J. Jpn. Bot. **94**(2): 96–102 (2019)

***Phaeocalicium polyporaeum* (*Mycocaliciaceae*, *Ascomycota*) New to Japan**

Hiroshi Masumoto[a,*], Yoshihito Ohmura[b] and Yousuke Degawa[a]

[a]Sugadaira Research Station, Mountain Science center, University of Tsukuba, 1278-294, Sugadairakogen, Ueda, Nagano, 386-2204 JAPAN;
[b]Department of Botany, National Museum of Nature and Science, 4-1-1, Amakubo, Tsukuba, 305-0005 JAPAN
*Corresponding author: masumoto.fungi@gmail.com

(Accepted on January 16, 2019)

Phaeocalicium polyporaeum (Nyl.) Tibell, a non-lichenized calicioid fungus, is reported as new to Japan. It was found on the surface of *Trichaptum abietinum*, a small polyporous fungus, growing on the trunks of *Pinus densiflora* and *Abies* sp. at an elevation of 1348 m in Nagano Pref., central Japan. The ITS rDNA and nrLSU sequences were obtained from the specimens of Japan and Far East Russia, in which the differences were one transition and one indel for ITS rDNA (ITS1 region) and one transition for nrLSU (D1/D2 region). The molecular phylogenetic position for our samples of *P. polyporaeum* among the related taxa based on the ITS rDNA sequences is also shown.

Key words: Calicioid fungus, eastern Asia, ITS rDNA, nrLSU, taxonomy, *Trichaptum abietinum*.

Phaeocalicium A. F. W. Schmidt (*Mycocaliciaceae*, *Ascomycota*) is a non-lichenized group of calicioid fungi and 17 species are recognized (Lumbsch and Lücking 2016). This genus is characterized by non-mazaediate apothecium and typically growing on the bark of branches, twigs and sapling of certain genera of tree or shrub, or parasitic on small polypores such as *Trichaptum biforme* and *T. abietinum* (Hawksworth et al. 2014, Selva 2014).

In Japan, three species of *Phaeocalicium* [i.e., *P. compressulum* (Nyl. ex Szatala) A. F. W. Schmidt, *P. flabelliforme* Tibell, and *P. triseptatum* Tibell] have been recorded before the present study (Tibell and Thor 2003, Tibell et al. 2014). During the course of mycobiota studies of Japan, an ecologically interesting *Phaeocalicium* species growing on fruiting bodies of *Trichaptum abietinum* was collected from Nagano Prefecture, central Japan. The morphology and molecular data confirm the identity as *Phaeocalicium polyporaeum* (Nyl.) Tibell that is new to Japan.

This study aims to describe the morphological and anatomical features of the Japanese material of this species and provide the molecular data of ITS rDNA (including ITS1, 5.8S rDNA, and ITS2 regions) and nrLSU (D1/D2 region).

Materials and Methods

Field investigations were carried out in 2017 and 2018 for Japanese material and in 2014 for Russian material. Voucher specimens are housed in the National Museum of Nature and Science

(TNS), Tsukuba, Japan, and the fungal cultures are deposited at NITE Biological Resource Center (NBRC), Kisarazu, Japan.

Morphological observation was made using a dissecting microscope (Olympus SZ61 and Olympus SZX16 equipped with an Olympus DP21 digital camera) and a differential interference contrast microscope (Olympus BX53 equipped with an Olympus DP73 digital camera). Anatomical examination was made on squash preparation mounted in water. Spore measurements are given as minimum–average–maximum (n = number of measurements).

For isolation of the fungus, spore suspension was spread onto 2.0% agar plate and each germinated spore was transferred onto 1.5% PDA plate to obtain a monosporic culture at room temperature (ca. 25 °C).

