Evolutionary history of the sequestrate genus *Rossbeevera* (*Boletaceae*) reveals a new genus *Turmalinea* and highlights the utility of ITS minisatellite-like insertions for molecular identification

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**Abstract** The sequestrate (truffle-like) basidiomycete genera *Rossbeevera*, *Chamonixia*, and *Octaviania* are closely related to the epigeous mushroom genera *Leccinum* and *Leccinellum*. In order to elucidate the properties and placement of several undescribed sequestrate taxa in the group and to reveal the evolutionary history of *Rossbeevera* and its allies, we conducted phylogenetic analyses based on three nuclear (ITS, nLSU, EF-1α) and two mitochondrial DNA loci (ATP6 and mtSSU) as well as precise morphological observations. Phylogenetic analyses of three nuclear loci suggest a complex evolutionary history with sequestrate fruiting bodies present in several clades, including a previously unrecognized sister clade to *Rossbeevera*. Here we propose a new sequestrate genus, *Turmalinea*, with four new species and one new subspecies as well as two new species of *Rossbeevera*. The three-locus nuclear phylogeny resolves species-level divergence within the *Rossbeevera-Turmalinea* lineage, whereas a separate phylogeny based on two mitochondrial genes corresponds to geographic distance within each species-level lineage and suggests incomplete lineage sorting (ILS) and gene introgression within several infraspecific lineages of *Rossbeevera*. Furthermore, topological incongruence among the three nuclear single-locus phylogenies suggests that ancient speciation within *Rossbeevera* probably involved considerable ILS. We also found an unusually long, minisatellite-like insertion within the ITS2 in all *Rossbeevera* and *Turmalinea* species. A barcode gap analysis demonstrates that the insertion is more informative for discrimination at various taxonomic levels than the rest of the ITS region and could therefore serve as a unique molecular barcode for these genera.

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**INTRODUCTION**

In recent years, molecular systematics has become increasingly popular and useful for examining the evolution of fruitbody morphology within the fleshy fungi in Ascomycota and Basidiomycota. Most types of mushroom fruitbodies, including agaricoid, gasteroid, resupinate, cup-shaped, and coralloid forms, have arisen multiple times due to convergent evolution (e.g., Hibbett et al. 1997, Bruns et al. 1998, Percudani et al. 1999, Pine et al. 1999, Peintner et al. 2001, Larsson et al. 2004, Hibbett 2004, Dentinger & McLaughlin 2006, Wilson et al. 2011, Miettinen et al. 2012). The sequestrate fruiting body form, which includes truffles, truffle-like fungi, and secotioid fungi, is characterized by fully or partially enclosed hymenia that render forcible spore discharge ineffective (Kendrick 1992). Sequestrate fungi have evolved dozens of times from other types of fruitbodies (e.g., Bruns et al. 1998, Percudani et al. 1999, Peintner et al. 2001, Læssøe & Hansen 2007, Lebel & Syme 2012; Lebel et al. 2015). Previous statistical studies suggest that evolution of the sequestrate-gasteroid form from other fruitbody forms is irreversible (Hibbett 2004, Wilson et al. 2011), and that this evolution may offer opportunities to adopt new ecological niches (Wilson et al. 2011). Many sequestrate fungi fruit belowground (hypogeous) and produce fruitbodies that are eaten by mammals, birds, or invertebrates that facilitate spore dispersal (Fogel & Trappe 1978, Maser et al. 1978, Claridge & May 1994, Danks 2011, Beever & Lebel 2014). Animal mycorphagy may also help to disperse spores directly into similar habitats with compatible mycorrhizal host plants (Frank et al. 2009, Trappe et al. 2009). Since most sequestrate fungi form mycorrhizas with plants, this animal interaction should be beneficial for efficient spore dispersal.

