Lignicolous freshwater ascomycota from Thailand: Phylogenetic and morphological characterisation of two new freshwater fungi: *Tingoldiago hydei* sp. nov. and *T. clavata* sp. nov. from Eastern Thailand

Li Xu¹, Dan-Feng Bao²,³,⁴, Zong-Long Luo², Xi-Jun Su², Hong-Wei Shen²,³, Hong-Yan Su²

¹ College of Basic Medicine, Dali University, Dali 671003, Yunnan, China ² College of Agriculture & Biological Sciences, Dali University, Dali 671003, Yunnan, China ³ Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand ⁴ Department of Entomology & Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

Corresponding author: Hong-Yan Su (suhongyan16@163.com)

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Abstract

Lignicolous freshwater fungi represent one of the largest groups of Ascomycota. This taxonomically highly diverse group plays an important role in nutrient and carbon cycling, biological diversity and ecosystem functioning. The diversity of lignicolous freshwater fungi along a north-south latitudinal gradient is currently being studied in Asia. In this paper, we introduce two novel freshwater taxa viz. *Tingoldiago hydei* sp. nov. and *T. clavata* sp. nov. which were collected from freshwater substrates in Eastern Thailand. Morphological comparison based on the size of ascomata, asci and ascospores, as well as multi-gene phylogenetic analyses based on LSU, SSU, ITS and TEF1-α DNA sequences, supports their placement in *Tingoldiago* (Lentitheciaceae). Descriptions and illustrations of these two new species are provided.

Keywords

2 new species, Lentitheciaceae, Freshwater fungi, phylogeny, taxonomy
Introduction

Freshwater fungi are those which the whole or part of their life cycle is found in a freshwater habitat (Thomas 1996, Wong et al. 1998) and they are an evolutionary important group (Vijaykrishna et al. 2006). The members of freshwater fungi can be saprobes, parasites, endophytes and mutualistic taxa (Vijaykrishna et al. 2005, Zhang et al. 2008, Swe et al. 2009, Jones et al. 2014, Huang et al. 2018). There is a wide range of organisms that can be freshwater fungi hosts, such as wood, plants, alga, foams, fish etc. (Sparrow 1960, Ellis and Ellis 1985, Jones et al. 2014). However, a lot of studies on freshwater fungi have focused on lignicolous freshwater fungi (Tsui et al. 2000, Cai et al. 2002, Luo et al. 2004, 2018, Jones et al. 2014, Hyde et al. 2016, Yang et al. 2017), which were defined as those fungi that grow on submerged woody debris in freshwater streams, ponds, lakes and tree hollows (Hyde et al. 2016). They also grow on submerged wood in peat swamps and dams (Pinnoi et al. 2006, Pinruan et al. 2007, 2014, Hu et al. 2010). Lignicolous freshwater fungi are a diverse group comprising species from different phyla (Aphelidiomycota, Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Monoblepharomycota, Mortierellomycota and Rozellomycota) (Shearer et al. 2007, Kagami et al. 2012, Zhang et al. 2012, Jones et al. 2014, Wijayawardene et al. 2018). The dominant groups of lignicolous freshwater fungi are Dothideomycetes and Sordariales (Jones et al. 2014, Hyde et al. 2016, Wijayawardene et al. 2017, 2018).

We are studying the diversity of lignicolous freshwater fungi in Thailand, in order to establish the phylogenetic relationships of lignicolous freshwater fungi, understanding the natural classification of this group and contributing to the biogeographical diversity of fungi (Hyde et al. 2016). The study on freshwater fungi in Thailand was first investigated by Tubaki et al. (1983) and they reported 40 freshwater fungal species from foam. Subsequently, mycologists started to study lignicolous freshwater fungi in Thailand and several taxa have been reported (Sivichai et al. 1998, 1999, 2000, 2002, 2010, Jones et al. 1999, Marvanová et al. 2000, Hu et al. 2010, Zhang et al. 2013, Luo et al. 2015, 2016, Bao et al. 2018).

