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Taxonomical revision of *Scolecostigmina chibaensis*

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Abstract: *Scolecostigmina chibaensis*, described from Japan, is a caulicolous and plant pathogenic fungal species on *Pinus* spp. In this study, a taxonomical revision of *S. chibaensis* based on the morphological characteristics and phylogenetic relationships using nrDNA ITS and LSU is conducted. As a result, *S. chibaensis* is transferred to the genus *Kirschsteiniothelia*. And, this is the first record of a *Kirschsteiniothelia* species in Japan.

Keywords: *Kirschsteiniothelia, new record, Pinus, phylogeny, plant pathogenic, Scolecostigmina, taxonomy*

INTRODUCTION

Scolecostigmina chibaensis described from Japan, is a caulicolous and plant pathogenic fungal species on *Pinus* spp. [Nakashima et al., 2007]. The host plant, *P. parviflora* Sieb. & Zucc., Pinaceae, is known as an endemic tree in Japanese forests. On the other hand, the number of individuals and the corresponding habitats are decreasing. The stem canker caused by *S. chibaensis* is mainly concerned with the decline of natural habitats and the natural regeneration of seedlings [Suzuki et al., 2001]. According to Yamada [2013], the disease caused by *S. chibaenisis* has been observed on species of the genus *Pinus* subgenus *Strobus*, including *P. parviflora*, *P. parviflora* Siebold & Zucc. var. *pentaphylla* (Mayr) A. Henry, *P. peuce* Griseb., *P. strobiformis* Engelm., *P. strobus* L., and *P. wallichiana* (Wall. ex D. Don) A.B. Jacks. in the fields, and the severity of the disease is different of the individual hosts.

The fungal genus *Scolecostigmina* U. Braun was established in Braun et al. [1999] based on *Scolecostigmina mangiferae* U. Braun & Mouch. (≡ *Cercospora mangiferae*) on *Mangifera indica* L., which is a stigmina-like fungus bearing filamentous conidia (scolecosporous). It is a small genus of 27 species [Mycobank, 2025; Robert et al., 2013]. However, its phylogenetic relationship is still unclear. In recent years, several species of the fungal genus *Kirschsteiniothelia* D. Hawksw. [Hawksworth, 1985] with anamorphs that are morphologically similar to *Scolecostigmina* species by having percurrently proliferating conidiogenous cells and dark-colored conidia with thickened walls, have been described from various habitats as saprophytes or phytopathogens. Along with the increasing of DNA sequencing data of *Kirschsteiniothelia* species on the DNA databank, the search results of the Basic Local Alignment Search Tool (NCBI BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi) using internal transcribed spacer regional sequences (ITS) of *S. chibaensis* have indicated a strong relationship to Kirschsteiniothelia species. This study constitutes a taxonomical revision of *S. chibaensis* based on the phylogenetic relationships.

MATERIAL AND METHODS

Fungal specimens and isolates: Type materials of *Scolecostigmina chibaensis* which have been deposited in the Herbarium, Phytopathology Laboratory, Graduate School of Bioresources, Mie University (TSU-MUMH), Tsu, Mie, Japan and Herbarium of Forest Mycology and Pathology, Forestry and Forest Products Research Institute (TFM), Tsukuba, Ibaragi, Japan, were examined. The isolates, including an ex-type culture, preserved in the culture collection, Phytopathology Laboratory, Graduate School of Bioresources, Mie University (MUCC), were examined [Nakashima et al., 2007]. Morphological characteristics of the conidia and conidiophores and mode of sporulation were reexamined under a compound microscope Axio Imager A1 (Zeiss, Göttingen, Germany).

DNA extraction and PCR amplification: Fungal mycelium of isolates (Tab. 1) was harvested with a sterilized toothpick, and the genomic DNA was isolated using the UltraClean Microbial DNA isolation kit (MoBio Laboratories, Inc., CA, USA), following the manufacturer's protocols. Two partial nuclear genes were

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Table 1. List of reference sequences and examined isolates in this study.