Genomic DNA was extracted from herbarium specimens (Y. Ohmura 11422, TNS) and a fungal culture (NBRC 113675) employing the procedure described by Izumitsu et al. (2012). PCR reactions were performed with the primer pairs ITS1F (Gardes and Bruns 1993) and LR5 (Vilgalys and Hester 1990) for ITS rDNA and nrLSU (D1/D2 region) sequences using KOD FX neo DNA polymerase (Toyobo, Osaka). Sequencing was carried out on an ABI 3130 genetic analyzer (Applied Biosystems) with BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions.

Sequence alignments for ITS rDNA and nrLSU were performed using MAFFT 7 (Katoh et al. 2017) and manually optimized using Seaview v.4.6.1 (Gouy et al. 2010). The ITS rDNA sequences were partitioned into ITS1, 5.8S rDNA and ITS2 by using ITSx v.1.1b (Bengtsson-Palme et al. 2013) to compare transitions and indels at each region.

The phylogenetic analyses of ITS rDNA were conducted using maximum-likelihood (ML) and Bayesian methods. The nrLSU was not applied to the phylogenetic analyses, because the sequences of the region were unavailable for many taxa of *Mycocaliciaceae*. The optimum substitution models for each data set were estimated using Kakusan4 software (Tanabe 2011) based on the Akaike information criterion (AIC; Akaike 1974) for the ML analysis and on the Bayesian information criterion (BIC; Schwarz 1978) for the Bayesian analysis. The ML analysis were performed with RAxML v8.2.10 on the CIPRES Science Gateway v3.3 webportal (Miller et al. 2010, Stamatakis 2014), using the GTR+GAMMA model containing the AICc4 parameter. Bootstrap proportions (BPs) were obtained using 1000 bootstrap replications. The Bayesian analysis was performed with MrBayes v.3.2.6 on the CIPRES Science Gateway (Miller et al. 2010, Ronquist et al. 2012), using the K80+GAMMA model containing the BIC4 parameter. Two simultaneous and independent Metropolis-coupled Markov Chain Monte Carlo (MCMC) runs were performed for 2,000,000 generations with the tree sampled for every 1,000 generations of the analyses. Convergence of the MCMC procedure was assessed from the average standard deviation of split frequencies (< 0.01) and effective sample size scores (all > 100) using MrBayes and Tracer v. 1.7.1 (Rambaut et al. 2018), respectively. The first 25% of the trees were discarded as burn-in, and the remainder were used to calculate the 50% majority-rule trees and to determine the posterior probabilities (PPs) for individual branches. *Byssochlamys laguncularlae* (U18361) and *Penicillium velutinum* (AF033411) were used as outgroups. These alignments were submitted to TreeBASE under study number S23354.

Results and Discussion

Phaeocalicium polyporaeum (Nyl.) Tibell, Publications from the Herbarium, University of Uppsala, Sweden **4**: 7 (1979).

The morphological features of the Japanese material (Fig. 1) agree well with the descriptions provided by Tibell (1981) (ascospores 9.8–12.5–17.4 × 2.7–3.7–4.9 µm), exsiccatae [James Colin