The *Boletaceae* is a family that primarily consists of soft-bodied, pileate-stipitate mushrooms with a poroid hymenophore (i.e., ‘boletes’). In recent years, many new genera of boletes have been proposed based on multigene phylogenetic analyses of epi-geous members of the *Boletaceae* (e.g., Halling et al. 2012a, b, Arora & Frank 2014, Gelardi et al. 2014, Li et al. 2014, Wu et al. 2014, Zeng et al. 2014). However, the family includes many sequestrate lineages and the number of sequestrate genera also continues to increase as more taxa are included in molecular phylogenies (Desjardin et al. 2008, 2009, Onihara et al. 2010, Trappe et al. 2013). The sequestrate genus *Rossbeevera* was erected to accommodate Australasian and Asian species of *Chamonixia* (Lebel et al. 2012a, b) that are phylogenetically close to, but distinct from, *Chamonixia* s.str. Species of *Rossbeevera* have fruitbodies with a whitish peridium that discolors bluish green to indigo and ellipsoid to fusiform basidiospores with 3–5 longitudinal ridges. Phylogenetically, this genus is part of a monophyletic lineage that also includes the epigeous bolete...
genera *Leccinum* and *Leccinellum* and the sequestrate genera *Chamonixia* and *Octaviania* (Lebel et al. 2012a, Orihara et al. 2012b) (i.e., the *leccinoid clade*). *Leccinum* and *Leccinellum* are well known because many species in these genera are prized edibles collected throughout the Northern Hemisphere (but some *Leccinum* species can cause gastrointestinal distress; Bessette et al. 2000). The leccinoid clade appears particularly rich in sequestrate fungi (> 40 spp.), even when compared with other groups of Boletales with many sequestrate taxa. A total of eight *Rossbeevera* species have thus far been reported from Australia (2 spp.), New Zealand (1 sp.), Southeast Asia (1 sp.), China (2 spp.), and Japan (2 spp.) (Lebel et al. 2012a, b, Orihara et al. 2012a).

For several decades, a sequestrate fungus that is macroscopically similar to *Rossbeevera* species but with pinkish fruitbodies and ellipsoid basidiospores with 6–10 longitudinal ridges has often been collected from western Honshu, Japan. Similarly, *Rossbeevera*-like fungi with different basidiospore morphology from *Rossbeevera* have also been formally and informally reported from other areas of Japan under alternative names (i.e., *Gautieria* sp. in Aoki (1978); *Hymenogaster* sp. in Yoshimi & Doi (1989)). All of these taxa have longitudinally ridged basidiospores and in some cases exhibit a bluish discolouration that is similar to *Rossbeevera* species. In order to better assess the diversity of sequestrate fungi in the leccinoid clade, it is important to clarify the morphology and phylogeny of these poorly documented taxa.

The aim of this paper is to provide well-resolved phylogenies based on three nuclear (ITS, nLSU, *EF-1α*) and two mitochondrial (ATP6 and mtSSU) DNA loci (472 nucleotide sequences in total) and topological comparisons of the resultant phylogenies as well as precise morphological observations to enhance our understanding of evolution within the leccinoid clade. We include known species of *Leccinum*, *Leccinellum*, *Chamonixia*, *Octaviania*, and *Rossbeevera* as well as species that have not been formally studied in the past. Based on a combination of morphological and molecular characterization, we describe two new species of *Rossbeevera* as well as four new species and one new subspecies within the new genus *Turmalinea*. We also examine the utility of a highly divergent minisatellite-like insertion found within the ITS2 rDNA of *Rossbeevera* and *Turmalinea* spp. for molecular delimitation within the *Rossbeevera-Turmalinea* lineage.

**MATERIALS AND METHODS**

**Materials examined and morphological observations**

Fresh fruitbodies were collected throughout Japan and from Australia, New Zealand, and Yunnan Province, China. After morphological observation and DNA extraction, the fruitbodies were air-dried or freeze-dried for later examination. These specimens are deposited in Kanagawa Prefectural Museum of Natural History, Japan (KPM), National Museum of Nature and Science, Japan (TNS) and the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (KUN, with HKAS accession numbers). Herbarium specimens were obtained from KPM, TNS, the Oregon State University Herbarium, Oregon, USA (OSC), the Mycological Herbarium of Università degli Studi di L'Aquila, L'Aquila, Italy (AQUI), the Western Australian Herbarium (PERTH), and the National Herbarium of Victoria, Royal Botanic Gardens Victoria, Australia (MEL).