Species name	Isolate	Status**	LSU	ITS
Asterina phenacis	TH589		GU586217	-
Homortomyces tamaricis	MFLUCC13-0441	T	NG_059495	NR_155161
Kirschsteiniothelia acutispora	MFLU21-0127	T	ON980758	OP120780
aethiops	CBS448.77		MH872853	MH861088
aquatica	MFLUCC16-1685		MH182594	MH182587
arasbaranica	IRAN2508C		KX621984	KX621983
atra	GZCC23-0731		PQ248936	PQ248940
bulbosapicalis	GZCC23-0732	T	PQ248933	PQ248937
cangshanensis	MFLUCC16-1350		MH182592	MH182584
chiangmaiensis	MFLU23-0358	T	OR575474	OR575473
crustacea	T201127		MW851854	MW851849
dendryphioides	KUNCC10431	T	NG_244359	-
ebriosa	ERA2017		LT985884	-
emarceis	MFLUCC10-0037	T	HQ441571	HQ441570
extensa	T201129		MW851855	MW851850
fluminicola	MFLUCC16-1263		MH182588	MH182582
guangdongensis	NDK2023a		OR164974	OR164946
inthanonensis	MFLUCC23-0277	T	NG_243932	NR_198652
lignicola	MFLUCC10-0036		HQ441568	HQ441567
longirostrata	GZCC23-0733		PQ248934	PQ248939
nabanheensis	HJAUPC2004	T	OQ023273	OQ023197
phoenicis	MFLUCC18-0216	T	MG860484	MG859978_
puerensis	ZHKUCC22-0271	T	OP451017	OP450977
ramus	GZCC23-0596	T	NG243331	NR_190260
rostrata	MFLU15-1154	T	KY697276	KY697280
saprophytica	MFLUCC23-0275	T	OR762783	OR762774
septemseptatum	MFLU21-0126	T	ON980757	OP120779
spatiosum	MFLU21-0128	T	-	OP077294
submersa	S601		MH182593	MH182585
tectonae	MFLUCC12-0050	T	KU764707	KU144916
thailandica	MFLUCC20-0116	T	NG_088170	NR_178154
thujina	JF13210		KM982718	KM982716
vinigena	FMR15668	T	NG_075229	-
xishuangbannaensis	ZHKUCC22-0220	T	OP303181	OP289566
zizyphifolii	MFLUCC23-0270	T	OR762776	OR762768
Scolecostigmina chibaensis	CBS122976=NBRC102148	T	MH874784	MH863260
	MUCC161*		**PV335805**	**PV341198**
	MUCC162		**PV335806**	**PV341199**
	MUCC163		**PV335807**	**PV341200**
	MUCC164		**PV335808**	**PV341201**
	MUCC165		**PV335809**	**PV341202**
Scolecostigmina mangiferae	CBS125467		MH875071	MH863595
Scortechinia conferta	CBS191.53		GU301814	-
Venturia populina	CBS256.38		GU323212	-

Note: *Newly obtained sequences were indicated as Bold. ** Status: "T" is an ex-type isolate.

subjected to PCR amplification and sequencing: internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS) of the nrDNA operon and large-subunit ribosomal DNA (LSU). The PCR amplifications were performed on a BioRad T100 Thermal Cycler (Bio-Rad Laboratories, Inc., CA, US). The PCR mixtures consisted of 1–10 ng genomic DNA, 1.25 uL 10× NH4 reaction buffer (Bioline, Luckenwalde, Germany), 2–2.5 mM MgCl2 (ITS: 2.5 mM, LSU: 2 mM), 40 µM of dNTPs, 0.8% dimethyl sulfoxide (DMSO; LSU), 0.16–0.2 µM of each primer (ITS: 0.16 µM, LSU: 0.20 µM) (Tab. 2) and 0.25–0.5 U Taq DNA polymerase (Bioline; ITS: 0.25 U, LSU: 0.5 U) in a total volume of 12.5 µL. The PCR cycling conditions for ITS were: initial denaturation (94°C, 5 min); 40 cycles amplification (denaturation 94°C for 45 s; annealing 48°C for 30 s; extension 72°C for 90 s), and final extension (72°C, 10 min). The PCR cycling conditions for LSU were: initial denaturation (96°C, 2 min); 10 cycles amplification (denaturation 94°C for 30 s, annealing 48°C for 30 s, extension 72°C for 60 s); 25 cycles amplification (denaturation 94°C for 45 s, annealing 54°C for 45 s, extension 72°C for 60 s; for each cycle 5 seconds extended the extension time), and final extension (72°C, 7 min). The resulting fragments were sequenced in both directions using the respective PCR primers and the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems Life Technologies, Carlsbad, CA, USA). DNA sequencing amplicons were purified through Sephadex G-50 Superfine columns (Sigma-Aldrich, St. Louis, MO) in MultiScreen HV plates (Millipore, Billerica, MA). Purified sequence reactions were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA). The DNA sequences were assembled and aligned with hitherto known sequences, retrieved from NCBI DNA GenBank (Tab. 2) using MEGA v. 7 [Kumar et al., 2016]. The matrices of DNA sequences were deposited to Figshere (10.6084/m9.figshare.28830464). All novel sequences obtained in this study were deposited in GenBank (Tab. 1).