Lentitheciaceae was introduced by Zhang et al. (2012) to accommodate Massarina-like species in the order Pleosporales. Presently, 13 genera are accepted in this family (Dayarathne et al. 2018, Hyde et al. 2018). Species in this family are widely distributed in the world (China, Egypt, Hungary, Italy, Japan, Russia, Saudi, Thailand, UK, Uzbekistan) and are commonly saprobic on stems and twigs of herbaceous and woody plants in terrestrial or aquatic habitats (Wanasinghe et al. 2014, 2018, Knapp et al. 2015, Wijayawardene et al. 2015, Luo et al. 2016, Tibpromma et al. 2017, Hyde et al. 2018). The genus Tingoldiago was established by Hirayama et al. (2010) with a single species Tingoldiago graminicola K. Hiray. & Kaz. Tanak, this species being originally treated as Massarina ingoldiana. Later, Hirayama et al. (2010) re-assessed the phylogeny of Massarina ingoldiana and introduced two new genera Tingoldiago and Lindgomyces to accommodate Massarina ingoldiana sensu lato, based on phylogenetic analyses. Currently, only one species is accepted in this genus.
In this paper, we introduce two new freshwater species of Tingoldiago (Lentitheciaceae), based on morpho-molecular studies. Detailed descriptions and illustrations of these two new species are provided.

Materials and methods

Collection, Isolation and morphological studies

Submerged decaying wood samples were collected from That Phanom, Nakhon Phanom, Thailand and brought to the laboratory in plastic bags. The samples were incubated in plastic boxes lined with moistened tissue paper at room temperature for one week. Specimen observations and morphological studies were conducted, following the protocols provided by Luo et al. (2018).

Pure cultures were obtained by single spore isolation followed by Chomnunti et al. (2014). Germinating ascospores were transferred aseptically to potato dextrose agar (PDA) plates and grown at 16–25 °C in daylight. Colony colour and other characters were observed and measured after three weeks. The specimens were deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand. Living cultures are deposited in the Culture Collection of Mae Fah Luang University (MFLUCC). Facesoffungi numbers and Index Fungorum numbers were obtained, following Jayasiri et al. (2015) and Index Fungorum (2019). New species have been established as recommended by Jeewon and Hyde (2016).

DNA extraction, PCR amplification and sequencing

Fungal mycelium was scraped from the surface of colonies grown on a PDA plate or MEA plate at 25 °C for 4 weeks, transferred into a 1.5 ml centrifuge tube and ground using liquid nitrogen. The EZ geneTM fungal gDNA kit (GD2416) was used to extract DNA from the ground mycelium according to the manufacturer’s instructions. The gene regions of the large subunit of the nuclear ribosomal DNA (LSU), the internal transcribed spacers (ITS), the small subunit of the nuclear ribosomal DNA (SSU) and the translation elongation factor (TEF1-α) RNA were amplified using the primer pairs LR0R/LR7 (Vilgalys and Hester 1990), ITS5/ITS4, NS1/NS4 (White et al. 1990) and 983F/2218R (Liu et al. 1999), respectively. The amplification reactions were performed in 25 μl of PCR mixtures containing 9.5 μl ddH₂O, 12.5 μl 2× PCR MasterMix (Tsingke Co., China), 1 μl DNA sample and 1 μl of each primer. The PCR thermal cycle programme for LSU, ITS, SSU and TEF1-α amplification were as follows: 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds, elongation at 72 °C for 1 minute and a final extension at 72 °C for 10 minutes and finally kept at 4 °C. PCR amplification was confirmed on 1% agarose electrophoresis gels.
stained with ethidium bromide. PCR products were sequenced using the same set of primers used in PCR in Beijing Tsingke Biological Engineering Technology and Services Co. Ltd. (Beijing, P.R. China).

**Sequencing and sequence alignment**

The sequence was assembled by using BioEdit and sequences with high similarity indices were determined from a BLAST search to find the closest matches with taxa in Lentitheciaceae and from recently published data (Dayarathne et al. 2018). All consensus sequences and the reference sequences were aligned using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), then checked visually and manually optimised using BioEdit v.7.0.9 (Hall 1999). Ambiguous regions were excluded from the analyses and gaps were treated as missing data. The phylogeny website tool “ALTER” (Glez-Peña et al. 2010) was used to convert the alignment fasta file to Phylip format for RAxML analysis and Clustalx BETA and PAUP 4.0 were used to convert the alignment fasta file to a Nexus file for Bayesian analysis. Phylogenetic analyses were obtained from Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian analysis.

**Phylogenetic analyses**

Maximum likelihood trees were generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis 2006, Stamatakis et al. 2008) in the CIPRES Science Gateway platform (Miller et al. 2010) using GTR+ I + G model of evolution which was estimated by MrModeltest 2.2 (Nylander et al. 2008). Maximum likelihood bootstrap values (ML), equal to or greater than 75%, are given above each node (Figure 1).