For microscopy hand-cut sections of fresh or dried specimens were mounted in water, 3 % KOH, lacto-glycerol, or 1 % phloxine B aqueous solution. To determine the amyloid reaction, dried material was stained with Melzer’s reagent. Basidiospore dimensions (e.g., range of spore length × spore width, length of hilar appendages) and their standard deviations (SD) were determined based on 50 measurements. The 95 % prediction intervals of basidiospore diameter are shown without parentheses in taxonomic descriptions. Both endpoints of the spore dimensions are shown in parentheses, but when the value is the same as the 95 % prediction interval, only the latter is shown. Two additional spore features are shown; the length to width ratio (Q) and the hilar appendage to spore length ratio (HA/S; Orihara et al. 2012a). Measurements include the hilar appendage but not spore ornamentation or the pedicel. Basidium sizes are presented as the range of the lengths × the range of the widths. Scanning electron microscopy (SEM) was performed according to Yanaga et al. (2012).

**DNA extraction, PCR amplification and sequencing**

DNA was extracted from fresh or dried fruitbodies using the FTA Classic Card or Indicating FTA Cards (Whatman International Ltd, Maidstone, England) based on the manufacturer’s protocol for plants (www.whatman.com/References/WGI_1397_Paint-Poster_V6.pdf). Dried fruitbody tissues were soaked in 99.5 % ethanol for 2 min before DNA extraction according to Orihara et al. (2012a). PCR amplification of ITS, nLSU, and *EF-1α* followed Orihara et al. (2012b) and the protocol for mtSSU was the same as for nLSU. PCR amplification of ATP6 was performed according to Kretzer & Brun (1999). PCR primers were ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) or ITS4B (Gardes & Bruns 1993), ITS5F1 and ITS2, and/or ITS3 (White et al. 1990) and ITS4B for the ITS region; LRR and LRS (Vilgalys & Hester 1990) for the nLSU; EF1-1983F and either EF1-2218R or EF1-1567R (Rehner & Buckley 2005) for *EF-1α*; ATP6-3 and ATP6-2 (Kretzer & Brun 1999) for ATP6, and MS1 and MS2 (White et al. 1990) for mtSSU. Unidirectional sequencing of the PCR products in forward and reverse directions were completed according to Orihara et al. (2012b). Sequences were edited and assembled with Sequence Scanner v. 1.0 (Applied Biosystems, Foster City, California, USA), BioEdit v. 7.0.9 (Hall 1999), SeaView v. 4 (Galtier et al. 1996) and Clustal X v. 1.83 (Thompson et al. 1997). A total of 362 sequences were obtained and were deposited in the International Nucleotide Sequence Databases (INSD).

**Multigene phylogenetic analyses**

Specimens and sequences used for phylogenetic analyses are listed in Table 1. A total of 111 collections and 467 sequences (composed of 362 new sequences for this study and 105 sequences from INSD) were included in the multigene datasets (nuclear dataset: ITS, nLSU, *EF-1α*; mitochondrial dataset: ATP6 and mtSSU). Identification of epigeous bolete taxa in the leccinoid clade that were included in the phylogenetic analyses were based not only on their sequence similarity to sequences in INSD but also on morphological descriptions by Den Bakker & Noordeloos (2005). Previous phylogenetic studies on boletoid mushrooms have shown that members of the leccinoid clade were closely related to *Austroboletus* spp., *Tylopilus vires*, *Harrya chromapes* (Binder & Hibbett 2006), *Petroboletus griseus* (Dentinger et al. 2010) or *Spongiforma thailandica* (Nuhn et al. 2013). *Spongiforma thailandica* is also closely related to *Borofutus dhakanus* and *Porphyrellus species* (Hosen et al. 2012). We therefore included taxa from all of these groups in the analyses to see which is supported as a sister to the leccinoid clade based on different loci. *Chalciporus piparius* and *Buchwaldboletus ligincola* were strongly supported as the earliest diverging lineage in the Boletaceae (Binder & Hibbett 2006, Dentinger et al. 2010, Halling et al. 2012a, b) and were therefore selected as outgroup taxa. Sequence alignments were performed using Clustal X with the data manually adjusted in SeaView v. 4.
Table 1  List of sequences used in this study. Newly generated DNA sequences are designated in **bold**. Taxa and sequences used only for the ITS analyses are indicated with asterisks (*). ITS sequence of **bold** was used only for comparison among genera.