Phylogenetic analysis: The individual matrix of each locus was aligned by the MAFFT online service [Katoh et al., 2018]. The best substitution model for each locus was determined by ModelTest-NG [Darriba et al., 2020]. Maximum-likelihood analysis (ML) was conducted on RAxML-NG [Kozlov et al., 2019] with 100 bootstraps. The resultant trees were drawn with FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Fungal specimens and isolates: Type materials of *Scolecostigmina chibaensis* deposited in TSU-MUMH and TFM have been examined (Fig. 1). The isolates, including ex-type cultures preserved in the MUCC, were examined.

Phylogenetic analysis: Scolecostigmina chibaensis has been suggested as a species of the genus *Kirschsteiniothelia* from a search result of BLAST search using the ITS region sequences. The LSU sequences of the examined isolates were combined with available sequences retrieved from *Kirschsteiniothelia* species and several Dothideomycetes fungi, viz. *Scolecostigmina mangiferae* of Mycosphaerellaceae, *Asterina phenacis* Syd. of Asterinaceae, *Homortomyces tamaricis* Wijayaw., Camporesi & K.D. Hyde of Homortomycetaceae, *Venturia populina* (Vuill.) Fabric. of Venturiaceae, *Scortechinia conferta* (Schwein.) Subram. & Sekar of Scortechiniaceae, and *Schismatomma decolorans* (Turner & Borrer) Clauzade & Vězda of Roccellaceae as out group, to grasp the phylogenetic position of the present fungus. The aligned matrix comprised 829 sites of 41 taxa and was analyzed with TN93+G4 option based on the BIC. The resultant tree is shown in Figure 2. Examined isolates and *Kirschsteiniothelia* species, except *K. emarceis* Boonmee & K.D. Hyde, formed a statistically well supported clade. The combined matrix of ITS and LSU sequences of *Kirschsteiniothelia* species was analyzed to reveal the phylogenetic relationship within the genus. The aligned matrix was 1421 sites composed of 41 taxa, including *Homortomyces tamaricis*

Table 2. List of primers used in this study.

Locus	Primer	Sequence (5′–3′)	Orientation	Annealing	Reference
ITS	V9G	TTA CGT CCC TGC CCT TTG TA	F	48	de Hoog and Gerrits van den Ende (1998)
	ITS4	TCC TCC GCT TAT TGA TAT GC	R	48	White et al. (1990)
LSU	LR0R	GTA CCC GCT GAA CTT AAG C	F	48	Rehner & Samuels (1994)
	LR5	TCC TGA GGG AAA CTT CG	R	48	Vilgalys & Hester (1990)
	LR7	TAC TAC CAC CAA GAT CT	R	48	Vilgalys & Hester (1990)

as out group, and analyzed with TIM2+I+G4 for ITS and TIM1+G4 for LSU option based on the BIC. The resultant tree is shown in Figure 3. Species of Kirschsteiniothelia were well recognized as an independent lineage in the tree, and the grouping was the same as in the LSU tree.

Taxonomy. ***Kirschsteiniothelia chibaensis*** (C. Nakash., Tak. Kobay. & Tosh. Yamada) C. Nakash., Yuk. Takah., T. Yamada, **comb. nov.** MB#858991 Fig. 1.

Basionym: *Scolecostigmina chibaensis* C. Nakash., Tak. Kobay. & Tosh. Yamada, Mycoscience 48: 250, 2007.