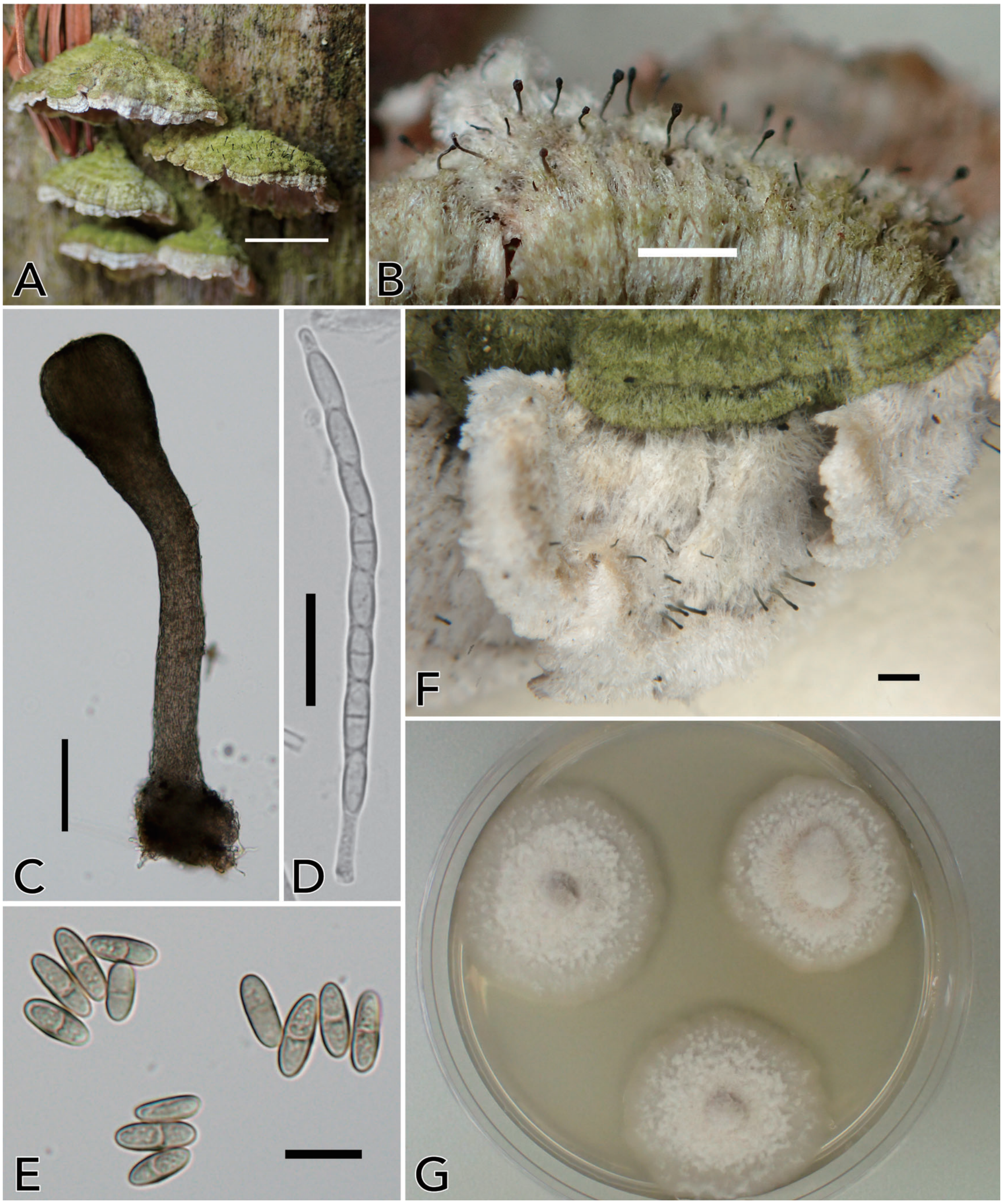

Fig. 1. *Phaeocalicium polyporaeum* collected in Japan. A. *Trichaptum abietinum* covered with aerial alga, the substrate of *P. polyporaeum*. B. Gregarious calicioid ascomata on *Trichaptum abietinum* (H. Masumoto 213, TNS). C. Ascoma. D. Ascus. E. Ascospores. F. Ascomata on freshly formed fruiting bodies of *T. abietinum* (H. Masumoto 268, TNS). G. Colony morphology of the fungal culture (NBRC 113675) incubated on PDA for one month. Scales: 5 mm (A), 1 mm (B), 0.5 mm (F), 100 μm (C), 20 μm (D), 10 μm (E).