| Taxon               | Locality                                                                 | Voucher No.       | GenBank No. | ITS  | nLSU | EF- ta | ATPe | mtSSU |
|---------------------|---------------------------------------------------------------------------|-------------------|-------------|------|------|-------|------|-------|
| *Rossbeevera* cryptocyanea | Japan, Kagoshima Pref., Amami-ohshima Isl., Yamato-son | KPM-NC 17843      | –           | KC552030 | KC552072 | KT581144 | KT581142 |
|                     | Japan, Kagoshima Pref., Kakeroma Isl.                                   | KPM-NC 23928      | KP222892 | KP222898 | KP222914 | KP222885 | KP222904 |
| *Rossbeevera* ecuycana    | Japan, Okinawa Pref., Ishigaki I., Eastern foot of Mt Omoto             | KPM-NC 23387      | KP222893 | KP222899 | KP222913 | KP222886 | KP222905 |
| *Rossbeevera* griseovelutina | Japan, Mie Pref., Kameyama-shi                                           | TUMH-40253        | HJ063874 | HJ063879 | JN378444 | KC552114 | KC552185 |
|                     | Japan, Tottori Pref., Tottori-shi, Ochidani (holotype)                  | TNS-F-36986       | HJ063875 | HJ063880 | KS52068  | KC552115 | KC552186 |
|                     | Japan, Tottori Pref., Tottori-shi, Ochidani                            | KC551981          | KC553026 | KS52069  | KC552116 | KC552191 |
|                     | Japan, Saga Pref., Nishimatsuura-gun, Arita-cho, near the Arita Dam    | TNS-F-36988       | KC551982 | KC552027 | KC552070 | KC552117 | KC552188 |
|                     | Japan, Okinawa Pref., Yanbaru                                          | KPM-NC 18043      | –           | –           | –           | –           | –           |
|                     | Japan, Kagoshima Pref., Amami-ohshima Isl., Setouchi-son               | KPM-NC 23865      | KP222890 | KP222896 | KP222915 | KP222883 | KP222902 |
|                     | Japan, Kagoshima Pref., Amami-ohshima Isl., Yamato-shi                 | KPM-NC 23911      | KP222891 | KP222897 | KP222916 | KP222884 | KP222903 |
|                     | Japan, Kagoshima Pref., Amami-ohshima Isl., Yamato-son                 | KPM-NC 17844      | KC551984 | KT581439 | KT581440 | KS52120  | KC552191 |
| *Rossbeevera* griseovelutina | Japan, Kyama Pref., Okaayama Prefectural Forest Park, Bunanodaira, under Fagus crenata | KPM-NC 18034      | –           | –           | –           | –           | –           |
|                     | Japan,Okayama Pref., Okaima Prefectural Forest Park, Bunanodaira, under Fagus crenata | TNS-F-36991 | HJ063876 | HJ063881 | KS52074  | KS552122 | KC552193 |
|                     | Japan, Kanagawa Pref., Odawara-shi, Iryuda, near Myorkiji temple, under Castanopsis cuspidata | KPM-NC 18044      | -           | -           | -           | -           | -           |
| *Rossbeevera* griseovelutina* | New Zealand, NZ South Isl., Te Anau Valley, Fiordland Nat. Park, Grebe Saddle. | MEL 2079341        | KJ001093* | –       | –       | –       | –       |
| *Rossbeevera* pachydemis | New Zealand, NZ South Isl., Te Anau to Milford Sound Rd, The Chasm trail | MEL 2079350        | HQ647138 | HQ647157 | –       | –       | –       |
|                     | New Zealand, NZ North Isl., Tōura National Park, Anwaniwa, Black Beech Track, under Nothofagus trees | KPM-NC 23334        | KJ001087 | KJ001094 | KJ001076 | –       | –       |
|                     | New Zealand, NZ North Isl., Te Urewera National Park, Aniwaniwa, Track to Lake Wakareiti, under Nothofagus trees | KPM-NC 23336        | KJ001088 | KJ001095 | KP222912 | KJ001064 | KJ001072 |
| *Rossbeevera* pachydemis* | New Zealand, NZ North Isl., Matata, Te Wera Reserve (Matata Conservation Area), under Nothofagus trees | KPM-NC 23347        | KJ001089 | KJ001096 | KJ001077 | KP222881 | KJ001073 |
| *Rossbeevera* paracyanea | Japan, Ehime Pref., Tobe-cho, Sasadani, under Quercus acutissima, under Castanopsis cuspidata | KPM-NC 17734        | KJ001093* | –       | –       | –       | –       |
| *Rossbeevera* paracyanea* | Japan, Nara Pref., Nara Park, under Quercus gluta | KPM-NC 17847        | KC552034 | KC552078 | KC552126 | KC552198 |
|                     | Japan, Nara Pref., near Mt Kasuga, under G. gluta                      | KPM-NC 18023      | KC552035  | –       | –       | –       | –       |
|                     | Japan, Nara Pref., near Mt Kasuga, under G. gluta                      | KPM-NC 18087      | KC550186 | KJ001086 | KJ001082 | KJ001068 | KJ001071 |
| *Rossbeevera* paracyanea* | Japan, Osaka Pref., Minoh-shi, Mt Minoh, in a Castanopsis-evergreen Quercus forest | KPM-NC 17848        | KC559989 | KC552036 | KC552079 | KC552127 | KC552200 |
