Ecological speciation of Japanese hedgehog mushroom: Hydnum subalpinum sp. nov. is distinguished from its sister species H. repando-orientale by means of integrative taxonomy

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Abstract

Hydnum repando-orientale is an East Asian species closely related to H. boreorepandum and H. repandum; all three species produce edible mushrooms. We identified two ecological groups of H. repando-orientale in Japan: a temperate group occurring in Fagaceae-dominated forest at < 1200 m a.s.l. (ROF) and a subalpine group occurring in coniferous forest in highland at > 1900 m a.s.l. (ROC). We re-examined the taxonomy of the two ecological groups of H. repando-orientale using integrative approaches. Phylogenies of the two ecological groups and other related species were inferred from the internal transcribed spacer (ITS) and gene portions encoding the large subunit of nc rRNA (LSU), translation elongation factor-1 alpha (TEF1), RNA polymerase II largest subunit (RPB1), and RNA polymerase II second-largest subunit (RPB2). The concatenated phylogenetic tree separated the two ecological groups into well-supported sister clades. Also, species delimitations based on the topological congruence (GCPSR) and coalescent models (GMYC and BP&P) supported to separate the two ecological groups.

Morphological analysis showed that ROC specimens had significantly larger basidiospores, compared with ROF specimens. Mon-mon mating tests using six ROF, three ROC, and three H. boreorepandum strains each showed independent incompatible groups, whereas one ROC strain showed compatibility with both ROC and ROF populations. Based on these results, we defined the ROC group as a new species, H. subalpinum. Because H. repando-orientale and H. subalpinum have smaller genetic divergence in nc rDNA and maintain slight sexual compatibility, they may have recently speciated in East Asia.

Keywords 1 new taxon · Ectomycorrhizal fungi · Hybrid incompatibility · Hydnaceae · Morphological description · Species delimitation

Introduction

Hydnum repandum L., the type species of the genus Hydnum L., is an ectomycorrhizal fungus (Donk 1956; Agerer et al. 1996; Harrington and Mitchell 2002; Niskanen et al. 2018). Its morphology characters include large and fleshy basidiomata with cream to orange ochraceous pileus-surface, spinaceous hymenophores, and robust stipe attaching decurrent spines; thin walled, smooth, hyaline, medium sized (7.0–8.5 × 6.2–7.5 μm), and subglobose to elongated subglobose basidiospores, produced on four-spored basidia; and monomitic hyphal system composed of clamped, oil-rich hyphae (Niskanen et al. 2018). This species was originally described from Sweden (Linnaeus 1753) but had long been considered a cosmopolitan species in the Northern Hemisphere (Rea 1922; Coker and Beers 1951; Hall and Stuntz 1971; Maas Geesteranus 1971; Harrison and Grund 1987); it also had been regarded as an economically important edible mushroom with many
vernacular names worldwide, including “Hedgehog-mushroom,” “Sweet-tooth-mushroom,” “Pied-de-mouton,” and “Kanoshita” (Kawamura 1913; Phillips 2005; Roberts and Evans 2011). However, recent molecular systematic analyses have suggested that fruiting bodies of true *H. repandum* occur only in Europe (Grebec et al. 2009; Olariaga et al. 2012; Yanaga et al. 2015; Feng et al. 2016; Niskanen et al. 2018; Swenie et al. 2018; Sugawara et al. 2022a). In the infragenic system established by Niskanen et al., *H. repandum* is regarded as the type species of the subgenus *Hydnum* L., which is composed of *H. boreorepandum* Niskanen, Liimat. & Niemelä; *H. olympicum* Niskanen, Liimat. & Ammirati; *H. repando-orientale* Liimat. & Niskanen; *H. repandum*; *H. svilenicum* Liimat. & Niskanen; *H. sphaericum* T. Cao & H. S. Yuan; *H. subolympicum* Liimat. & Niskanen; *H. vagabundum* Swenie, Ovrebo & Matheny; and *H. washingtonianum* Ellis & Everh. (= *H. neorepandum* Niskanen & Liimat.; Swenie et al. 2018) (Niskanen et al. 2018; Swenie et al. 2018; Cao et al. 2021b). The subg. *Hydnum* was further separated into two sections (i.e., *Hydnum* L. and Olympica Niskanen, Liimat. & Ammirati) on the basis of phylogeny (Niskanen et al. 2018). Because most species of the subg. *Hydnum* produce similar basidiomata, recent studies have strongly recommended molecular approaches for accurate species identification (Niskanen et al. 2018; Sugawara et al. 2022a).

*Hydnum repandum* has been also reported from Japan (Kawamura 1913, 1929, 1954; Yasuda 1913; Asahina 1939; Ito 1955; Imazeki and Hongo 1957; Kikuhara 1987; Yanaga 2015; Yanaga et al. 2015; Sugawara et al. 2019). However, most of them were re-identified as *H. alboluteum* R. Sugaw. & N. Endo; *H. albobalidum* R. Sugaw. & N. Endo; *H. cremeoalbum* Liimat. & Niskanen; and *H. repando-orientale* (Yanaga 2015; Yanaga et al. 2015; Niskanen et al. 2018; Sugawara et al. 2022a)—of these, only *H. repando-orientale* has not been re-classified into another subgenus. Currently, only two species of subg. *Hydnum* (i.e., *H. boreorepandum* and *H. repando-orientale*) have been proven to be distributed in Japan (Niskanen et al. 2018; Sugawara et al. 2022a). *Hydnum boreorepandum* and *H. repando-orientale* were recognized as sister species of *H. repandum* in the phylogeny inferred from the nc rDNA internal transcribed spacer (ITS) sequences (Niskanen et al. 2018); these three species were distinguished by their geographical distribution patterns in Eurasia and by forest habitats. *Hydnum repandum* occurs in Europe, *H. repando-orientale* occurs in East Asia, and *H. boreorepandum* occurs in both areas; *H. boreorepandum* prefers coniferous forests in boreal climate, whereas *H. repandum* has a wider range of hosts and climate regions (Niskanen et al. 2018). We discovered two ecological groups of *H. repando-orientale* in Japan: a temperate group (ROF) which occurs in temperate Fagaceae-dominated forests at ≥ 1200 m a.s.l. and a subalpine group (ROC) which occurs in subalpine coniferous-dominated forests at ≥ 2000 m a.s.l. (Sugawara et al. 2022a). A previous study suggested small differences between ROC and ROF groups in terms of morphological characters (basidiospore size) and sequence data (ITS of nc rDNA operon and translation elongation factor 1-alpha (*TEF1*)) because we have not found intermediate types in Japan, the two groups may have adapted to their different ecological niches. We tentatively concluded that the ROC and ROF groups are conspecific (= *H. repando-orientale* s. lat.) because of very small variations in ITS sequences between them (< 0.3%); these variations are within the range of common intraspecific variations in the genus *Hydnum* (i.e., 0–1% (or 1.5%) for intra-specific variations and > 1% (or > 1.5%) for species delimitation (Niskanen et al. 2018)). However, mating incompatibility among ROC and ROF groups could not be assessed because of the slow growth of their monospore isolates; thus, the taxonomic designations of the two ecological groups of *H. repando-orientale* require further investigation.

The *Hydnum* species classification emphasizes on the phylogenetic relationships inferred from ITS sequences (Niskanen et al. 2018; Swenie et al. 2018; Cao et al. 2021b; Sugawara et al. 2022a); however, a phylogeny of the non-coding ITS region alone sometimes leads to unreliable data because it depends on the evolutionary history of a single-DNA sequence, rather than a species. For this reason, the use of multiple molecular markers is strongly recommended in a taxonomic framework (Lücking et al. 2020; Aime et al. 2021; Cao et al. 2021a). Molecular data from multiple loci contribute to species delimitations based on topological congruence and the multispaces coalescent model. The genealogical concordance phylogenetic species recognition (GCPSR) approach proposed by Taylor et al. (2000) is used in *Fungi* to define phylogenetic species based on congruent clades from multiple genealogies. Coalescent-based species delimitation using multiple loci is a powerful method for estimating evolutionary lineages that involve ancestral polymorphism which lacks reciprocal monophyly among alleles (Fujita et al. 2012). Unfortunately, species delimitation approaches have not been used in the current classification systems in the genus *Hydnum*. However, these approaches enable the recognition of cryptic (or pseudocryptic) species as part of an integrative taxonomy, together with other species concepts such as morphology, ecophysiology, and hybrid incompatibility (Fujita et al. 2012; Looney et al. 2020; Cao et al. 2021a).