Symptoms on twigs are swellings, cancerous, rugged, gray to grayish black, cracking. *Caespituli* blackish, loose to dense. *Stromata* lacking or small to well developed, blackish brown to black, epidermal, erumpent through cortex, 2–13(–85) µm in diam. *Conidiophores* solitary to densely fasciculate, erected from stromata, straight to curved, blackish brown to olivaceous brown, smooth to rough, multi-septate, variable in width, 30–60 × 4–8 µm; conidiogenous cells terminal, integrated in conidiophores, proliferating percurrently with conspicuous annellation or rarely sympodially, with unthickened loci at the apex. *Conidia* solitary, occasionally catenulate, brown to blackish brown, phragmo- to scolecosporous, thick-walled, smooth to verrucous, truncate and thin at the basal end, obtuse and dull at the apex, often germinate (rostrate-like), various in width, 65–136 × 9–13 µm, with 5–13 euseptate.

Holotype: on *Pinus parviflora* Sieb. & Zucc. (Pinaceae), Japan, Chiba, Kamogawa, Kiyosumi, The University of Tokyo Forest, 19 May 2006, collected by Toshihiro Yamada (holotype, Herbarium of Forest Mycology and Pathology, Forestry and Forest Products

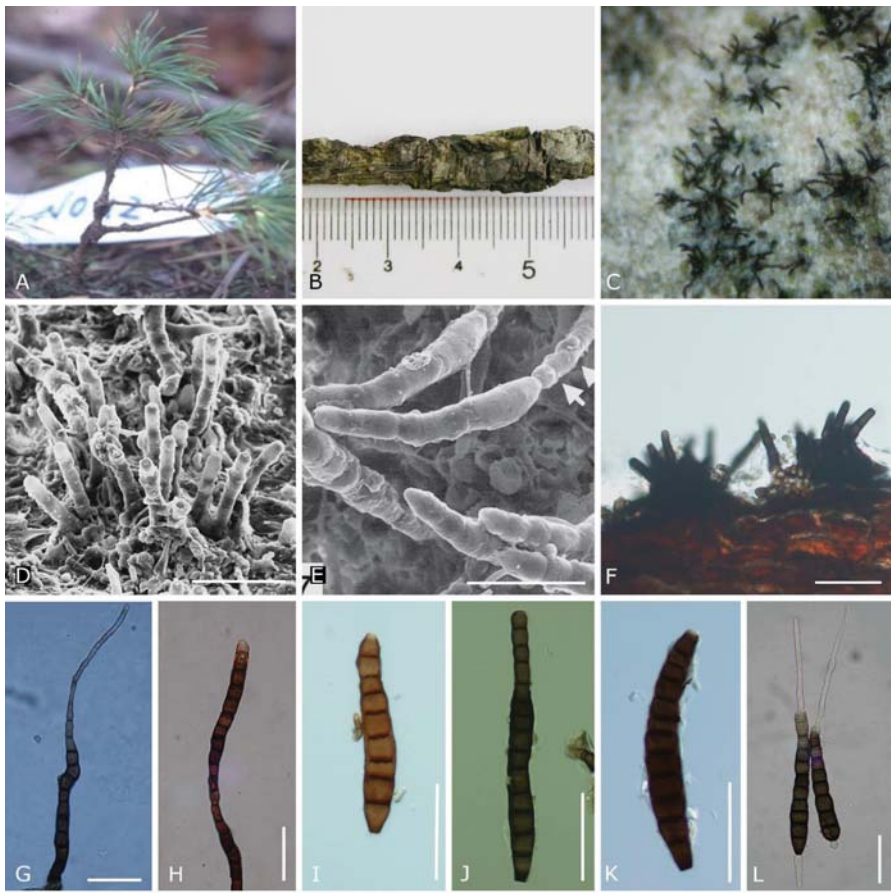

Figure 1. A: Symptoms on a seedling of *Pinus parviflora* var. *pentaphylla*. B: Magnified symptom on the twig. C: Caespituli on the symptom. D. SEM micrograph of caespituli on the symptom. E. SEM micrograph of conidiogenous cells and conidia at the terminal of conidiophores. F. Stromata. G. Conidiophore with a shoulder by sympodial proliferation. H. Conidiophore with annellations by percurrent proliferation. I–K. conidia. L. Germinating conidia trapped in the field. D–F. The original plates are Nakashima et al. [2007]. Bars = 20 µm.