|                     | Japan, Nara Pref., near Mt Kasuga, under G. gluta                      | KPM-NC 23940      | KP222894* | –       | –       | –       | –       |
| *Rossbeevera* vittatispora | Victoria, Eastern Highlands region                                       | A.W. Claridge 2137 | –       | KC552022 | KC552063 | KC552105 | KC552176 |
|                     | Victoria, Gippsland region                                             | A.W. Claridge 2785 | –       | KC552023 | KC552064 | KC552106 | KC552177 |
| *Rossbeevera* vittatispora | Australia, Tasmania, Mount Field, Lyrebird Track, Mt Field Nat. Park. | MEL 2078292        | –       | KC552024 | KP222910 | KC552107 | KC552178 |
|                     | Australia, Victoria, East Gippsland, Eumundar National Park, along Orbost-Bendoc Rd. | TO-AUS-72          | –       | KC552025 | KC552065 | KC552108 | KC552179 |
| *Rossbeevera* vittatispora | Australia, Victoria, Midlands, Lilton, Miltain (Forest Park) edge of picnic area | MEL 207834 | –       | KC551075 | KJ001075 | KJ001065 | KJ001074 |
|                     | Australia,Victoria, Snowfields, Mount Baffalo                          | MEL 232058         | –       | KJ001086 | KP222895 | KP222911 | KP222882 |
| *Rossbeevera* vittatispora* | Australia, Australia, Nangong, Nangong Forest Reserve, South western island Swamp, J. Packer, 22 July 2009 (AD56098) | AD56098 (Tb646)       | –       | –       | –       | –       | –       |
| *Rossbeevera* aff. vittatispora | Australia, New South Wales, Genoa National Park.                      | OSC61484          | KC551976 | JN378506 | JN378446 | KC552109 | KC552180 |
| *Rossbeevera* westraliensis | Australia, New South Wales, 5 km south of the junction between Prince's highway and Eden rd. | OSC61484          | KC551978 | JN378507 | JN378447 | KC552110 | KC552181 |
| *Rossbeevera* westraliensis | Australia, Western Australia, Beedup National Park, Anzac Rd.           | OSC61484          | –       | –       | –       | –       | –       |
| *Rossbeevera* westraliensis | Australia, WA, Darling, Dryandra, Tomingley Rd under Allocasuarina.      | MEL 2219011        | HC647160 | HC647160 | KS52066  | KS552112 | KS552183 |
| *Rossbeevera* westraliensis | Australia, WA, Darling, Jarramahale, Jarramahale Rd., under Casuarina haseriana and Eucalyptus marginata | MEL 2322708        | –       | –       | –       | –       | –       |
| Taxon                          | Locality                                                                 | Voucher No.                                                                 | ITS GenBank No. | nLSU GenBank No. | ATP6 GenBank No. | mtSSU GenBank No. |
|-------------------------------|--------------------------------------------------------------------------|----------------------------------------------------------------------------|-----------------|------------------|------------------|-------------------|
| *Rossbeevera yunnanensis*     | China, Yunnan Prov., Chu Xang Pref., Mt Zi Xi                            | KPM-CN 17850                                                             | KC551900        | JN879437         | KC552080         | KC552128          |
| *Turmalinea chrysocarpa*      | China, Yunnan Province, Manhao, Geju, alt. 860 m, in a broad-leaf forest. | KHCAS 70061 (Ge3098)                                                     | KC552003        | KC552051         | KC552080         | KC552126          |
| *Turmalinea mesomorpha*       | Japan, Aomori Pref., Towada, Ohorona                                      | KPM-CN 18012                                                             | KC551999        | JN55047          | KC552090         | KC552139          |
| *Turmalinea persicina*        | Japan, Iwate Pref., Appi-Kogen                                          | KPM-CN 18014                                                             | KC552000        | JN879437         | KC552048         | KC552120          |
| *Turmalinea yunnanensis*      | Japan, Aomori Pref., Towada, Tsuta-onsen                                  | KPM-CN 18059                                                             | KJ001090        | JN879437         | –                | KC552109          |
| *Turmalinea mesomorpha*       | Japan, Eihime Pref., Safo-shi, Mt Isbuzuchi, in a Fagus crenata forest.  | KPM-CN 18016                                                             | KC552001        | JN55047          | KC552092         | KC552141          |
| *Turmalinea persicina*        | Japan, Mie Pref., Kamaya-machi-shi                                       | KPM-CN 17743                                                             | KC552002        | JN55047          | KC552091         | KC552142          |