Here, we aimed to (1) reconsider species boundaries among *H. repando-orientale* and related species in Japan by integrative taxonomic approaches, and (2) present a taxonomic treatment for the ROC group of *H. repando-orientale* s. lat. under the nomenclature. Multi-locus molecular phylogeny was inferred from the sequences of the ITS and the large subunit (LSU) of nc rDNA, *TEF1*, RNA polymerase II largest subunit (*RPB1*), and RNA polymerase II second-largest subunit (*RPB2*). In addition, species delimitations were performed.
using the GCPSR approach and the coalescent model based on the generalized mixed Yule coalescent (GMYC) and Bayesian framework (BP&P). A mating test using monospore isolates was performed to evaluate mating compatibility among the ROC and ROF groups, as well as \( H. \ repandum \) and \( H. \ boreorepandum \), a sister species of \( H. \ repando-orientale \) s. lat. Finally, we performed morphological characterization of basidiomata including a statistical analysis of mean basidiospore size. Based on findings indicating that the ROC and ROF groups are distinct species, we provide a detailed description of the ROC group as a new species.

### Materials and methods

#### Basidiomata specimens

We examined 33 basidiomata specimens of \( H. \ boreorepandum \) and \( H. \ repando-orientale \) s. lat. (Table 1): 23 were collected in our studies in 2016–2020 (Sugawara et al. 2019, 2022a); 4 were collected in this study; 2, including holotype of \( H. \ repando-orientale \) (TUMH 60745), were loaned from the Tottori University Mycological Herbarium (TUMH), Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University; 4 were loaned from the TNS herbarium of the National Museum of Nature and Sciences, Tokyo. These specimens were collected at 25 sites in Honshu, mainland Japan, at 10 to 2500 m a.s.l. (Fig. 1). We defined \( H. \ repando-orientale \) s. lat. as the two ecological groups (ROC and ROF) based on the recorded altitude and forest habitat (Table 1).

#### Morphological analysis

The morphological characterization was conducted in accordance with the method in our previous report (Sugawara et al. 2022a). We observed macroscopic characters of fresh basidiomata and used the color code of the Methuen Handbook of Colour (Kornerup and Wanscher 1978). Microscopic characters of dried specimens were observed in 3% KOH or Melzer’s reagent using differential interference contrast microscopy. Thirty basidiospores were measured from each of 21 specimens, and previous measurements were reused for 19 specimens. We calculated length/width ratio of each basidiospore (\( Q \)), its mean of a specimen (\( Q_m \)), and statistics of basidiospore length and width (5% smallest value (a), mean–SD (b), mean + SD (c), 5% highest value (d)), in which we described each highest or lowest value among all specimens as “(a)–(d)” Length or width of other tissues were expressed as range.

Because the ROC group showed slightly larger basidiospores, compared with the ROF group, we statistically analyzed the mean basidiospore sizes (MBS) by unpaired two-sample Wilcoxon tests. We also included eight MBS values of \( H. \ repandum \) and \( H. \ boreorepandum \) measured by Grebenc et al. (2009), Olariaga et al. (2012), and Niskanen et al. (2018); overall, our analysis included measurements of 5 \( H. \ boreorepandum \), 7 \( H. \ repandum \), 11 ROCs, and 16 ROFs. Using the “stats” package in R v. 4.0.5 (R Core Team 2021), the length (L) and width (W) of MBS were compared among ecological/phylogenetic groups (ROC, ROF, \( H. \ boreorepandum \), and \( H. \ repandum \)) by the function “pairwise.wilcox.test” and the Bonferroni method.

#### Monospore isolation

Fresh basidiomata materials were used for monospore isolation, in accordance with the method proposed by Sugawara et al. (2019). Basidiospores from each hymenophore were collected on an axenic plastic Petri dish, suspended in sterile distilled water, and inoculated onto modified Norkrans’ C medium (MNC; Yamada and Katsuya 1995) solidified with 1.5% gellan-gum (MNC1.5G; Sugawara et al. 2019). Using a platinum loop, inoculated spores were streaked in zigzag to create a spore concentration gradient; they were then incubated at 15 °C in the dark (MIR-254-PJ, Panasonic Healthcare, Japan, Tokyo). Infrequent (ca. < 1%) basidiospore germination was observed 1–2 months after incubation. When mycelial colonies reached approximately 2-mm diameter, each was transferred to MNC medium solidified with 1.5% agar (MNC1.5A). To conduct mating tests, we selected monospore isolates that exhibited better growth and lacked clamp connections on hyphal septa. In total, three strains from three ROC basidiomata, six strains from four ROF basidiomata, and three strains from three \( H. \ boreorepandum \) basidiomata were isolated and established from September 2020 to March 2021.

#### Mating tests

We used only newly obtained strains for mating tests. Monokaryotic strains of the ROC group showed poor growth on MNC1.5A medium (e.g., 0.5-cm diameter per 1-month incubation at 20 °C); for this reason, we conducted mating tests three times on different dates and under different culture conditions. The first test involved nine monokaryotic strains that showed better growth on MNC1.5A medium from April 27 to August 4, 2021. Mycelial agar blocks (ca. 3 × 3 mm) pre-cultured for 1–2 months at 15 °C were cut and placed near a pair-strain for 3-mm spacing on MNC1.5A medium. Next, each mating pair was incubated at 15 °C for 2 months, then at 20 °C for 2 months. For the second and third mating tests, each mycelium was pre-cultured in MNC liquid medium for 6 weeks and subsequently transferred to MNC medium plates. From this pre-culture, we could obtain enough mycelial biomass from all monokaryotic strains. Then, second and third tests were conducted using MNC1.5A and one-tenth
### Table 1  Specimens of *H. boreorepandum* and *H. repando-orientale* s. lat. (ROC and ROF populations) examined in this study

| Ecological/phylogenetic group | Personal nos. | Herbarium nos. | Collection information | Examination in this study | Examinations in this study |
|------------------------------|---------------|----------------|------------------------|---------------------------|----------------------------|
| **H. boreorepandum**         | SuR20191130-01| TUMH 64005     | Nagano Pref., Azumino City, Mount Orensbo | Conifer (Pp) | + + + + + |
|                             | SuR20200922-103| TUMH 64006     | Nagano Pref., Chino City, Mount Aka | Mixed (Av, Bsp) | + + + + + |
|                             | SuR20200923-007| TUMH 64007     | Nagano Pref., Minamisakai Dist., Sakuho Town, Maruyama | Conifer (Av) | + + + + + |
| ROC                         | SuR20200920-007| TUMH 64012     | Nagano Pref., Chino City, Mount Aka | Conifer (Am) | + + + + + |
|                             | SuR20200920-012| TUMH 64013     | Nagano Pref., Chino City, Mount Aka | Conifer (Am) | + + + + + |
|                             | SuR20210907-002| TUMH 64630     | Nagano Pref., Chino City, Toyohira | Conifer (Pp, Av, Td) | + + + + + |
|                             | SuR20200926-002| TUMH 64016     | Nagano Pref., Chino City, Tsuboniotic | Conifer (Av, Td, Pp) | + + + + + |
|                             | SuR20201121-201| TUMH 64017     | Nagano Pref., Chino City, Kitayokodake | Conifer (Am) | + + + + + |
|                             | SuR20191130-02| TUMH 64011     | Nagano Pref., Minamisakai Dist., Kawakami Village, Jumonjigote | Td (2000 m) | + + + + + |
|                             | SuR20200923-101| TUMH 64014     | Nagano Pref., Minamisakai Dist., Sakuho Town, Maruyama | Conifer (Am, Td, Pa, Be) | + + + + + |
|                             | SuR20200923-202| TUMH 64015     | Nagano Pref., Minamisakai Dist., Sakuho Town, Maruyama | Conifer (Am, Td) | + + + + + |
|                             | SuR20210907-001| TUMH 64629     | Nagano Pref., Suwa Dist., Fujimi Town, Fujimi, Mount Kamanashi | Conifer (Av, Td, Be) | + + + + + |
|                             | TNS-F-80714   |                | Yamanashi Pref., Minami-Alps City, Kitazawatoge | N.D. (2000 m) | + + + + + |