Lendemer, Lichens of Eastern North America Exsiccati 203 (TNS) (ascospores 9.9–12.4–15.2 × 2.7–3.5–4.8 µm, n = 50); Leif Tibell, Caliciales Exsiccatae 45 (TNS) (ascospores 8.8–10.8–14.7 × 2.8–3.6–4.4 µm, n = 50), 169 (TNS) (ascospores 9.6–11.6–14.6 × 2.8–4.0–5.6 µm, n = 28); Clifford M. Wetmore; Lichenes Exsiccati 47 (ascospores 10.5–11.8–14.4 × 3.1–3.6–4.6 µm, n = 31)], and a specimen from the Russian Far East (Y. Ohmura 11422, TNS) (ascospores 8.5–10.1–14.8 × 2.6–3.5–4.8 µm, n = 87). The measured values of morphological features for the Japanese material fell within the range of the known sizes, and the other features are summarized as follows: Ascomata black, stalked, solitary to gregarious on host pileus (Fig. 1A, B); apothecial cup not laterally-compressed, 75–190 µm wide; stipe 450–800 µm in length, 100–160 µm thick at the base, and 35–60 µm at the narrowest part (Fig. 1C). Asci persistent but often evanescent when aged, cylindrical, 80–105 × 3.4–5.0 µm with a thickened apex (Fig. 1D). Ascospores uniseriate, pale brown, oblong to cylindrical with obtuse ends, 8.5–10.5–14.5 × 2.8–3.7–4.5 µm (n = 160), primarily one-septate but occasionally aseptate (Fig. 1E).

The ascospores of *P. polyporaeum* germinated in 48 h on the 2% agar plate at room temperature (ca. 25 °C) and formed a cream velvet colony with ca. 3 cm diam. on PDA plate in one month (Fig. 1G). Neither ascoma nor conidium was formed on PDA the plate.

Phaeocalicium polyporaeum is morphologically similar to the other three *Phaeocalicium* species occurring in Japan but it is distinguished by the 0–1 septate spore and the obconical capitula. In addition, the habitat on the fruiting bodies of polyporous fungi is characteristic for *P. polyporaeum* while others are saprobic or parasitic on twig or bark of certain genera of trees such as *Alnus*, *Betula*, and *Sorbus*.

The Japanese collection of *P. polyporaeum* was found on the surface of the fruiting bodies of *Trichaptum abietinum*, a small polyporous fungus, grown on trunks of planted *Abies* sp. and *Pinus densiflora* at an elevation of 1348 m in Nagano Prefecture, central Japan (Fig. 1A). Mature fruiting bodies of *Trichaptum abietinum* were often covered with aerial green algae such as *Apatococcus* sp., *Coccomyxa* sp., and *Stichococcus* sp. However, it seems that *P. polyporaeum* would not be lichenized because no direct morphological interaction was recognized among them and we observed that it grew on the freshly formed fruiting bodies of *T. abietinum* before being covered with algae (Fig. 1F).

This species has been reported from North America, Caucasus, Siberia, and European Russia (e.g., Hutchison 1987, Selva 2014, Davydov and Konoreva 2017). Although the type locality is in Hungary (Nylander 1875), this species was seldom collected in Europe (Tibell 1981). It is reported here for the first time from Japan.

Exsiccata examined: Leif Tibell, *Caliciales Exsiccatae* 45, 169 (TNS); James Colin Lendemer, Lichens of Eastern North America Exsiccati 203 (TNS); Clifford M. Wetmore; Lichenes Exsiccati 47 (TNS).

Specimens examined: **JAPAN**. Honshu. Prov. Shinano (Pref. Nagano): Sugadaira Research Station, Sugadairakogen, Ueda-city (36.52625N, 138.34888E), on *Trichaptum abietinum* on *Pinus densiflora*, elevation 1348 m, 5 December 2017, H. Masumoto 213 (TNS); ditto, 14 August 2018, H. Masumoto 268 (TNS); ditto, on *Trichaptum abietinum* on *Abies* sp., 14 August 2018, H. Masumoto 269 (TNS). **RUSSIA**. Primorsky Territory: ca. 8 km S of Mt. Snegnaya, Olginsky District (43°39′44″N, 134°24′32″E), on *Trichaptum abietinum* on Abies sp., elevation 830 m, 15 September 2014, Y. Ohmura 11422 (TNS).