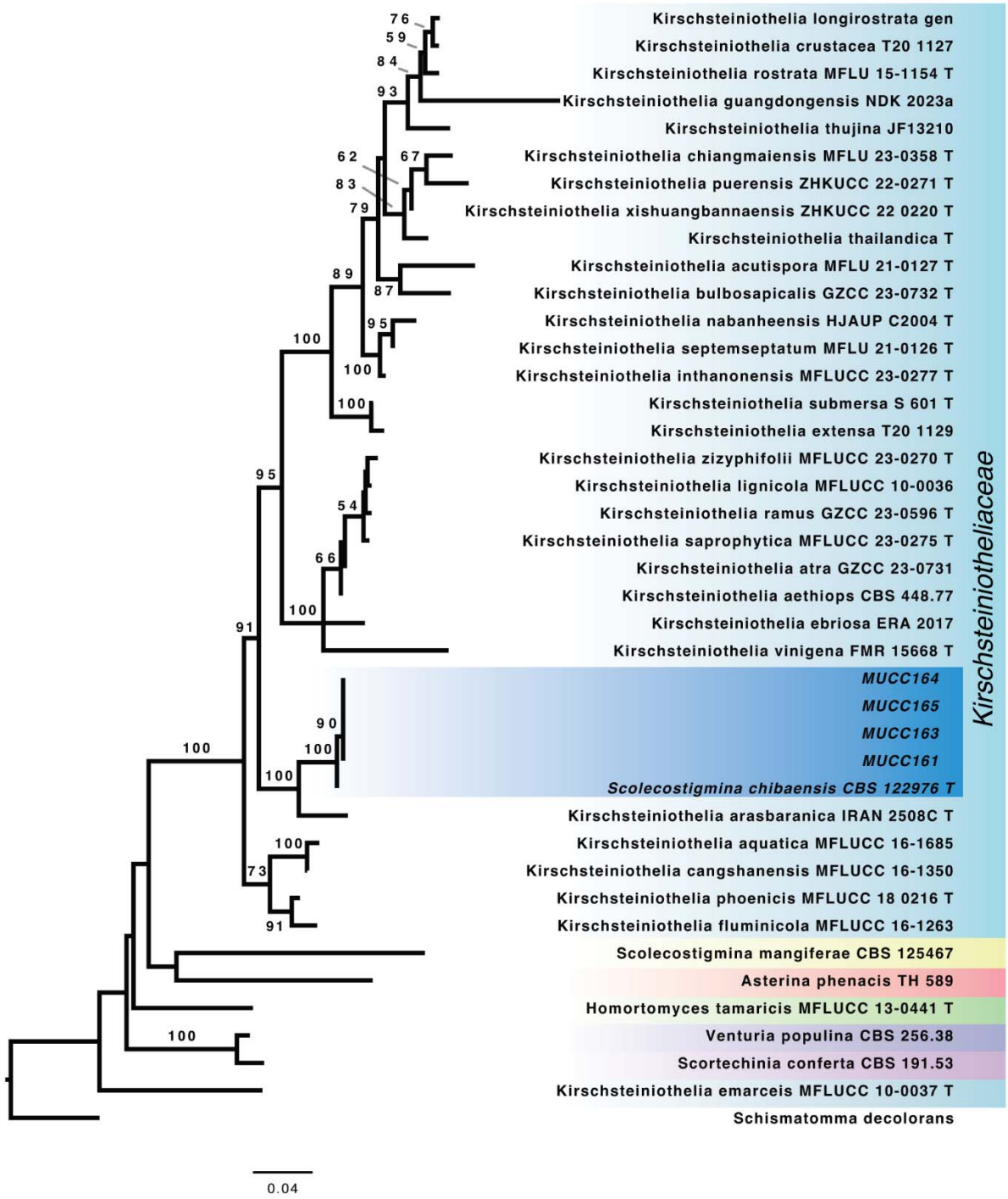

Figure 2. Maximum-likelihood (ML) phylogenetic tree of Kirschsteiniotheliaceae and some Dothideomyces fungi constructed by using LSU. The bootstrap of ≥ 50 was indicated near branch as BS. *Schismatomma decolorana* was used as an outgroup. Legend refers to nucleotide substitution per site.

Research Institute, TFM: FPH-7858 (MBT#125502); isotype, Mie University Mycological Herbarium, MUMH 10314) (ex-type cultures: NBRC 102148 = MUCC408 = CBS 122976).

Isolates examined in this study: on *Pinus parviflora* var. *pentaphylla*, Fukushima, 6 August 2004 (MUCC161, MUCC162); Chiba, 19 June 2003 (MUCC165); on *Pinus parviflora*, Chiba, 8 May 2003 (MUCC163, MUCC164).