Ecological/phylogenetic group: personal nos. = personal numbers; herbarium nos. = herbarium numbers; collection site in Japan = collection site in Japan; forest = forest; canopy tree species = canopy tree species; altitude = altitude; site code = site code; morphological analysis = morphological analysis; ITS phylogeny = ITS phylogeny; concatenated phylogeny = concatenated phylogeny; species delimitations = species delimitations; mating tests = mating tests.
| Ecological/ | Personal nos. | Herbarium nos. | Collection information | Examinations in this study |
|---|---|---|---|---|
| | | | Collection site in Japan | Forest↓ Canopy tree species↓ Altitude Date Site code↓ Morphological analysis ITS phylogeny Concatenated phylogeny Species delimitations Mating tests |
| | | | | | | | | | | |
| – | TNS-F-85326 | Yamanashi Pref., Minamitsuru Dist., Narusawa Village, Okuniwa | Mixed Be, Td Am | 2200 m 20 Sep 2018 15 | + | + | + | + |
| ROF | – | TNS-F-36899 | Ehime Pref., Hyogo Pref., Kobe City | Deciduous Fe, Qc Mixed Qs, Pd N.D. 2 Nov 2010 16 | + | + | | |
| | – | TUMH 60744 | | | | | | | | |
| SuR20191026-309 | TUMH 64072 | Nagano Pref., Kamiina Dist., Minowa Town, Kayanokogen | Mixed Af, Qs | 1200 m 26 Oct 2019 18 | + | + | + | + |
| | SuR20191001-001 | TUMH 64071 | Nagano Pref., Kamiina Dist., Tatsumo Town, Yokokawa | Mixed Pd, Qc, Bp | 1000 m 28 Sep 2019 19 | + | + | + | + |
| | – | TNS-F-78326 | Nagano Pref., Kitaazumi Dist., Otori Village, Kamaike | N.D. N.D. 1100 m 27 Sep 2017 20 | + | + | + | + |
| SuR20201025-201 | TUMH 64073 | Nagano Pref., Nagano Pref., City, Mount Iizuna | Mixed Qc, Pd | 1200 m 25 Oct 2020 21 | + | + | + | + |
| | – | TUMH 64070 | Tottori Pref., Saitoku Dist., Daisen Town | Deciduous Fe, Qc | 900 m 14 Oct 2017 22 | + | + | + | + |
| | – | TUMH 60745 | Tottori Pref., Saitoku Dist., Daisen Town | Deciduous Fe, Qsp | N.D. 2 Oct 2010 23 | + | + | | |
| SuR20171014-27-01 | TUMH 63125 | Tottori Pref., Kurayoshi City, Nakano Town, Utsukukiyama | Hardwood Cs | 150 m 14 Oct 2017 24 | + | + | + | + |
| | SuR20181020-17 | TUMH 64070 | Tottori Pref., Kurayoshi City, Nakano Town, Utsukukiyama | Hardwood Cs | 150 m 20 Oct 2018 25 | + | + | | |
| | – | Tottori Pref., Kurayoshi City, Nakano Town, Utsukukiyama | Hardwood Cs | 150 m 7 Nov 2020 26 | + | | | |
| | SuR20201109-001 | | | | | | | | | |
| | TUMH 64074 | Tottori Pref., Tottori City, Hirodomeno | Deciduous Fe | N.D. 7 Nov 2020 27 | + | | + | |
| | NaoE20161106-01 | TUMH 62678 | Tottori Pref., Tottori City, Kokufu Town, Okamasu | Hardwood Cs, Qg | 60 m 6 Nov 2016 28 | + | | | |
| Ecological/phylogenetic group | Personal nos. | Herbarium nos. | Collection information | Examinations in this study |
|-------------------------------|---------------|----------------|------------------------|---------------------------|
|                              |               |                |                        | Morphological analysis    | ITS phylogeny | Concatenated phylogeny | Species delimitations | Mating tests |
|                              |               |                | Forest<sup>2</sup> | Canopy tree species<sup>2</sup> | Altitude | Date | Site code<sup>3</sup> |                        |                     |                     |                     |
| **Forest**                    | SuR20161112-18 | TUMH 62860     | Hardwood              | Cs, Qg                 | 60 m     | 12 Nov 2016 | 29 | + | + | + | + |
|                              | SuR20171026-04 | TUMH 63127     | Hardwood              | Cs, Qg                 | 60 m     | 26 Oct 2017 | 30 | + | + |               |
|                              | SuR20201024-101 | –              | Hardwood              | Cs, Qg                 | 60 m     | 24 Oct 2020 | 31 | + |                     |
|                              | SuR20171026-13 | TUMH 64069     | Hardwood              | Qv, Cs                 | 10 m     | 26 Oct 2017 | 32 | + | + | + | + |
|                              | –              | TUMH 60743     | Mixed                 | Pd, Fc                 | 60 m     | 27 Oct 2010 | 33 | + | + |               |

1. Forest vegetation of dominant host trees
2. Canopy tree species of nearby specimens. Dominant trees is shown in bold. Coniferous trees: Af, *Abies firma*; Am, *A. mariesii*; Av, *A. veitchii*; Lk, *Larix kaempferi*; Pd, *Pinus densiflora*; Pk, *P. koraiensis*; Pa, *P. parviflora* var. *pentaphylla*; Pp, *P. pumila*; Td, *Tsuga diversifolia*. Broadleaved trees: Be, *Betula ermanii*; Bp, *B. platyphylla*; Bsp, *Betula spp.*; Cs, *Castanopsis sieboldii*; Fc, *Fagus crenata*; Qg, *Quercus glauca*; Qc, *Q. mongolica* subsp. *crispyula*; Qs, *Q. serrata*; Qv, *Q. variabilis*; Qsp, *Quercus spp.*; N.D., no available data
3. Site code number indicates collection site in Fig. 1

- personal number or herbarium number absent. +: specimen or its monospore isolate was examined in each experiment
concentration of MNC medium solidified with 2.0% agar (1/10MNC2.0A), respectively. Each test was started at 6 August 2021 or 12 August 2021 and incubated for 2 months at 20 °C.

The formation of clamp connections was observed under a light microscope at 200× magnification with a long working-distance objective lens and at 1000× magnification on slide glasses mounted with distilled water. Some strains showed possible crossing between ecological/phylogenetic groups and resulted in intermediate mating incompatibility groups; therefore, we also examined the nuclear phases of hyphae that formed clamp-like cells to determine whether dikaryotization occurs. A portion of mycelium was mounted in a mixture of 4',6-diamidino-2-phenylindole (DAPI; Fujifilm Wako Pure Chemical, Japan, Osaka) 0.04% (v/v) and Calcofluor White (Fujifilm Wako Pure Chemical, Japan, Tokyo) equipped with a mercury lamp (Intensilight C-HGFI, Nikon Imaging). To assess the viability of descendant mycelium in artificial medium, a clamped mycelium was inoculated onto a MNC1.5A plate and incubated at 20 °C.

**DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing**

DNA extraction, PCR amplification, and sequencing analyses were performed as described by Sugawara et al. (2022a). ITS and LSU amplicons were obtained using universal primers for basidiomycetes: ITS1F/LBW (Gardes and Bruns 1993; Tedersoo et al. 2008) and CTB6/LR5F (Garbelotto et al. 1997; Tedersoo et al. 2008). TEF1 and RPB1 amplicons were obtained using primers for Hydnum species (RPB1: Hrpbl1F or Hrpbl1-4F/Hrpbl1-2R or Hrpbl1R (Feng et al. 2016)) (TEF1: HydTef1-F/ HydTef1-R (Sugawara et al. 2022a)). For PCR amplification of RPB2, we first amplified and sequenced several Hydnum and ectomycorrhizal Sistotrema (H. itachiharitake, S. aff. confluens, and other four Sistotrema spp.) using universal primers (fRPB2-5F or bRPB2-6F/ RPB2-b7R2 or RPB2-b7.1R (Kretzer and Bruns 1999; Liu et al. 1999)), then obtained the consensus sequence of their multiple alignment along with three RPB2 sequences in the International Nucleotide Sequence Database (INSD): DQ234553 (H. albomagnum), DQ381834 (S. confluens), and DQ987616 (S. confluens). The new primers were designed based on the consensus sequence using GENETYX v. 15.1.0 (Genetyx Corp., Japan, Tokyo): HRPB2-5.5F (forward: 5’-GNAAYTGGGGBGACCAGAAG-3’), HRPB2-5.6F (forward: 5’-AAGGCWWGYTGRCCAGGT-3’), and HRPB2-6.9R (reverse: 5’-GGRTGRATCRCAATGTGTCGA-3’). The primer pairs HRPB2-5.5F or HRPB2-5.6F/HRPB2-6.9R were designed to amplify 820 bps and 783 bps of a coding region respectively and enabled the amplification of all known Japanese Hydnum species (data not shown). The PCR protocols are shown in Table S1. The PCR products were directly sequenced with the same or nested primers; bidirectional sequences were assembled using ClustalW (Thompson et al. 1994). We attempted DNA extraction and PCR amplification of protein-coding genes from the holotype specimen of H. repando-orientale (TUMH 60745), but we could not successfully extract DNA using the cetyltrimethylammonium bromide (CTAB) method (Gardes and Bruns 1993) or the E.Z.N.A. HP Fungal DNA Kit (Omega Bio-Tek, Norcross, GA, USA). We obtained 113 sequences and deposited them in the INSD under the accession numbers in Tables 2 and S2.