Key to Japanese taxa of the genus *Phaeocalicium*

1. Spores 3 septate .... *P. triseptatum* Tibell
1. Spores 0–2 septate 2
2. Capitula obconical; on *Trichaptum abietinum* (or possibly allied polyporous fungi) .... *P. polyporaeum* (Nyl.) Tibell
2. Capitula strongly flattened when mature; on

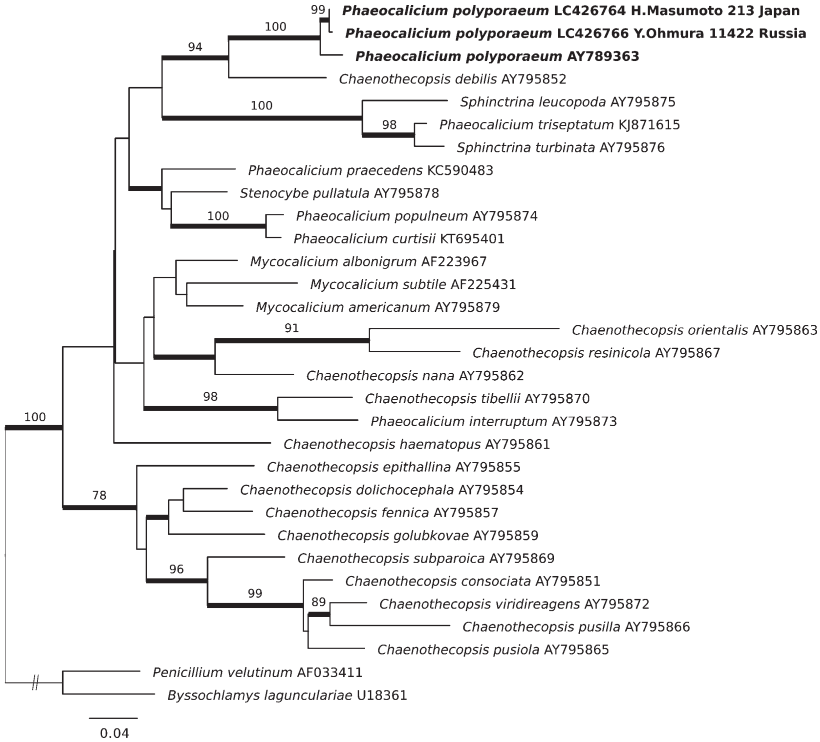

Fig. 2. Molecular phylogeny of *Phaeocalicium polyporaeum* in *Mycocaliciaceae* based on a maximum-likelihood (ML) tree based on the ITS rDNA sequences. Only a ML bootstrap proportion ≥ 75% is shown. Branches supported by Bayesian posterior probabilities ≥ 0.95 are indicated with bold lines. The scale bar represents the nucleotide substitutions per site.

smooth bark of *Alnus*, *Betula*, *Sorbus* 3
3. Spore non-septate .... *P. compressulum* (Nyl. ex Szatala) A. F. W. Schmidt
3. Spore 1-septate (rarely 2-septa) .... *P. flabelliforme* Tibell

Molecular analyses

Sequences of ITS rDNA (570 bp) and nrLSU (738 bp) of *Phaeocalicum polyporaeum* were successfully obtained in this study. The differences between Japanese and Russian samples were one transition and one indel for ITS1 of ITS rDNA and one transition for D1/D2 region of nrLSU. The BLAST identity of them was 99%. In comparison, between the sequence of Japanese sample and the *P. polyporaeum* sequence registered in Genbank (deposited as *Mycocalicium polyporaeum*; accession number AY789363), there were eight transitions and five indels in ITS1, three transitions in ITS2, and one transition in D1/D2 region of nrLSU, respectively, and the BLAST identity of ITS rDNA was 97%.