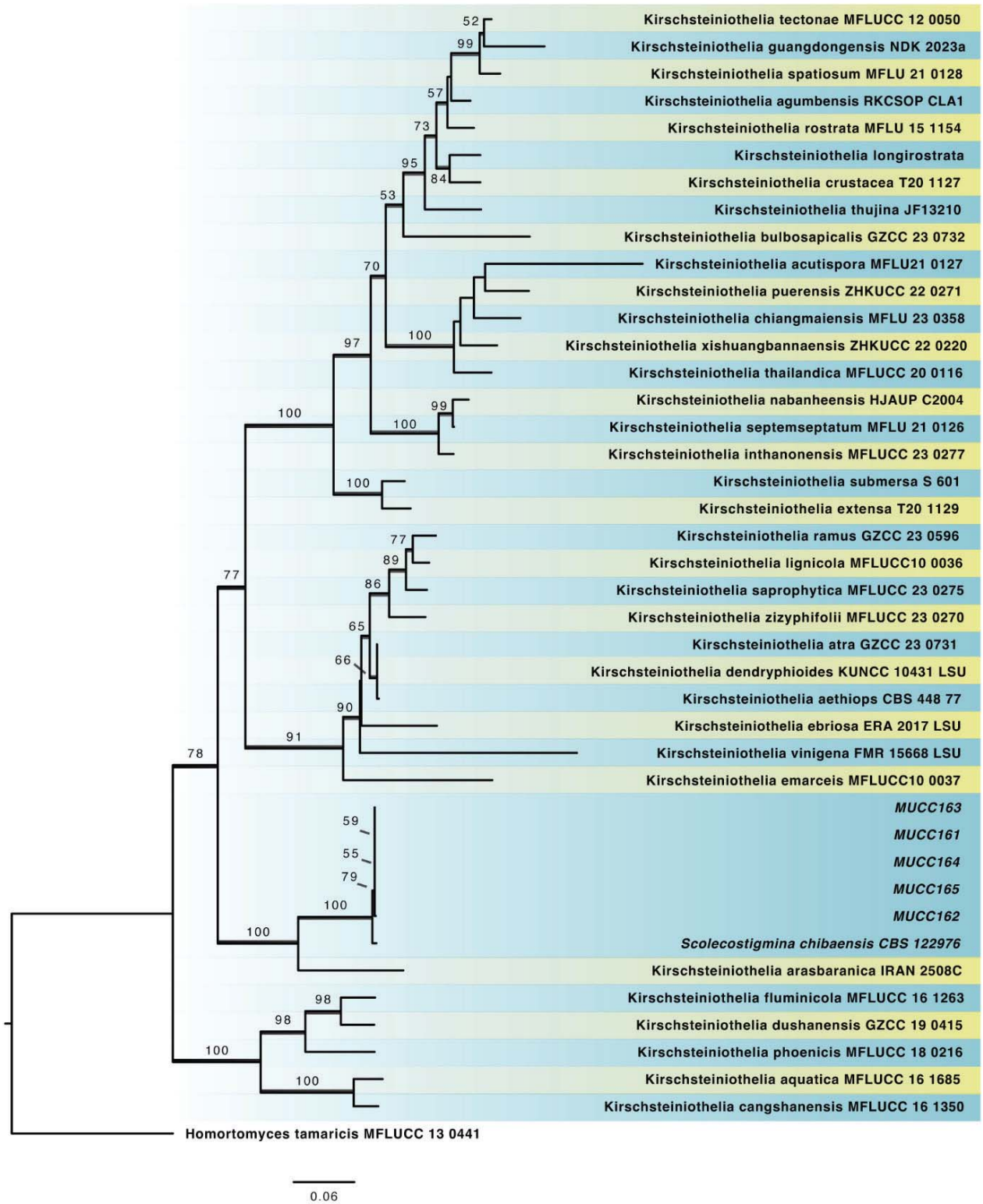

Figure 3. Maximum-likelihood (ML) phylogenetic tree of the genus *Kirschsteiniothelia* constructed by a concatenated matrix composed of ITS and LSU. The bootstrap of ≥ 50 was indicated near branch as BS. *Homortomyces tamaricis* was used as an outgroup. Legend refers to nucleotide substitution per site.