**Phylogenetic analyses**

Phylogenetic analyses were performed based on the (i) ITS dataset and (ii) concatenated dataset of five loci (ITS, LSU, TEF1, RPB1, and RPB2). The ITS dataset included sequences of Holarctic species of the genus Hydnum downloaded from the INSD/UNITE databases, which comprised > 60 phylogenetic species (Table S2; Olariaga et al. 2012; Yanaga et al. 2015; Niskanen et al. 2018; Swenie et al. 2018; Cao et al. 2021b; Sugawara et al. 2022a). As an outgroup, we selected mycorrhizal Sistotrema spp. (Yanaga et al. 2015; Niskanen et al. 2018; Sugawara et al. 2022a); we excluded S. confluens Pers. And S. subconfluens L.W. Zhou because they showed extremely high genetic divergence in the ITS sequences (Niskanen et al. 2018; Sugawara et al. 2022b). The 175 ITS sequences including the outgroup were sampled and automatically aligned using MAFFT online v. 7 (Katoh et al. 2019). The alignment was manually refined, and the best substitution model for RAxML program was estimated using ModelTest-NG v. 0.2.0 (Flouri et al. 2015; Darriba et al. 2020). We identified the best maximum likelihood tree using the rapid bootstrap algorithm in RAxML v. 8.2.10 (Stamatakis 2014) on the raxmlGUI v. 2.0.5 platform (Edler et al. 2021). This analysis was computed under a HKYGAMMA substitution model with 1000 replications of bootstrap analyses (MLBS).

A more detailed phylogenetic analysis was performed using the sequences of five loci (ITS, LSU, TEF1, RPB1, and RPB2) obtained from 24 materials of H. boreorepandum, the ROC group, and the ROF group. Other taxa belonging to the subg. Hydnum were included in the dataset if ≥ 3 sequences of the five loci to be analyzed were available in the INSD. Furthermore, we used newly obtained 45 sequences of eight Hydnum species and four Sistotrema species (Table 2). Thus, 51 total specimens was included in the second analyses.

First, we annotated each locus and constructed independent phylogenies. The ITS and LSU sequences were contiguously connected and subsequently annotated using ITSx v. 1.1.3 (Bengtsson-Palme et al. 2013) on the PlutoF workbench (Abarenkov et al. 2010). The portions of protein-coding genes (TEF1, RPB1, and RPB2) were independently aligned and
annotated based on the following references: DQ234568 (H. albomagnum) for TEF1 (Matheny et al. 2007), EF014376 and KU612731 (H. repandum) for RPB1 (Liu et al. 2006; Feng et al. 2016), and DQ234553 (H. albomagnum) for RPB2 (Matheny et al. 2007). TEF1 includes three coding (ca. 500 bp) and two intronic regions (ca. 100 bp); RPB1 comprises mostly a coding region (ca. 850 bp) with a small intronic region (ca. 15 bp); RPB2 has only a coding region (ca. 800 bp). The RAxML phylogenies were independently constructed using each alignment as described above. The following substitution models were selected as recommended by ModelTest-NG: GTRGAMMAI for ITS+LSU, HKYGAMMAI for TEF1, GTRGAMMAI for RPB1, and GTRGAMMA for RPB2. For phylogenetic analysis of the concatenated dataset using MrBayes v. 3.2.7 (Ronquist et al. 2012), we determined the partitioning schemes and substitution models using PartitionFinder 2 v. 1.1 (Lanfear et al. 2017) under a “greedy” scheme search algorithm (Lanfear et al. 2012) and AICc criteria. By this analysis, we set nine subsets that included independent substitution models as shown in Table 3. Because the TEF1 alignment showed partition scheme trends that differed from the RPB1 and RPB2 alignments, we performed further model estimation and scheme search. Next, PartitionFinder 2 analysis was performed on only the TEF1 alignment under an “all” scheme search algorithm, in which all possible combinations of data blocks were analyzed. This analysis yielded the same partitioning scheme as greedy option; thus, we adopted the scheme in Table 3. The MrBayes analysis was computed with 2,000,000 generations of two iterations of four Markov chain Monte Carlo (MCMC) chains, where trees were sampled every 100 generations.

Chain convergence was confirmed by both visualization by Tracer v. 1.7.2 (Rambaut et al. 2018) and a small value (< 0.01) of average standard deviation of split frequencies (ASDSF). After burn-in of the first 25% generations, the consensus topology was constructed based on the 50% majority-rule of whole topologies. We also constructed concatenated gene trees based on the RAxML and maximum-parsimony methods. The RAxML phylogeny was computed under the GTRGAMMAI substitution model with 1000 replications of bootstraps. The maximum-parsimony trees were inferred by Subtree-Pruning-Regrafting (SPR) methods with 1000 replications of bootstraps using MEGA 7 v. 0.26 (Kumar et al. 2016), and a bootstrap consensus tree was constructed from the resulting 10 unrooted trees.

The alignments of ITS and concatenated dataset are provided in the Supplementary Data. Numerical information concerning the alignments (e.g., numbers of parsimony-informative sites and distinct alignment patterns) is shown in Table S3.

Species delimitations

Because there was a little conflict among topologies constructed from single alignments, we performed species delimitation using GCPSR (Taylor et al. 2000), GMYC (Pons et al. 2006), and BP&P (Yang 2002; Rannala and Yang 2003) approaches. The GCPSR approach defines a congruence of multi-gene genealogies as a phylogenetic species. In the GCPSR protocol proposed by Dettman et al. (2003, 2006), congruent clades were recognized as genealogical concordance and/or genealogical non-discordance; they were then defined
as phylogenetic species via exhaustive subdivision, in which all individuals were required to be placed within a phylogenetic species without creating conflicts with other phylogenetic species. Here, genealogical concordance was defined as the same monophyletic clade recognized in most topologies ($\geq 3$ of 4 of ITS-LSU, TEF1, RPB1, and RPB2 phylogenies); genealogical non-discordance was defined as the recognition of supported-monophyly (MLBS $\geq 70$) in $\geq 1$ topology, the clustering of which is never contradicted with the same level of support in other topologies. Finally, specimens were defined as the smallest phylogenetic groups that did not create discordance.

The GMYC approach explores the switching of branching by speciation under the Yule model (interspecific) to neutral coalescent within a species (intraspecific) based on the topology of an ultrametric tree (Pons et al. 2006; Fujisawa and Barraclough 2013). The species delimitation based on GMYC was analyzed using “splits” package in R v. 4.0.5. We generated an ultrametric tree for each locus (ITS-LSU, TEF1, RPB1, and RPB2) under Bayesian inference implemented in BEAST 2 v. 2.6.6 (Bouckaert et al. 2019). We set a strict clock model for estimating branch lengths and tree priors under the Yule model. The MCMC analysis was performed for 10,000,000 generations and sampled every 1000 steps. The convergence of chain was confirmed by higher values ($\geq 200$) of effective sample size (ESS) for each parameter on Tracer, and a consensus topology was summarized after a 25% burn-in. For each locus, we removed an outgroup (i.e., Sistotrema spp.) from the topology using “ape” package (Paradis and Schliep 2019) and assigned the topology by GMYC analysis with a single threshold using “splits” package (Thomas et al. 2021).

The BP&P is a Bayesian MCMC program that infers species tree and species delimitation under the multispecies coalescent model using multiple-locus alignments (Yang 2002; Rannala and Yang 2003). We performed an unguided species delimitation of “A11” algorithm in BP&P v. 4.3., which performs joint species delimitation and species tree inference using the reversible-jump MCMC algorithm (Yang and Rannala 2014; Yang 2015). We assigned 26 individuals for four inferred species as a prior ($H. repandum$, $H. boreorepandum$, ROC, and ROF); four alignments (ITS-LSU, TEF1, RPB1, RPB2) were assigned as multiple loci. Because we could not provide the corroborated values of the population size parameters ($\theta$s) and the divergence time at the root of the species tree ($\tau_0$) for each inverse gamma distribution, we assigned four combinations of rate parameters between higher (0.1) and lower (0.01) values as a prior in multiple analyses (Košťáthová et al. 2020); overall, “A11” analyses were independently run under priors [$\theta$s = 0.01 and $\tau_0 = 0.01$], [$\theta$s = 0.01 and $\tau_0 = 0.1$], [$\theta$s = 0.1 and $\tau_0 = 0.01$], and [$\theta$s = 0.1 and $\tau_0 = 0.1$]. The shape parameter 2 was assigned to each inverse gamma distribution. The remaining divergence parameters ($\tau$s) were assigned to the Dirichlet prior (Yang and Rannala 2010: equation 2). These analyses were run twice to confirm consistency between iterations.

The evolutionary divergences in each gene were analyzed to estimate overlapping of inter/intraspecific variations within/ between each group. This analysis is useful for determining the optimal DNA barcode (e.g., Harder et al. 2013; Li et al. 2017; Wang et al. 2018). Using MEGA 7, the mean evolutionary divergence “within groups” and “net between groups” were estimated for each locus (ITS, LSU, TEF1, RPB1, and RPB2). The maximum-composite-likelihood method with 1000 replications of bootstrap analysis was implemented to analyze pairwise distances. The patterns among lineages were set as gamma distribution rates, including site heterogeneity.