MP analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BS) analysis with 1000 replicates, each with ten replicates of random stepwise addition of taxa (Hillis and Bull 1993).

The Bayesian analysis was performed with MrBayes v3.2 (Ronquist et al. 2012), with the best-fit model of sequence evolution estimated with MrModeltest 2.2 (Nylander et al. 2008) to evaluate posterior probabilities (PP) (Rannala and Yang 1996, Zhaxybayeva and Gogarten 2002) by Markov Chain Monte Carlo (MCMC) sampling. Six simultaneous Markov chains were run for 10,000,000 generations, trees were sampled every 1000<sup>th</sup> generation and 1,0000 trees were obtained. Based on the tracer analysis, the first 1,000 trees representing 10% were discarded as the burn-in phase in the analysis. The remaining trees were used to calculate posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01).
Table 1. Taxa used in this study and their GenBank accession numbers, the newly generated sequences are indicated with * and the type strains are indicated in bold.

| Taxa                          | strain     | GenBank accession number |
|-------------------------------|------------|--------------------------|
|                              | LSU        | SSU                      | ITS          | TEF1        |
| *Bambusicola bambusae*        | MFLUCC 11–0614 | JX442035               | JX442039   | NR121546   | KP761722   |
| *B. irregulispora*            | MFLUCC 11–0437 | JX442036               | JX442040   | NR121547   | KP761723   |
| *B. massarinia*               | MFLUCC 11–0389 | JX442037               | JX442041   | NR121548   | –           |
| Binuria novaezelandiae        | AFTOL 1D931 | –                        | –           | –           | DQ471087   |
| *Byssothecium circinans*      | CBS67592   | GU205217                 | GU205235   | –           | GU349061   |
| Corynespora cassiicola        | CBS100822  | GU301808                 | GU296144   | –           | GU349052   |
| C. smithii                    | CAB15649b  | GU323201                 | –           | –           | GU349018   |
| *Dacampia engeliana*          | 72868      | KT383791                 | –           | –           | –           |
| D. hoekeri                    | 74269      | KT383793                 | –           | –           | –           |
| D. hoekeri                    | 81840      | KT383795                 | –           | –           | –           |
| *Darksidea alpha*             | CBS 135650 | KP184019                 | KP184049   | NR137619   | KP184166   |
| D. beta                       | CBS 135637 | KP184023                 | KP184049   | NR137597   | KP184189   |
| D. delta                      | CBS 135638 | –                        | NR137075   | –           | –           |
| D. epsilon                    | CBS 135658 | KP184029                 | KP184070   | NR137959   | KP184186   |
| D. gamma                      | CBS 135634 | KP184031                 | KP184073   | NR137587   | KP184188   |
| D. zeta                       | CBS 135640 | KP184013                 | KP184071   | NR137958   | KP184191   |
| Falciiformispora lignatilis   | BCC 21117  | GU371826                 | GU371834   | –           | GU371819   |
| F. lignatilis                 | BCC 21118  | GU371827                 | GU371835   | –           | GU371820   |
| Halobyssothecium obiones      | 27AY2385   | –                        | –           | –           | XX263864   |
| H. obiones                    | MFLUCC 15–0381 | MH376744              | MH376745   | MH377060   | MH376746   |
| *Helicascus nypae*            | BCC36752   | GU479789                 | GU479755   | –           | GU479855   |
| Kalmusia scabrispora          | KT2202     | AB524594                 | AB524453   | –           | AB539107   |
| Karstenella rhodostoma        | CBS69094   | GU301821                 | GU296154   | –           | GU349067   |
| Katumotoa bambusicola         | KT1517a    | AB524595                 | AB524454   | LCO14560   | AB539108   |
| Keizeriella brevisaca         | KT649      | AB807588                 | AB797298   | –           | AB808567   |
| K. culmifida                  | KT2642     | AB807592                 | AB797302   | LCO14562   | –           |
| K. gloeospora                 | KT829      | AB807589                 | AB797299   | LCO14563   | –           |