DISCUSSION

Based on the morphological characteristics of the fungus on the symptomatic stem of *Pinus* spp., it had been recognized as belonging to the genus *Scolecostigmina* [Nakashima et al., 2007]. The genus *Scolecostigmina* was established for stigmina-like hyphomycetes (*Mycosphaerella anamorphs*) with phragmo- to scolecosporous and transverse euseptate and thick-walled conidia [Braun et al., 1999]. As shown in Figure 2, the phylogenetic position of S. chibaensis was recognized as a member of *Kirschsteiniothelia* and sister to *K. arasbaranica* Mehrabi, R. Hemmati & Asgari, a species described from Iran on a dead branch of *Quercus petraea* (Matt.) Liebl. [Mehrabi et al., 2017]. The grouping of OTUs was similar to each other between the trees using LSU and LSU+ITS regions. The genus *Kirschsteiniothelia* is a member of Kirschsteiniotheliaceae Boonmee [Boonmee, 2012], and it is known to be widespread in tropical regions, where it commonly inhabits dead wood [Mahrabi et al., 2017]. Its asexual morphs, previously assigned to *Dendryphiopsis* S. Hughes, are characterized by having long-stalked and transversely septated conidiophores and cylindrical dark colored conidia [Wijayawardene et al., 2014]. Boonmee [2012] showed that *K. aethiops* (Sacc.) D. Hawksw. grouped with *D. atra* (Corda) S. Hughes, the type species of Dendryphiopsis, and *K. lignicola* Boonmee & K.D. Hyde and *K. emarceis* have Dendryphiopsis anamorphs. Moreover, Luo et al. [2025] indicated that *Kirschsteiniothelia* has two types of asexual morphs, namely dendryphiopsis-like and sporidesmium-like. The dendryphiopsis-like asexual morph is characterized by determinate or percurrently extending conidiophores with mono- to polytretic conidiogenous cells and acrogenous, solitary or catenate, septate conidia. The sporidesmium-like asexual morph is characterized by unbranched conidiophores with monoblastic or monotretic, determinate or irregularly extending conidiogenous cells and acrogenous, solitary or catenate, septate conidia with or without a mucilaginous sheath. The morphological characteristics of *S. chibaensis* were categorized as sporidesmium-like rather than dendryphiopsis-like. On the other hand, Mehrab et al. [2017] pointed out that *K. emarceis* using LSU sequences belonged to Pleosporales. The LSU sequence of *K. emarceis* (HQ441571) depositing in GenBank indicates a relationship to *Phaeoseptum* species (Pleosporales), even though in a comprehensive tree of the genus *Kirschsteiniothelia* published by Luo et al. [2025], using a combined matrix composed of SSU, ITS, and LSU, *K. emarceis* was clearly located in the Kirschsteiniothelia clade together with other species of this genus.

From these results, *Scolecostigmina chibaensis* must be transferred to the genus *Kirschsteiniothelia*. Furthermore, this is the first report of a species of *Kirschsteiniothelia* from Japan.

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***Scolecostigmina chibaensis* növünün taksonomik təftişi**

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Yaponiyadan *Pinus* spp. üzərində təsvir edilən *Scolecostigmina chibaensis* gövdədə rast gəlinən bitki patogenidir. Bu işdə nrDNA ITS və LSU istifadə etməklə *S.chibaensis* növünün morfoloji xüsusiyyətləri və filogenetik əlaqələri əsasında taksonomik təftişi aparılır. Nəticə etibarı ilə *S. chibaensis* növü *Kirschsteiniothelia* cinsinə keçirilib və bu Yaponiyada *Kirschsteiniothelia* növünün ilk sənədləşdirilməsidir.

Açar sözlər: *Kirschsteiniothelia, yeni sənədləşmə, Pinus, filogeniya, bitki patogeni, Scolecostigmina, taksonomiya*

Таксономическая ревизия вида *Scolecostigmina chibaensis*

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Scolecostigmina chibaensis, является патогеном растений, выявлено на стволе *Pinus* spp. из Японии. В данном исследовании проведена таксономическая ревизия вида *S. chibaensis* на основе морфологических характеристик и

филогенетических связей с использованием ярДНК ITS и LSU. В результате вид *S. chibaensis* был внесен в род *Kirschsteiniothelia*, что является первым документальным подтверждением вида из рода *Kirschsteiniothelia* в Японии.

Ключевые слова: *Kirschsteiniothelia, новая документация, Pinus, филогения, фитопатоген, Scolecostigmina, таксономия*