**Results**

**Phylogenetic analyses**

The RAxML phylogram obtained from the ITS dataset showed topology similar to the phylogram in our previous study (Fig. 2; Sugawara et al. 2022a). Sequences of ROC, ROF, $H. repandum$, and $H. boreorepandum$ formed a monophyletic clade with high support (MLBS = 89) within the subg. Hydnum (MLBS = 75). Hydnum repandum sequences formed a paraphyletic group with $H. boreorepandum$ and $H. repando-orientele$ s. lat. Hydnum boreorepandum sequences formed a paraphyletic group with $H. repando-orientele$ and $H. boreorepandum$ including European and East Asian specimens showed monophyly with strong support (MLBS = 91). Hydnum repando-orientele s. lat. (i.e., the assemblage of ROC and ROF groups) formed a monophyletic clade with strong support (MLBS = 100). Among them, all 18 sequences in the ROF group, including holotype of $H. repando-orientele$ (TUMH 60745), were slightly separated from the sequences in the ROC group as a subclade with low support (MLBS = 61). Three specimens in the ROC group formed a further subclade with the other eight sequences in the ROC group with moderate support (MLBS = 81).

Independent phylogenies of ITS-LSU, TEF1, RPB1, and RPB2 showed slightly different topologies regarding the relationships among ROC, ROF, and $H. boreorepandum$. The ITS-LSU dataset showed a paraphyletic relationship between the ROC and ROF groups (MLBS = 100 in ROF + ROC; MLBS = 69 in ROF alone), corresponding to the ITS alone dataset described above (Fig. S1). The TEF1 phylogeny had strong support for monophyly in each clade of $H. boreorepandum$ (MLBS = 100) and ROC (MLBS = 95). Most ROF specimens formed a monophyletic clade, but one ROF specimen (TUMH 63126) was outside the ROC and ROF clades (Fig. S2). The RPB1 phylogeny showed two monophyletic clades of ROC (MLBS = 99) and ROF (MLBS = 100), together with a paraphyletic position of $H. boreorepandum$ (MLBS = 92); the assemblage of ROC
| Genus/subgenus | Species | Herbarium/personal nos. | Locality | Accession nos. |
|---------------|---------|------------------------|----------|----------------|
| **Hydnum** subg. Alba | *H. albomagnus* | AFTOL-ID-471 | USA | DQ218305 DQ234568 – DQ234553 |
| | *H. cremeoalbum* | TUMH 64024 | Japan | LC621823 LC717912 LC622458 LC717839 LC717872 |
| **subg. Hydnum** | *H. aff. sphaericum* | HKAS78334 | China | KU612589 – KU612768 KU612733 – |
| | *H. boreorepandum* | TUMH 64005 | Japan | LC621814 LC717880 LC622449 LC717806 LC717840 |
| | *H. boreorepandum* | TUMH 64006 | Japan | LC621815 LC717881 LC622450 LC717807 LC717841 |
| | *H. boreorepandum* | TUMH 64007 | Japan | LC621816 LC717882 LC717873 LC717808 LC717842 |
| | *H. boreorepandum* | TUMH 64008 | Japan | LC621817 LC717883 LC622451 LC717809 LC717843 |
| | *H. repandum* | 03129A | Slovenia | KU612574 KU612655 KU612770 KU612732 – |
| | | HKAS93253 | Germany | KU612581 – KU612769 KU612731 – |
| | *H. sphaericum* | Wei 10243 | China | MW980563 MW979549 – MW999470 MW999444 |
| | *H. subolymicum* | F1188765 | USA | KU612599 KU612653 – KU612741 – |
| | *Hydnum* sp. 2 | HKAS55410 | China | KU612596 KU612654 KU612771 KU612729 – |
| | | HKAS82558 | China | KU612595 – KU612772 KU612730 – |
| | ROC (*H. subalpinum*) | TUMH 64011 | Japan | LC621867 LC717866 LC622493 LC717812 LC717846 |
| | | TUMH 64012 | Japan | LC621868 LC717887 LC622494 LC717813 LC717847 |
| | | TUMH 64013 | Japan | LC717913 LC717888 LC717874 LC717814 LC717848 |
| | | TUMH 64014 | Japan | LC621869 LC717889 LC622495 LC717815 LC717849 |
| | | TUMH 64015 | Japan | LC621870 LC717890 LC622496 LC717816 LC717850 |
| | | TUMH 64016 | Japan | LC621871 LC717891 LC622497 LC717817 LC717851 |
| | | TUMH 64017 | Japan | LC621872 LC717892 LC622498 LC717818 LC717852 |
| | | TUMH 64629 | Japan | LC717914 LC717893 LC717875 LC717819 LC717853 |
| | | TUMH 64630 | Japan | LC717915 LC717894 LC717876 LC717820 LC717854 |
| | | TNS-F-80714 | Japan | LC621865 LC717884 LC622488 LC717810 LC717844 |
| | | TNS-F-85326 | Japan | LC621866 LC717885 LC622489 LC717811 LC717845 |
| | ROC (*H. repando-orientale*) | TUMH 62860 | Japan | LC377883 LC717900 LC622490 LC717826 LC717860 |
| | | TUMH 63125 | Japan | LC377886 LC717901 LC622491 LC717827 LC717861 |
| | | TUMH 63126 | Japan | LC377887 LC717902 LC622492 LC717828 LC717862 |
| | | TUMH 64069 | Japan | LC621873 LC717903 LC622499 LC717829 LC717863 |
| | | TUMH 64071 | Japan | LC621875 LC717896 LC622500 LC717822 LC717856 |
| | | TUMH 64072 | Japan | LC621876 LC717897 LC622501 LC717823 LC717857 |
| | | TUMH 64073 | Japan | LC621877 LC717898 LC622502 LC717824 LC717858 |
| | | TUMH 64074 | Japan | LC621878 LC717899 LC622503 LC717825 LC717859 |
| | | TNS-F-78326 | Japan | LC621864 LC717895 LC622487 LC717821 LC717855 |
| | **subg. Pallida** | *H. albopallidum* | TUMH 63997 | LC621807 LC717904 LC622442 LC717830 LC717864 |
| | | *H. pallidomarginatum* | Yuan 13928a | China | MW980566 MW979552 – MW999473 MW999447 |
| | **subg. Rufescencia** | *H. mulsicolor* | TUMH 63094 | Japan | LC377892 LC717911 LC622472 LC717838 LC717871 |
| | | *H. itachiharitake* | TUMH 64032 | Japan | LC621829 LC717905 LC622461 LC717831 LC717865 |
| | | *H. jussii* | Yuan 14008 | China | MW980553 MW979539 – – MW999436 |
| | | *H. longibrasidium* | Wei 10383 | China | MW980556 MW979541 – MW999464 MW999438 |
| | | *H. pallidocrocceum* | Yuan 14023 | China | MW980568 MW979554 – – MW999449 |
| | | *H. umbilicatum* | TUMH 63128 | Japan | LC377891 LC717909 LC622516 LC717836 LC717869 |
| | | *H. ventricosum* | Yuan 14536 | China | MW980561 MW979547 – MW999468 MW999442 |
| | | *H. flavilocarnum* | Yuan 13903a | China | MW980559 MW979545 – MW999466 MW999441 |
| | | *H. minus* | TUMH 64050 | Japan | LC621842 LC717910 LC622470 LC717837 LC717870 |
| | | *H. orientalbidium* | TUMH 62998 | Japan | LC377875 LC717908 LC622478 LC717835 LC717868 |
| | | *H. tomaense* | TUMH 64086 | Japan | LC621885 LC717907 LC622509 LC717834 LC717867 |
| | **Subgenus Incertae sedis** | *S. aff. alboballescens* | TUMH 62071 | Japan | LC621901 LC667373 LC622522 – LC667370 |
and ROF groups formed a moderately supported clade (MLBS = 75) (Fig. S3). The RPB2 phylogeny showed monophyletic clades of ROC (MLBS = 97), ROF (MLBS = 96), and *H. boreorepandum* (MLBS = 99); the assemblage of ROC and ROF groups formed a strongly supported clade (MLBS = 96; Fig. S4).

The Bayesian inference tree of the concatenated dataset showed that ROC, ROF, *H. boreorepandum*, and *H. repandum* specimens formed independent clades within the subg. *Hydnum* clade (Fig. 3). The maximum likelihood and maximum-parsimony trees were almost consistent with the Bayesian tree; thus, four species-level clades were supported by all approaches. The maximum likelihood bootstrap, maximum-parsimony bootstrap (MPBS), and Bayesian inference posterior probability (BIP) values for each branch were 100/100/1 in ROC, 99/99/1 in ROF, 100/100/1 in *H. boreorepandum*, and 100/100/1 in *H. repandum*. In addition, the ROF clade has a subclade, comprising three specimens with moderate support (MLBS/MPBS/BIP = 70/96/0.99). ROC formed a sister clade of ROF (MLBS/MPBS/BIP = 100/100/1), and *H. boreorepandum* was positioned as a sister clade of the assemblage of ROC and ROF groups (MLBS/MPBS/BIP = 100/100/1).