| K. poagena                    | CBS136767  | KJ869170                 | –          | KJ869112   | –           |
| K. quadriripetata             | KT2292     | AB807593                 | AB797303   | AB811456   | AB808572   |
| K. taminensis                 | KT571      | AB807595                 | AB797305   | LCO14564   | AB808574   |
| K. trichophoricola            | CBS 136770 | AB807595                 | AB797305   | LCO14566   | AB808515   |
| Lentithecium clionina         | KT1149A    | AB807540                 | AB797250   | LCO14566   | AB808515   |
| L. fluviatile                 | CBS 123090 | FJ795450                 | FJ795492   | –           | –           |
| L. pseudoliminum              | KT1111     | AB807544                 | AB797254   | AB809632   | AB808520   |
| *Massarina cisti*             | CBS 266 62 | FJ795447                 | FJ795490   | LCO14568   | AB808514   |
| M. eburna                     | CBS 473 64 | GU301840                 | GU296170   | –           | GU349040   |
| Montagnula opulenta           | AFTOLID1734 | DQ678086              | AF164370   | –           | –           |
| Morosphaeria                  | JK5304B    | GU479794                 | GU479790   | –           | –           |
| ramunculicola                 |            |                         |             |             |             |
| *Murilentinhectium clematidis*| IT1078     | KM408758                 | KM408760   | KM408756   | –           |
| M. clematidis                 | MFLUCC 14–0562 | KM408759            | KM408761   | KM408757   | KM454445   |
| Neophytophaerella sassaica    | KT1706     | AB524599                 | AB524458   | LCO14577   | AB539111   |
| Palmisacoma gregariacomum     | MFLUCC 11–0175 | KP744495             | KP753958   | KP744452   | –           |
| Paraconiothyrium brasiliense  | CBS100299  | JX496124                 | AY642523   | JX496011   | –           |
| Paraphaeosphaeria michotii    | MFLUCC 13–0349 | JX939282            | JX939285   | JX939279   | –           |
| P. minutans                   | CBS122788  | EU754173                 | EU754074   | –           | GU349083   |
| Phaeodatis winterti           | CBS18258   | –                        | GU296183   | –           | –           |
| Phragnocamarosporium platani  | MFLUCC 14–1191 | KP842915            | KP842918   | –           | –           |
| Taxa                  | strain   | GenBank accession number |
|----------------------|----------|-------------------------|
| **Pleurophoma ossicola** | CBS139905 | KR476769 – KR476736 – |
| **P. ossicola**       | CPC24985  | KR476770 – NR137992 – |
| **Pleurophoma pleurospora** | CBS130329 | JF740327 – – – |
| **Poacea coma aquaticum** | MFLUCC 14-0048 | KT324690 KT324691 – – |
| **P. halophila**      | MFLUCC 15-0949 | MF615399 MF615400 – – |
| **P. helicoides**     | MFLUCC 11-0136 | KP998462 KP998463 KP998459 KP998461 |
| **Pseudomurilentithecium camporeisi** | MFLUCC 14-1118 | MN638846 MN638850 MN638861 – |
| Seto septoria arundinacea | KT600 | AB807575 AB797285 LC014595 AB808551 |
| S. magniarundinacea   | KT1174  | AB807576 AB797286 LC014596 AB808552 |
| **S. phragmitis**     | CBS 114802 | KF251752 – KF251249 – |
| **S. scirpi**         | MFUCC 14-0811 | KY770982 KY770980 MF939637 KY770981 |
| Stagonospora macropycnida | CBS 114202 | GU301873 GU296198 – GU349026 |
| Tingo diago graminicola | KH155 | AB521745 AB521728 LC014599 AB808562 |
| T. graminicola        | KH68    | AB521743 AB521726 LC014598 AB808561 |
| T. graminicola        | KT891   | AB521744 AB521727 – AB808563 |
| *T. hydei*            | MFLUCC 19-0499 | MN857177 – MN857181 – |
| *T. clavata*          | MFLUCC 19-0496 | MN857178 MN857186 MN857182 – |
| *T. clavata*          | MFLUCC 19-0498 | MN857179 MN857187 MN857183 – |
| *T. clavata*          | MFLUCC 19-0495 | MN857180 MN857188 MN857184 – |
| Towyspora aestuari     | MFLUCC 15-1274 | KU248852 KU248853 NR148095 – |
| Trematosphaeria pertusa | CBS 122368 | FJ201990 FJ201991 NR132040 KF015701 |
| **T. pertusa**        | CBS 122371 | GU301876 GU348999 KF015669 KF015702 |

The phylograms were visualised in FigTree 1.4.2 (Rambaut 2014) and made in Adobe Illustrator CS5 (Adobe Systems Inc., USA). All newly generated sequences of this study have been submitted in GenBank.

