica gale* 4
- Occurring on dead culms of grasses ..... *I. densiseptatum*
4. Occurring on dead leaves ..... *I. foliicola*
- Occurring on dead twigs 5
5. Having hyaline ectal excipulum ..... *I. sulphurellum*
- Having brownish ectal excipulum ..... *I. pseudosulphurellum*
6. Occurring on dead leaves of ericaceous plants 7
- Occurring on plants except for Ericaceae 8
7. Croziers present at the basal septa of asci ..... *I. longispineum*
- Croziers absent at the basal septa of asci ..... *I. virtembergense*
8. Occurring on dead stems of tall herbaceous plants ..... *I. sinegoricum*
- Occurring on other plants 9
9. Croziers present at the basal septa of asci 10
- Croziers absent at the basal septa of asci 11
10. Occurring on dead leaves of *Fagus* ..... *I. radiatum*
- Occurring on dead leaves or culms of grasses ..... *I. hakonechloae-macrae*
11. Spores <12 µm long ..... *I. saccardoi*
- Spores ≥12 µm long 12
12. Occurring mainly on dead leaves of fagaceous plants ..... *I. ciliare*
- Occurring on dead leaves of *Dryas octopetala* ..... *I. uralense*

Acknowledgments

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