| *Turmalinea persicina*        | Japan, Kyoto Pref., Saiko-ku, wakura-agura-cho                            | KPM-CN 18001                                                             | KC551991        | JN55047          | KC552038         | KC552120          |
| *Turmalinea persicina*        | Japan, Kyoto Pref., Higashiyama-ku, Kiyomizu Shrine                      | KPM-CN 1807                                                              | KC551995        | JN55047          | KC552042         | KC552120          |
| *Turmalinea persicina*        | Japan, Oita Pref., Saiki-shi, Shiroyama Park                              | KPM-CN 18005                                                             | –               | JN55047          | KC552039         | –                 |
| *Turmalinea persicina*        | Japan, Oita Pref., Saiki-shi, Ume-Oh-aza, Shigeoka, 1.3 km southwest of Sorato Station | KPM-CN 18008                                                             | KC551996        | JN55047          | KC552041         | KC552129          |
| *Turmalinea persicina*        | Japan, Hyogo Pref., Shigahara                                            | KPM-CN 18053                                                             | KC551997        | JN55047          | KC552045         | KC552120          |
| *Turmalinea persicina*        | Japan, Muroi361                                                           | –                                                                         | –               | –                | –                | –                 |
| *Turmalinea yunnanensis*      | Japan, Kaboshima Pref., Amami-Oshima Isl.                                | KPM-CN 18011                                                             | KC551998        | JN55047          | KC552046         | KC552128          |
| *Chamonixia caespitosa*        | USA (locality of the ATP6 sequence is unknown.)                          | –                                                                         | –               | –                | –                | –                 |
| *Leccinellum crocipodium*     | France, ITS (nLSU), Canada, New Brunswick, Fundy Natural Park, Point Wolfe (mtSSU) | –                                                                         | –               | –                | –                | –                 |
| *Leccinellum aff. c. crocipodium* | Japan, Tottori Pref., Yazu-cho, Hatto                                  | KPM-CN 18041                                                            | KC552008        | JN378508         | KC552059         | KC552165          |
| *Leccinellum aff. griseum*    | Japan, Tottori Pref., Tottori-shi, Ouchidani                             | KPM-CN 18032                                                            | KC552009        | JN378509         | KC552060         | KC552165          |
| *Leccinellum sp.*             | China, CAS 5022                                 | –                                                                         | –               | –                | –                | –                 |
| *Leccinum 'albellum'*         | USA, NY, Erie County                                                     | MB680404                                                               | –               | –                | –                | –                 |
| *Leccinum scabrum*            | Austria                                                                  | –                                                                         | –               | –                | –                | –                 |
| *Leccinum scabrum*            | UK, Scotland, Aberdeenhireshire, Burn O'Vat                               | KPM-CN 17840                                                            | KC552012        | JN378515         | KC552065         | KC552170          |
| *Leccinum aff. castanedolium*  | Japan, Hyogo Pref., Uwano                                                | KPM-CN 17813                                                            | KC552008        | JN378508         | KC552060         | KC552165          |
| *Leccinum aff. chiristophillum* | UK, Scotland, Aberdeenhireshire, Burn O'Vat                              | KPM-CN 17841                                                            | KC552011        | JN378513         | KC552065         | KC552165          |
| *Leccinum aff. speculum*      | Japan, Iwate Pref., Appi-Kogen                                          | KPM-CN 17839                                                            | KC552011        | JN378513         | KC552065         | KC552165          |