**Results**

**Phylogenetic analyses**

The aligned sequence matrix comprises LSU, SSU, ITS and TEF1-α sequence data for 69 taxa, with Corynespora smithii and Corynespora cassiicola as out-group taxa. The dataset comprises 3334 characters after alignment including gaps (LSU: 1–897; SSU: 898–1920; ITS: 1921–2522; TEF1-α: 2523–3479). The topologies of RAxML, MP and Bayesian are similar and the bootstrap support values for Maximum Likelihood (ML), Maximum Parsimony (MP) higher than 75% and Bayesian posterior probabilities (PP) greater than 0.95 are given above the nodes. Maximum parsimony analyses indicated that 2,442 characters were constant, 232 variable characters parsimony un-informative and 805 characters are parsimony-informative. The RAxML analysis of the combined dataset yielded the best scoring tree (Figure 1) with a final ML optimisation likelihood value of -21568.71378. The matrix had 1322 distinct alignment patterns,
The novel species Tingoldiago hydei and T. clavata, introduced in this paper, are supported by multi-phylogenetic analyses. Four newly generated strains clustered together within Tingoldiago with strong statistical support (100 ML/95 MP/1.00 PP, Figure 1). Three strains of T. clavata clustered together and sister to T. hydei with strong bootstrap support (99 ML/97 MP/1 PP, Figure 1).
Taxonomy

*Tingoldiago hydei* D.F. Bao, Z.L. Luo & H.Y. Su, sp. nov.

Index Fungorum No: IF557047

Facesoffungi No: FoF07082

Figure 2

**Etymology.** Referring to Kevin D. Hyde for his contributions in fungal taxonomy.

**Holotype.** THAILAND, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B-126 (MFLU 19–2842, holotype), ex-type living culture, MFLUCC 19–0499.

**Description.** Saprobic on submerged decaying wood. **Sexual morph:** Ascomata 180–280 × 330–470 μm (x = 400 × 420 μm, n = 10), immersed to semi-immersed, erumpent, gregarious, scattered, depressed globose to conical with a flattened base, dark brown to black, as dark spots on host surface. Ostioles central, papillate, short, crest-like, dark brown. Peridium 33.5–50 μm wide, comprising 4–6 layers, brown to dark brown cells of textura anngularis. Hamathecium comprising 2–2.5 μm (n = 30) wide, numerous, branched, septate, hyaline, cellular pseudoparaphyses. Asci 95–164 × 18–22 μm (x = 129 × 20 μm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical-clavate, rounded at apex, with a short pedicellate. Ascospores 37.5–42 × 7.5–9 μm (x = 40 × 8 μm, n = 30), overlapping, 2–3-seriate, clavate with round ends, straight, unisepatate, deeply constricted at septum, with broad and short upper cells 17.5–20 × 7–8.7 μm (x = 18.7 × 7.9 μm, n = 30), narrow and long lower cells 20.6–23.3 × 5.9–7.4 μm (x = 21.9 × 6.7 μm, n = 30), tapering towards the end, with short appendages at the septum, hyaline, guttulate, smooth, surrounded by a fusiform gelatinous sheath.

**Asexual morph:** Undetermined.

**Culture characteristics.** Ascospores germinating on PDA within 24 hours. Colonies on MEA effuse, greyish-white to dark brown from above and below, reaching 3–4 cm diameter within 30 days at room temperature under natural light, composed of subhyaline to pale brown, septate, smooth hyphae.

**Notes.** Phylogenetic analysis showed that *Tingoldiago hydei* is related to *T. clavata*; however, they are in different lineages with significant support (99 ML/97 MP/1.00 PP, Figure 1). *Tingoldiago hydei* resembles *T. clavata* in having bitunicate, cylindrical-clavate asci and clavate, hyaline, unisepatate, ascospores with broad and short upper cells, narrow and long lower cells, tapering towards the end, surrounded by a gelatinous sheath. However, *Tingoldiago hydei* can be distinguished from *T. clavata* in having longer and narrower asci (95–164 × 18–22 vs. 110–148 × 20–27 μm) and smaller ascospores (37.5–42 × 7.5–9 vs. 48–51 × 7.5–8.5 μm). Moreover, ascospores of *T. clavata* have longer appendages at the septum, while the appendages of *T. hydei* are much shorter than *T. hydei*.