ML and Bayesian phylogenetic analyses were conducted using an aligned sequence dataset comprising 404 bp from ITS rDNA. The ML tree with the highest log likelihood (−4636.49) is shown in Fig. 2. The Bayesian likelihood score was −4689.53. The topology recovered by Bayesian analysis was identical to that of the ML tree. The sequences of Japanese and Russian samples and the sequence in Genbank (AY789363) formed a monophyletic clade with high support value [100 (BP)/1.00 (PP)], which suggests that they belong to the same species. However, the difference between the sequence AY789363 and our data from Japan and Russia was larger (97% BLAST identity) than that between Japan and Russia (99%). We could not discuss the morphological consistency between our samples and the sample of AY789363 because morphological information of the voucher specimen has never been shown in Wang et al. (2005) or in other studies (e.g., Tuovila et al. 2014). It is highly desirable to show morphological information especially when the sequence of a taxon is first registered into Genbank in order to ensure the accuracy of species identification or to compare with other data.

The phylogenetic tree (Fig. 2) indicates polyphyly of the genus *Phaeocalicium* as also shown by other studies (Tibell and Vinuesa 2005, Tuovila et al. 2014). Further taxonomic studies are needed to elucidate the generic concept of *Phaeocalicium* as well as other non-lichenized calicioid genera.

References

Akaike H. 1974. A new look at the statistical model identification. IEEE Trans. Automat. Contr. **19**: 716–723.

Bengtsson-Palme J., Ryberg M., Hartmann M., Branco S., Wang Z., Godhe A., de Wit P., Sánchez-García M., Ebersberger I., Sanli K., de Souza F., Kristiansson E., Abarenkov K., Eriksson M. and Nilsson R. H. 2013. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods. Ecol. Evol. **4**: 914–919.

Davydov E. A. and Konoreva L. A. 2017. New data on lichens from Salair province in Altaisky krai (Siberia, Russia). Turczaninowia **20**: 185–197.

Gardes M. and Bruns T. D. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. **2**: 113–118.

Gouy M., Guindon S. and Gascuel O. 2010 SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molec. Biol. Evol. **27**: 221–224.

Hawksworth D. L., Aguirre-Hudson B. and Ainsworth A. M. 2014. *Sphinctrina tigillaris*, an overlooked species of *Chaenothecopsis* growing on *Perenniporia meridionalis*, a polypore new to the UK. Lichenologist **46**: 729–735.

Hutchison L. J. 1987. Studies on *Phaeocalicium polyporaeum* in North America. Mycologia **79**: 786–789.

Izumitsu K., Hatoh K., Sumita T., Kitade Y., Morita A., Gafur A., Ohta, A., Kawai M., Yamanaka T., Neda H., Ota Y. and Tanaka C. 2012. Rapid and simple preparation of mushroom DNA directly from colonies and fruiting bodies for PCR. Mycoscience **53**: 396–401.

Katoh K., Rozewicki J. and Yamada K. D. 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. bbx108. doi:10.1093/bib/bbx108.

Lumbsch H. T. and Lücking R. 2016. Mycocaliciomycetidae. *In*: Jaklitsch W., Baral H. O., Lücking R., Lumbsch H. T. and Frey W. (eds.), Syllabus of plant families 1/2 *Ascomycota*. p.111. Gebr. Borntraeger Verlagsbuchhandlung, Stuttgart.

Miller M. A., Pfeiffer W. and Schwartz T. 2010. "Creating the CIPRES Science Gateway for inference of large phylogenetic trees" in Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA: 1–8.

Nylander W. 1875. Addenda nova ad lichenographiam europaeam. Cont. XIX. Flora **58**: 6–15.

Rambaut A., Drummond A. J., Xie D., Baele G. and Suchard M. A. 2018. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. Syst. Biol. **67**: 901–904.

Ronquist F., Teslenko M., van der Mark P., Ayres D. L., Darling A., Höhna S., Larget B., Liu L., Suchard M. A. and Huelsenbeck J. P. 2012. MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**: 539–542.