| *Leccinum versipellum*        | UK, Scotland, Dennet Oakwood National Nature Reserve                    | KPM-CN 17834                                                            | KC552013        | JN378516         | KC552065         | KC552165          |
| *Octaviania carpinensis*      | Spain                                                                     | –                                                                         | –               | –                | –                | –                 |
| *Octaviania asahimontana*     | Japan, Hokkaido, Kamikawa-cho, Mt Daisetsu                               | KPM-CN 17824                                                            | JQ619178        | JN378489         | KC552154         | KC552228          |
| *Octaviania asterosperma*     | Spain, Sella Covallera                                                   | Trappe 2337                                                             | JN257998        | JN378497         | –                | KC552238          |
| *Octaviania ashiimontana*     | Italy, Provincia L'Aquila, Comune di Cappadocia                          | AQU 3899                                                                | –               | KC552052         | KC552093         | KC552234          |
| *Octaviania celatifila*       | Japan, Nara Pref., Nara-shi, Nara Park - Mt Kasuga                      | KPM-CN 17776                                                            | JN257997        | KJ223200         | KC552045         | KC552120          |
| *Octaviania cyanescens*       | OR, Lane Co., Lamb Butte south of English Mountain.                     | KPM-CN 18082 (nLSU)                                                    | –               | –                | –                | –                 |
| *Octaviania decimae*          | Japan, Kyoto Pref., Mt Hiei                                              | KPM-CN 17763                                                            | KC552006        | JN378502         | KC552046         | KC552125          |
| *Octaviania durianelloides*   | Japan, Kanagawa Pref., Minami-ashigara-shi                              | KPM-CN 17829                                                            | JQ619177        | JQ619188         | KC552107         | KC552227          |
| *Octaviania etchuenas*        | Japan, Toyama Pref., Nakashingga-gun, Tetsuaya-cho                      | KPM-CN 17822                                                            | JQ619182        | JN378492         | KC552125         | KC552230          |
| *Octaviania flavofascilus*    | Japan, Kanagawa Pref., Zushi-shi                                        | KPM-CN 17793                                                            | JQ619173        | JN378490         | KC552125         | KC552225          |
| *Octaviania japonimontana*    | Japan, Akita Pref., near Lake Towada                                     | KPM-CN 17797                                                            | JQ619174        | JN378483         | KC552125         | KC552225          |
| *Octaviania kobayasii*        | Japan, Nara Pref., Nara-shi, Mt Kasuga                                   | KPM-CN 17785                                                            | JQ619170        | JN378478         | KC552125         | KC552222          |
| *Octaviania mortae*           | Japan, Kyoto Pref., Uji-shi                                              | KPM-CN 17770                                                            | JQ619171        | JN378477         | KC552125         | KC552225          |
| *Octaviania mori*             | Japan, Tottori Pref., Hie Shrine                                        | KPM-CN 17775                                                            | JQ619172        | JN378476         | KC552125         | KC552225          |
| *Octaviania nonae*            | Japan, Kagoshima Pref., Amami-ohshima Isl.                              | KPM-CN 17748                                                            | JQ619175        | JN378475         | KC552125         | KC552225          |
For testing topological congruence among different loci, we directly compared statistically supported topologies (BS ≥ 75 %) in ML trees constructed with RAxML v. 7.2.6 (Stamatakis 2006), setting the number of bootstrap replicates to 1 000. Introns of EF-1α were carefully adjusted in SeaView and also included in the analyses. Approximately 80 % of nucleotides were variable in the mtSSU dataset and we therefore included only 106 bp of variable sites in the phylogenetic analyses.