*Tingoldiago clavata* is similar to the type species, *T. raminicola* in having immersed to semi-immersed, depressed globose to conical ascomata with flattened base, bitunicate, fissitunicate, cylindrical-clavate asci and clavate, straight, unisepatate ascospores.
Figure 2. Tingoldiago hydei (MFLU 19–2842, holotype). a–c Ascomata on wood d section of ascoma e peridium f, g pseudoparaphyses h ostiole i–l asci m–r ascospores s germinating ascospore t vegetative hyphae in culture u, v culture on PDA from surface and reverse. Scale bars: 50 μm (d, e, h), 20 μm (f–g, m–t), 30 μm (i–l).
However, *T. clavata* differs from *T. raminicola* in having longer asci (95–164 × 18–22 vs. 87.5–122 × 18.25–25 μm) and smaller ascospores (37.5–42 × 7.5–9 vs. 43.5–53 × 7.5–11 μm). Moreover, ascospores of *T. clavata* have short appendages at the septum while ascospores of *T. raminicola* lack appendages. In addition, we compared the base pairs of ITS regions between these two species and there were 25 base pairs without gaps (5.1%) differences. Therefore, we introduce our isolate as a new species based on both phylogeny and morphological characters.

*Tingoldiago clavata* D.F. Bao, L. Xu & H.Y. Su, sp. nov.
Index Fungorum No: IF557048
Facesoffungi No: FoF07083
Figure 3

**Etymology.** Referring to the clavate ascospores of this fungus.

**Holotype.** THAILAND, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B-161 (MFLU 19–2843, holotype), ex-type culture, MFLUCC 19–0496.

**Description.** Saprobic on submerged decaying wood. **Sexual morph:** Ascomata 145–210 × 145–195 μm (x = 175 × 169 μm, n = 10), immersed to semi-immersed, gregarious, scattered, erumpentia, depressed globose to conical with a flattened base, dark brown to black, as dark spots on host surface. **Ostiole** central, round to papilate, short, crest-like, dark brown. **Peridium** 28–47 μm wide, comprising several layers, pale brown to brown cells of textura anngularis. **Hamathecium** comprising 1.5–2.0 μm (n = 30) wide, numerous, branched, septate, hyaline, cellular pseudoparaphyses. Asci 110–148 × 20–27 μm (x = 129 × 23 μm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical-clavate, rounded at apex, with a short pedicellate. **Ascospores** 48–51 × 7.5–9 μm (x = 50.5 × 8.5 μm, n = 30), overlapping, 2–3-seriate, clavate, with round ends, straight, uniseptate, deeply constricted at septum, hyaline, with broad and short upper cells 16.6–18.9 × 7.8–9.0 μm (x = 17.7 × 8.4 μm, n = 30), narrow and long lower cells 30–32.9 × 6.5–8.0 μm (x = 31.5 × 7.3 μm, n = 30), tapering towards the end, guttulate, smooth, 2–4 equatorial appendages at the septum and surrounded by a fusiform gelatinous, sheath. **Asexual morph:** Undetermined.

**Culture characteristics.** Ascospores germinating on PDA within 24 hours. Colonies on MEA effuse, velvety, greyish-white to dark brown from above and below, reaching 2.5–3 cm diameter within 30 days at room temperature under natural light, composed of subhyaline to brown, septate, smooth hyphae.

**Additional specimens examined.** THAILAND, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B160 (paratype: MFLU 19–2844; living culture, MFLUCC 19–0498); THAILAND, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B136 (paratype: MFLU 19–2845; living culture, MFLUCC 19–0495)
Lignicolous freshwater ascomycota from Thailand

Figure 3. Tingoldiago clavata (MFLU 19–2843, holotype). a–c ascomata on wood d section of ascoma e ostiole f peridium g pseudoparaphyses h–l asci m–r ascospores s vegetative hyphae in culture t, u culture on PDA from surface and reverse. Scale bars: 50 μm (d, e), 20 μm (f–l), 10 μm (m–s).
Notes. *Tingoldiago clavata* resembles the type species, *T. graminicola* in having bitunicate, cylindrical-clavate asci with a short pedicellate and clavate, hyaline, 1-septate, ascospores with broad upper cells, narrow lower cells. However, we can distinguish them by the size of ascomata and asci and the colour, septate and appendages of ascospores. *Tingoldiago clavata* has smaller ascomata (110–148 × 145–195 vs. 150–250 × 250–450 μm) and larger asci (110–148 × 20–27 vs. 87.5–122 × 18.25–25 μm). Moreover, ascospores of *T. clavata* are hyaline, uniseptate, with 2–4 equatorial appendages at the septum, while ascospores of *T. graminicola* are brown and 3-septate at maturity and lacking appendages at the septum. In addition, a comparison of the 491 nucleotides across the ITS gene region of *T. clavata* and *T. graminicola* reveals 25 base-pair differences and therefore provides further evidence to introduce *T. clavata* as a new species as recommended by Jeewon and Hyde (2016).