### Species delimitations

A summary of each species delimitation is shown in Fig. 4. The GCPSR criterion supported both genealogical concordance and non-discordance of ROC, ROF, and *H. boreorepandum*, respectively (see "C/nDC" on branches in Fig. 4). A subclade of the ROF group containing three specimens (TNS-F-78326, TUMH 63125, and TUMH 64069) exhibited genealogical non-discordance. Finally, the exhaustive subdivision process (Dettman et al. 2006) in the GCPSR approach recognized ROC, ROF, and *H. boreorepandum* as three phylogenetically distinct species and the subclade in ROF as an intraspecific variation.

The GMYC analyses of each topology rejected the null model (likelihood ratio test *p*-value < 0.001) and supported the assumption that all *H. boreorepandum* specimens belonged to a single group. The unity of a mixed group of ROC and ROF specimens was supported by the ITS-LSU topology (AICc-supported-value = 1.00) but not by the TEF1, RPB1, and RPB2 topologies (< 0.15). ROC and ROF specimens were clearly separated as two distinct groups based on the RPB1 and RPB2 topologies. In the TEF1 topology, most ROC and

| Scheme nos. | Subsets<sup>a</sup> | Substitution model | Total sites including gaps | Base positions |
|-------------|----------------------|--------------------|---------------------------|---------------|
| 1           | ITS                  | GTR+G              | 520                       | 1–246; 404–677 |
| 2           | 5.8S                 | K80+I              | 157                       | 247–403       |
| 3           | 28S                  | GTR+I+G            | 895                       | 678–1572      |
| 4           | TEF1-int, RPB1-int   | GTR+I              | 157                       | 1573–1586; 3368–3440; 3578–3647 |
| 5           | RPB1-c1, RPB2-c1     | GTR+I              | 561                       | 1587–2470/3; 2471–3268/3 |
| 6           | RPB1-c2, RPB2-c2     | HKY                | 561                       | 1588–2470/3; 2472–3268/3 |
| 7           | RPB1-c3, RPB2-c3     | GTR+G              | 560                       | 1589–2470/3; 2473–3268/3 |
| 8           | TEF1-c1, TEF1-c3     | GTR+I+G            | 342                       | 3269–3367/3; 3441–3577/3; 3648–3924/3; 3271–3367/3; 3443–3577/3; 3650–3924/3 |
| 9           | TEF1-c2              | HKY+I              | 171                       | 3270–3367/3; 3442–3577/3; 3649–3924/3 |

| Overall       | 3924 sites           |

<sup>a</sup>ITS, ITS1, and ITS2 regions. 5.8S and 28S, 5.8S and 28S of nc rDNA, respectively. C1, c2, and c3, first, second, and third positions of the coding region (TEF1, RPB1, and RPB2), respectively. Int, intronic regions of RPB1 and TEF1

**Bold,** newly obtained sequences. –, sequence not available

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**Table 3** Optimal partitioning scheme and substitution models for MrBayes analysis using PartitionFinder 2

| Genus/subgenus | Species | Herbarium/personal nos. | Locality | Accession nos. |
|----------------|---------|-------------------------|----------|----------------|
|                |         |                         |          | ITS | LSU | TEF1 | RPB1 | RPB2 |
| *S. aff. muscicola* | TUMH 63116 | Japan | | LC621902 | LC667374 | LC622523 | **LC717833** | LC667372 |
| *S. aff. confluens* | SuR20201011-303 | Japan | | **LC717916** | **LC717906** | **LC717877** | **LC717832** | **LC717866** |
| *S. chloroporum* | TUMH 64409 | Japan | | LC642034 | LC642057 | **LC717878** | – | **LC667369** |
| *S. flavorhizomorphae* | TUMH 64399 | Japan | | LC642049 | LC642067 | **LC717879** | – | **LC667371** |

Bold, newly obtained sequences.
ROF specimens were assigned to distinct two groups; one ROF specimen (TUMH 63126) formed a separate group.

The BPP&P analysis under all combinations of θs and τ0 priors supported a four-species model composed of *H. boreorepandum*, *H. repandum*, ROC, and ROF, with the highest posterior probabilities (0.98–1.00). These analyses supported the assumption that the ROC and ROF groups were two distinct species with high posterior probability support (≥0.98) in the all priors set; they did not support a single species model for ROC and ROF. Therefore, all species delimitation approaches supported the assumption that ROC and ROF are distinct species, rather than a single species.

For all loci (ITS, LSU, *TEF1*, *RPB1*, and *RPB2*), the ranges of evolutionary divergences within each ecological group estimated by the maximum-composite-likelihood method were 0.000–0.001 in ROC, 0.000–0.004 in ROF, and 0.000–0.001 in *H. boreorepandum* (Table 4). The variation of ITS sequences among *H. boreorepandum*, ROC, and ROF was considerably higher (0.012–0.014) than in *H. boreorepandum* alone (0.000–0.001); however, the variation between ROC and ROF (0.001) overlapped with the variation within each group (0.000–0.001). Compared with ITS, LSU sequences showed lower values of evolutionary divergences in all groups; thus, three groups could not be distinguished by ITS and LSU markers. However, each of the *TEF1*, *RPB1*, and *RPB2* genes showed substantially higher variation among the three groups, compared to within the three groups: divergences of single group vs. all three groups were [≤0.004 vs. 0.008–0.019] in *TEF1*, [≤0.001 vs. 0.006–0.010] in *RPB1*, and [0.000 vs. 0.005–0.014] in *RPB2* (Table 4).

**Morphological analysis**

With the exception of basidiospore size, no diagnostic microscopic morphologic character could distinguish among ROC, ROF, and *H. boreorepandum*. The basidiospore length or width of three species overlapped; however, the MBS was slightly larger in the ROC group (Fig. 5): [ave. 8.0–9.1 × 6.9–8.0 μm, Qm = 1.08–1.16] in ROC, [ave. 7.2–8.3 × 6.5–7.5 μm, Qm = 1.09–1.17] in ROF (Sugawara et al. 2022a), and [ave. 7.8–8.7 × 6.9–7.3 μm, Qm = 1.10–1.19] in *H. boreorepandum* (Sugawara et al. 2022a). Additionally, MBS of ROC specimens were slightly larger than basidiospores of *H. repandum* (ave. 7.3–8.4 × 6.2–7.1 μm; Grebenc et al. 2009; Olariaga et al. 2012; Niskanen et al. 2018). Pairwise Wilcoxon rank-sum tests showed that basidiospore length and width were significantly larger in ROC than ROF \( p = 0.000 \) (L)/0.001 (W) and *H. repandum* \( p = 0.005 \) (L)/0.005 (W) (Table S4). There was no significant difference between ROC and *H. boreorepandum* in terms of basidiospore length \( p = 0.368 \), whereas ROC showed a significantly larger basidiospore width \( p = 0.019 \).

**Mating incompatibility tests**

Because clamped hyphae were observed (although infrequently) compared to absent in the original simple-septate hyphae, mating compatibility was evaluated based on the presence of clamp connection at junctions between two confronting colonies. Mating tests showed mating compatibility exists in each ecological/phylogenetic group (Table 5). Although most strains did not show mating compatibility with a strain of a different group, two strains showed mating compatibility beyond their potential incompatibility group (Table 5): one ROC strain (SuR20200920-007 ST03: TUMH 60412) formed clamps with all ROF strains, and one *H. boreorepandum* strain (SuR20201011-301 ST03: TUMH 64408) formed clamps with several ROF strains (Fig. 5). The clamped hyphae between ROC and ROF strains showed dikaryotization of hyphal cells (Fig. S5e), and we successfully isolated it as a dikaryotic culture strain (SuR20201024-101 ST01 × SuR20200920-007 ST03). Because the clamped hyphae generated by crossing of *H. boreorepandum* and ROF strains were very rare and sparse, their nuclear phase could not be observed and they could not be isolated in culture; we thus presumed that their dikaryotization failed or the dikaryon lost viability on culture medium. In conclusion, ROC, ROF, and *H. boreorepandum* groups belong to different mating incompatibility groups but retain partial mating ability; one ROC strain potentially has the ability to form a hybrid with ROF; one *H. boreorepandum* strain contingently forms clamp-like structures by crossing with ROF but has the lost normal mating ability.

**Taxonomy**

*Hydnum* L. subg. *Hydnum* L.

*Hydnum* L. sect. *Hydnum* L.

*Hydnum subalpinum* R. Sugaw. & N. Endo, sp. nov. (Fig. 6).

MycoBank no.: 844782

**Diagnosis:** *Hydnum subalpinum* is a sister species of *H. repando-orientale* but differs in that its basidiospores are slightly larger (ave. 8.0–9.1 × 6.9–8.0 μm) and it occurs in subalpine forest habitats associated with Gymnosperm. This species is also related to *H. boreorepandum*: *H. subalpinum* and *H. boreorepandum* show similar morphologies and ecologies but differs in terms of phylogeny and biological isolation, as indicated by in vitro mating incompatibility.