Schwarz G. 1978. Estimating the dimension of a model. Ann. Stat. **6**: 461–464.

Selva S. B. 2014. The calicioid lichens and fungi of the Acadian Forest ecoregion of northeastern North America, II. The rest of the story. Bryologist **117**: 336–367.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics **30**: 1312–1313.

Tanabe A. S. 2011. Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. Mol. Ecol. Resour. **11**: 914–921.

Tibell L. 1981. Comments on *Caliciales* exsiccatae II. Lichenologist **13**: 51–64.

Tibell L., Frisch A. and Thor G. 2014. Additions to the calicioid flora of Japan and Korea, with the descriptions of two new species. Ann. Bot. Fenn. **51**: 189–194.

Tibell L. and Thor G. 2003. Calicioid lichens and fungi of Japan. J. Hattori Bot. Lab. **94**: 205–259.

Tibell L. and Vinuesa M. 2005. *Chaenothecopsis* in a molecular phylogeny based on nuclear rDNA ITS and

LSU sequences. Taxon **54**: 427–442.
Tuovila H., Davey M. L., Yan L., Huhtinen S. and Rikkinen J. 2014. New resinicolous *Chaenothecopsis* species from China. Mycologia **106**: 989–1003.
Vilgalys R. and Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J. Bacteriol. **172**: 4238–4246.
Wang Z., Binder M. and Hibbett D. S. 2005. Life history and systematics of the aquatic discomycete *Mitrula* (*Helotiales*, *Ascomycota*) based on cultural, morphological, and molecular studies. Amer. J. Bot. **92**: 1565–1574.

升本 宙[a]，大村嘉人[b]，出川洋介[a]：**日本新産の *Phaeocalicium polyporaeum*（チビピンゴケ科ノミピンゴケ属）**

Phaeocalicium polyporaeum (Nyl.) Tibell コシカケノムシピン（新称）が長野県上田市（標高 1348 m）のアカマツ樹幹に生えたシハイタケの子実体表面に確認されたので，日本新産種として報告する．本種は微小なピンゴケ様の黒色の子嚢果を多数形成し，柄の長さは 450–800 µm，子嚢果の横幅は 75–190 µm，柄の基部の太さは 100–160 µm，柄の最も細い部分の太さは 35–60 µm であった．子嚢は円柱形で 80–105 × 3.4–5.0 µm．子嚢胞子は長楕円形から円柱形で両端は鈍形，淡褐色，8.5–10.5–14.5 × 2.8–3.7–4.5 µm，おもに 1 隔壁であるが無隔壁の場合もあった．本種はこれまでに北米を中心にヨーロッパロシアや極東ロシア，コーカサス地方で報告されていた．日本で既に報告されている同属の 3 種 *P. compressulum* (Nyl. ex Szatala) A. F. W. Schmidt（ノミピンゴケ），*P. flabelliforme* Tibell（ニセノミピンゴケ，新称），および *P. triseptatum* Tibell（ミツカベノミピンゴケ，新称）から本種は胞子の隔壁が 0–1 で子実体が倒円錐形という点で区別されるが，他種はいずれも樹木の枝や樹皮上に着生するので生育場所からも容易に区別することができる．

日本産およびロシア産の試料より得られた塩基配列を比較したところ，両者の間では ITS rDNA で 2 塩基，nrLSU で 1 塩基の違いがみられた．ITS rDNA にもとづく系統解析では，日本産試料はロシア産試料および Genbank に *P. polyporaeum* として登録された配列と同一クレードを形成し，種としての独立性が確認された．ただし，*Phaeocalicium* はすでに指摘されているように多系統の属であるため，*P. polyporaeum* の帰属については関連属の分類学的検討もあわせて今後行っていく必要がある．

([a]筑波大学大学院生命環境科学研究科，
[b]国立科学博物館植物研究部)