Discussion

During the last decade, freshwater fungi in Thailand have been mainly reported from north, south and northeast of Thailand (Jones et al. 1999, Marvanová and Hywel-Jones 2000, Sivichai and Boonyuen 2010, Sivichai and Hywel-Jones 1999, Sivichai et al. 1998, 2000, Sri-indrasutdhi et al. 2010). No freshwater fungi from Eastern Thailand have been reported so far. In this study, two new freshwater species, viz. *Tingoldiago hydei* and *T. clavata* from Eastern Thailand, are introduced, based on morphology and phylogeny. *Tingoldiago hydei* and *T. clavata* satisfied the generic concept of the genus *Tingoldiago* (Hirayama et al. 2010). They comprise globose to conical, immersed to erumpent ascomata, cellular pseudoparaphyses, bitunicate, fissitunicate asci and clavate ascospores with a median primary septum and a large fusiform gelatinous sheath around the ascospore (Hirayama et al. 2010). Morphologically, *T. hydei* and *T. clavata* are quite similar as they have similar shape of asci and ascospores; however, we can distinguish them by the size of ascomata, asci and ascospores (Table 2). In addition, we also compared the morphological differences of these two species with the type species, *T. graminicola*. Ascospores of *T. hydei* and *T. clavata* are hyaline, uniseptate, with appendages at the septum and the upper cells are broader and shorter than the lower cells, while the ascospores of *T. graminicola* are hyaline, uniseptate, but becoming brown and 3-septate with age, lacking appendages at the septum, upper cells and lower cells are similar lengths. Phylogenetic analyses showed that our two new isolates clustered together and are sister to the type species, *Tingoldiago graminicola* with strong bootstrap support (100 ML/92 MP/1.00 PP). This evidence strongly supports our two isolates to be the new species.

Hyde et al. (2020) introduced a new genus, *Pseudomurilentithecium* in Lentitheciaceae. In their phylogenetic analysis, *Pseudomurilentithecium* clustered with *Poaceascoma* and was basal to Lentitheciaceae. However, in our phylogenetic analysis, *Pseudomurilentithecium* grouped with the members of Massarinaceae, rather than Lentitheciaceae. Therefore, further investigation is required to confirm the placement of the genus.
Tingoldiago is a well-resolved genus in this family with a stable clade within Lentitheciaceae. The genus can be distinguished from other genera in this family by having hyaline, uniseptate, upper cells are broad and basal cells are narrow ascospores with a large fusiform gelatinous sheath. The sheath is considered to be an adaptation by the genus that enables ascospores to attach to the substrates in moving water (Shearer 1993, Hyde and Goh 2003, Jones 2006, Devadatha et al. 2019). It is reported that the genus Tingoldiago is exclusively found in freshwater habitats (Hirayama et al. 2010) and our two new species were collected from lotic habitats of Mekong River.

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| Taxa            | Distribution | Ascomata (μm) | Pseudoparaphyses (μm) | Asci (μm) | Ascospores (μm) | References                        |
|-----------------|--------------|---------------|------------------------|-----------|----------------|-----------------------------------|
| Tingoldiago     |              |               |                        |           |                | Hirayama et al. 2010              |
| graminicola     | Japan, UK    | 150–250 × 250–450 | 1.5–4                   | 87.5–122 × 18.25–25 | 43.5–53 × 7.5–11 |                                   |
| T. hydei        | Thailand     | 180–280 × 330–470 | 1.8–2.5                 | 95–164 × 18–22   | 37.5–42 × 7.5–9  | This study                       |
| T. clavata      | Thailand     | 145–210 × 145–195 | 1.4–2.0                 | 110–148 × 20–27  | 48–51 × 7.5–8.5  | This study                       |

Table 2. The morphological comparisons of Tingoldiago species discussed in this study.
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