**Type:** JAPAN, Nagano Pref., Chino City, Tsuboniwa, 2250 m, on the ground under *Abies homolepis*, *Tsuga diversifolia*, and *Pinus pumila* individuals, 26 Sep. 2020, R. Sugawara SuR20200926-002 (TUMH 64016).

Gene sequences ex-holotype: LC621871 (ITS), LC717891 (LSU), LC622497 (*TEF1*), LC717817 (*RPB1*), LC717851 (*RPB2*)
Fig. 2 RAxML phylogram inferred from ITS sequences of Holarctic species of the genus *Hydnum*. Branches show statistical support in terms of maximum likelihood bootstrap (MLBS ≥ 60). In total, 175 sequences were included. *Hydnum* species of the subgenera *Alba* s. lat. (including *Alba* Niskanen & Liimat.), *Pallida* Niskanen & Liimat., *Rufescentia* Niskanen & Liimat., and *Vagabundum* H. Subayama were collapsed. Sequence color shows collection site: orange, East Asia; blue, Europe; green, Northern to Central America; yellow, South America.
Etymology: *subalpinum*, from its distribution range.

Japanese name: Takane-kanoshita

Macroscopic characters: Basidiomata medium to large-sized, 5–10 cm high, robust, solitary or gregarious. Pileus 3–7.5 cm diam, round to reniform, convex to plano-convex, infundibuliform when old; surface glabrous, smooth, sometimes depressed at center; whitish cream, cream to pale yellow (4A2–4A4), partly tinged yellowish orange (4A8), sometimes coloring orange ochraceous (6B6–8); margin incurved when young, becoming straight to undulant, concolor to surface. Spines conical to spathulate, slightly distant, up to 9 mm long, whitish cream to cream (4A2–4A4), adnate to clearly decurrent. Stipe robust, 20–60 × 9–16 mm, central or eccentric, equal to slightly enlarged at the base, solid, glabrous, whitish cream to cream, not turning color where scratched. Context flesh, whitish cream to cream, when young turning yellowish where scratched. Rhizomorphs abundant, white. Odor mild, strong.

Microscopic characters: Basidiospores (7.5)7.7–9.8(10.4) × (6.1)6.5–8.6(9.4) μm, Q = (1.00)1.03–1.23(1.32), Qm = 1.08–1.16 [mean, 8.0–9.1 × 6.9–8.0 μm, Qm = 1.11], thin-walled, smooth, subglobose to broadly ellipsoid, hyaline in 3% KOH, containing subhyaline oily droplets, inamyloid. Basidium 36.5–66 × 7.5–12 μm, 3–5-spored, clavate to subumbiform, thin-walled, smooth, including subhyaline oily droplets, sterigma 3.5–7.5 μm.

Fig. 3  Bayesian phylogeny based on the concatenated dataset. Branches show statistical support in term of maximum likelihood bootstrap (MLBS ≥ 60), maximum-parsimony bootstrap (MPBS ≥ 60), and Bayesian inference posterior probability (BIP ≥ 0.90). Bold branches indicate strong support (MLBS ≥ 95, MPBS ≥ 95, BIP ≥ 95). Pie charts at left show forest habitats of the *H. repandum* and closely related species, referring to available collection information (broadleaf-dominated, coniferous-dominated, or mixed forests) and sequence data (Grebenc et al. 2009; Oliariga et al. 2012; Yanaga et al. 2015; Niskane et al. 2018; Sugawara et al. 2022a).
Fig. 4  Species delimitations based on GCPSR, GMYC, and BP&P illustrated on the RAxML topology. Contrasting bars on left show best species boundaries. Branch shows phylogenetic concordance (C) and non-discordance (nDC) determined using the GCPSR approach.

Table 4  Average evolutionary divergences within/between groups

| Phylogenetic/ecological groups | ITS   | LSU   | TEF1  | RPB1  | RPB2  |
|-------------------------------|-------|-------|-------|-------|-------|
| Within group                  |       |       |       |       |       |
| ROC                           | 0.001 | 0.000 | 0.001 | 0.000 | 0.000 |
| ROF                           | 0.000 | 0.000 | 0.004 | 0.000 | 0.000 |
| \textit{H. boreorepandum}     | 0.001 | 0.001 | 0.000 | 0.001 | 0.000 |
| Between group                 |       |       |       |       |       |
| ROC vs. ROF                   | 0.002 | 0.000 | 0.008 | 0.010 | 0.005 |
| ROC vs. \textit{H. boreorepandum} | 0.015 | 0.002 | 0.019 | 0.006 | 0.014 |
| ROF vs. \textit{H. boreorepandum} | 0.017 | 0.002 | 0.019 | 0.007 | 0.014 |

The average evolutionary divergences estimated by the maximum-composite-likelihood method using MEGA 7. ROC and ROF showed higher interspecific divergences in \textit{TEF1}, \textit{RPB1}, and \textit{RPB2} (in bold), compared to ITS and LSU.
long. Hyphae of spines 2.5–4.5 μm wide, thin-walled, smooth, hyaline, sometimes including brownish cytoplasmic pigment; hyphal end cylindric to clavate, 3–7 μm wide. Pileipellis mixocutis, subhyaline; hyphae 5–10 μm wide, cylindric to slightly inflate at apex. Stipitpellis mixocutis, subhyaline; hyphae 3–4.5 μm wide, cylindric, without colored pigment. Cystidium absent. Rhizomorphs composed of hyphae 2.5–5.5 μm wide, cylindric, thin-walled, smooth, containing subhyaline oily droplets, including ampullate inflation at hyphal septum, 5.5–8 μm wide. Clamp connection present in all tissues.

Ecology and distribution: On ground in conifer-dominated forest of Abies, Pinus, and Tsuga, including some Betula in subalpine climate (1900–2500 m a.s.l.). High mountain in Nagano and Yamanashi Pref. in Japan.

Additional specimens examined: JAPAN. Nagano Pref.: Minamisaku Dist., Sakuho Town, Maruyama, 2180 m, under Abies mariesii in coniferous forest of Abies, Tsuga, Pinus with some Betula, 23 Sep. 2020, R. Sugawara SuR20200923-101 (TUMH 64014); 2100 m, under A. mariesii near Abies and Tsuga trees, 23 Sep. 2020, R. Sugawara SuR20200923-202 (TUMH 64015); Kawakami Village, Jumonjitoge, 2000 m, under T. diversifolia, 1 Oct. 2019, W. Aoki SuR20191130-02 (TUMH 64011); Kitayokodake, under A. mariesii, 9 Sep. 2020, W. Aoki SuR20201121-201 (TUMH 64017); Mount Aka, 2400 m, in fir forest of A. mariesii, 20 Sep. 2020, R. Sugawara SuR20200920-007 (TUMH 64012); R. Sugawara SuR20200920-012 (TUMH 64013); Toyohira, 2070 m, in coniferous forest of P. koraiensis, A. veitchii, T. diversifolia, 1 Sep. 2021, A. Koyama SuR20210907-002 (TUMH 64030); Suwa Dist., Fujimi Town, Mount Kamanashi, 1890 m, on the ground in A. veitchii dominated forest with some T. diversifolia and Betula ermanii, 3 Sep. 2021, A. Koyama SuR20210907-001 (TUMH 64029). Yamanashi Pref.: Minamitsuru Dist., Narusawa Village, Okuniwa, 2200 m, in mixed forest of B. ermanii, T. diversifolia, and A. mariesii, 20 Sep. 2018, E. Imura 199 (TNS-F-85326, labeled as “Hydnum rufescens”); Minami-Alps City, Kitazawatoge, 2000 m, 28 Aug. 2017, H. Uehara 122 (TNS-F-80714, labeled as “Hydnum rufescens”).

Remarks: Hydnum subalpinum morphologically resembles most species in the subg. Hydnum; namely, H. boreorepandum, H. olympicum, H. repando-orientale, H. repandum, H. slovenicum, H. vagabundum, and H. washingtonianum. Of
these eight species, *H. boreorepandum*, *H. repando-orientale*, and *H. subalpinum* can be found in Japan; therefore, the remaining five species were distinguished by their geographic distributions in Europe and Northern to Southern America (see Fig. 2). Morphological differences among these species were very poor; however, *H. subalpinum* shows the largest basidiospores along with *H. olympicum* (8.0–9.2 × 6.5–7.5 μm; Niskanen et al. 2018). *Hydnium sphaericum* is a species in the subg. *Hydnium* but differs in a smaller pileus (20–35 mm wide) and slightly narrower basidiospores ([6.0]6.5–7.5(8.0) μm wide, Qm = 1.20–1.23; Cao et al. 2021b). The most diagnostic feature of *H. subalpinum* is the forest habitat, where they occur in conifer-dominated subalpine forest located at high elevation (>1900 m) in Japan. Compared to other species in this subgenus, *H. sphaericum*, *H. subolympicum*, and *H. repando-orientale* occur in broadleaved forests, whereas *H. boreorepandum*, *H. olympicum*, *H. slovenicum*, and *H. washingtonianum* occur in coniferous forests (Niskanen et al. 2018; Swenie et al. 2018; Cao et al. 2021b; Sugawara et al. 2022a). Morphology and forest habitat of *H. subalpinum* are very similar to Japanese population of *H. boreorepandum* (Sugawara et al. 2022a), but all genomic DNA markers support their distinct biological isolation.

Pileal color of *H. boreorepandum* and *H. subalpinum* was slightly whiter, compared with *H. repandum* and *H. repando-orientale* (Niskanen et al. 2018; Sugawara et al. 2022a); we presumed that this was affected by environmental condition. Indeed, older basidiomata of *H. boreorepandum* were tinged with orange hues (Sugawara et al. 2022a). This notation is supported by two TNS specimens of *H. subalpinum* that were labeled as “H. rufescens” in the subg. *Rufescentia* Niskanen & Liimat. because of their brownish-orange color. Yanaga et al. (2015) showed that *H. repando-orientale* (as “H. repandum var. repandum” and “H. repandum var. album”) has color variations of the pileus, even at the same collection site.

### Discussion

In this study, integrative taxonomic approaches verified that two ecological groups of *Hydnium repando-orientale* s. lat. constituted two independent species. Phylogeny inferred from five loci demonstrated the distinct divergence between temperate ROF (*H. repando-orientale*) and subalpine ROC (*H. subalpinum*) groups. While *H. repando-orientale* and *H. subalpinum* have not yet accumulated sufficient variations in ITS, LSU, and *TEF1*, all species delimitation analyses (GCPSR, GMYC, and BP&P) separated them into two distinct phylogenetic clades; this genetic divergence implies reproductive isolation derived from their different ecological niches. Contrary to our expectations, mating tests among monospore isolates in these two species did not show complete incompatibility between species—one strain showed intermediate incompatibility. However, the other strains exhibited mating incompatibility with other species. Morphological difference between these two species are scarce, but *H. subalpinum* specimens have significantly larger basidiospores, compared with specimens of *H. repando-orientale*. We suspect that these two species have lost gene flow because of local adaptation to different vegetation/climate conditions; genetic divergence accumulated independently, resulting in mating incompatibility and phenotypic differences.

One strain of *H. subalpinum* (SuR20200920-007 ST03: TUMH 64012) clearly showed in vitro dikaryotization with *H. repando-orientale* strains. This hybridization ability indicates recent speciation into *H. repando-orientale* and *H. subalpinum*. However, although the sister species have not established genetic hybrid incompatibility, they presumably have other isolating barriers such as immigrant inviability or ecological hybrid inviability (Nosil et al. 2005) because of their allopatric distribution and niche divergence. In many basidiomycetes, the crossing of different species is frequently observed in mating tests (Le Gac and Giraud 2008). A typical
case is that of *Flammulina* species (e.g., *F. filiformis*, *F. velutipes*, and *F. rossica*); they show incomplete reproductive isolation in mating tests (Petersen et al. 1999; Ripková et al. 2010), but natural basidiomata retain an independent monophyletic lineage and have a separate genetic structure in each species (Wang et al. 2018). Evidence of hybridization is occasionally detected in natural basidiomata as interspecific heterozygosity (Garbelotto et al. 1996; Hughes and Petersen 2001; Kausurud et al. 2007; Ripková et al. 2010; Harder et al. 2013; Hughes et al. 2013; Li et al. 2017; Sillo et al. 2019). However, in most cases, *F*₁ hybrids rarely occur in nature because their low hybrid viability hinders the production of *F*₂ or higher progeny (Hughes et al. 2013). Therefore, the hybridization among close species is occasionally observed in basidiomycetes when the potential pre/post-zygotic isolation events do not occur. In this study, crossing between *H. repando-orientale* and *H. subalpinum* was probably an artifact of the unnatural in vitro conditions, which enforces mating between ecologically allopatric species.

Another case that suggests stable natural hybridization in basidiomycetes was reported from *Heterobasidion irregulare* Garbel. & Otrosina and *H. occidentale* Otrosina & Garbel., plant pathogens in the order *Russulales* (Garbelotto et al. 1996; Garbelotto and Gonthier 2013; Sillo et al. 2019).
Heterobasidion irregulare and H. occidentale have distinct host ranges but a parapatric or sympatric distribution in North America (Otrosina and Garbelotto 2010; Garbelotto and Gonthier 2013). These two species can easily cross in nature and in vitro, and fruiting of the natural hybrid occurs on intermediate host plants. In this case, repeated backcrossing has caused H. irregulare, H. occidentale, and its hybrids to have greater genetic variations in their fungal DNA (ITS, TEF1, RPB2, and glyceraldehyde-3-phosphate dehydrogenase (GPD)) because of the recombination of different alleles from distinct species (Sillo et al. 2019). In the present study, H. repando-orientale and H. subalpinum showed lower intraspecific genetic variations, supporting the notion of minimal gene flow between the two populations. Furthermore, mating incompatibility among most H. repando-orientale and H. subalpinum strains indicates deeper reproductive isolation by genetic drift. This rapid generation of hybrid incompatibility is explained as reinforcement between different ecological populations (Dobzhansky 1940; Noor 1999).

We also clarified the species boundaries of H. repandum, H. boreorepandum, and H. repando-orientale. Hydnum boreorepandum and H. subalpinum co-occur in the same forests; however, they showed hybrid incompatibility and clear intra/inter-specific evolutionary divergence in all genomic markers (Table 4). Furthermore, each species delimitation approach recognized H. boreorepandum as a single species separate from H. repando-orientale s. l. Therefore, our findings support strict species boundaries among H. boreorepandum, H. repando-orientale, H. repandum, and H. subalpinum, despite smaller interspecific variations in ITS sequences (1%; Niskanen et al. 2018). The ITS sequences do not indicate a convincing topology between H. repandum and related species; notably, H. repando-orientale and H. subalpinum show overlapping intra/inter-specific variations in ITS and LSU sequences. Thus, these nr rDNA markers are unsuitable for species delimitation and identification for the sect. Hydnum. Alternatively, RPB1 and RPB2 sequences enabled phylogenetic delimitation among H. boreorepandum, H. repando-orientale, and H. subalpinum. The RPB2 primers designed here enabled PCR amplification of all Japanese Hydnum and mycorrhizal Sistotrema species; therefore, we recommend use of the RPB2 gene fragment as the second DNA barcode for this genus. The RPB1 gene is also a useful molecular marker to identify this taxon, but the RPB1 primers for Hydnum designed by Feng et al. (2016) did not enable PCR amplification of some Hydnum and Sistotrema species.

The concatenated phylogeny strongly indicates that H. boreorepandum, H. repando-orientale, H. repandum, and H. subalpinum share a common ancestor, which might have experienced speciation in relation to geographic isolation along with adaptation to host and temperature. Additionally, H. repandum and its closely related species have more informative taxonomic characteristics in terms of ecogeographical traits (geographic distribution, temperature of habitat, and host association), rather than morphology. Hydnum washingtonianum, a sister clade of these four species, also has the diagnostic ecogeography of a North American distribution and coniferous forest habitats (Niskanen et al. 2018; Svenne et al. 2018). In contrast, there have been few revelations concerning the biogeography of most lineages in subg. Hydnum. Currently, with evidence of molecular tools, basidiomata of H. repandum are found only from European countries but one ITS sequence (JQ063050) from mycorrhizal root tip of Pakaraimaea dipterocarpacea in Venezuela implies a wider distribution of H. repandum in South America (Feng et al. 2016; Niskanen et al. 2018). Besides, Japanese specimens of H. boreorepandum showed slightly wider range of morphological variations in pileal color, number of sterigmata, and basidia length, compared with the original description from European specimens (Sugawara et al. 2022a). The biogeographic histories of these taxa can provide insight into the worldwide dispersal and diversification of ectomycorrhizal species in Agaricomycetes. To understand the biogeography of species in the subg. Hydnum, there is a need for species identification and delimitation using integrative approaches, including multiple DNA markers, ecogeographical information, morphological analysis, and mating incompatibility testing.

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Author contribution Ryo Sugawara, Naoki Endo, and Akira Nakagiri contributed to the study conception and design. Materials’ preparation was performed by Ryo Sugawara, Akira Nakagiri, and Akiyoshi Yamada. Data collection and analyses were performed by Ryo Sugawara. The original draft of the manuscript was written by Ryo Sugawara and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Voucher specimens and culture collections have been deposited in the Tottori University Mycological Herbarium (TUMH), Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University. The newly generated sequences have
been submitted in INSD with the accession numbers listed in Tables 2 and S2. The alignments for phylogenetic analyses are provided in Supplementary Data. All other data generated or analyzed in this research available from the corresponding author on requests.

Declarations

Ethics approval and consent to participate Not applicable.

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