In the ITS dataset, a large part of the ITS1 and ITS2 regions were too divergent to be aligned across the leccinoid clade and the allied taxa. Therefore, we first tested whether to include unambiguously aligned sites using the Gblocks algorithm (Casatresana 2000) in SeaView v. 4. This allowed smaller final blocks, gap positions within the final blocks, and less strict flanking positions. The resultant dataset was 263 bp in length and many phylogenetically informative positions that contained gaps were excluded from the analysis based on the results produced by Gblocks. Consequently, the ML tree generated from the dataset by RAxML was poorly resolved with evidence of long-branch attraction of *B. dhakanus* and *S. thailandica* within the *Leccinum* clade. Because of these inconsistent results we abandoned the Gblocks dataset and manually and carefully selected phylogenetically informative sites within ITS using SeaView, allowing inclusion of the sites that contained gaps (up to approximately 40 % of the individuals at any given site). The final ITS dataset for the multi-locus analyses was 462 bp in length, and the resultant ML tree had much higher resolution without obvious evidence of long-branch attraction.

Subsequently, two multigene datasets that consisted of three nuclear (i.e., ITS, nLSU, and *EF-1α*) and two mitochondrial (i.e., *ATP6* and mtSSU) DNA loci, respectively, were created to examine the phylogeny of the leccinoid clade and to check for topological incompatibility between nuclear and mitochondrial phylogenies. In both datasets, we included only specimens with sequences for at least two loci (i.e., specimens represented by only ITS were omitted in the nuclear dataset; Table 1). Gaps were treated as ‘missing’ data for all analyses. The nuclear and mitochondrial combined datasets are deposited in TreeBASE (accession URL: http://purl.orgphylo/treebase/phylows/study/ TB2:15097).

For each dataset, Bayesian analysis was conducted with MrBayes 3.2 (Ronquist & Huelsenbeck 2003). The combined dataset was partitioned by regions for the non-coding sequences and by codons for the coding sequences, and the best-fit likelihood models were estimated for the resultant 10 partitions with MrModeltest 2.3 (Nylander 2004). The GTR+I+G model was used for all the nuclear partitions (i.e., ITS1-5.8S, mtSSU, and the 1st and 3rd codons of *nLSU*, and the 1st, 2nd, and 3rd codons and introns of ITS2, *nLSU*, and the 1st, 2nd, and 3rd codons and introns of *EF-1α*) and the F81 model for the 2nd codon of *ATP6*, the GTR+G model for mtSSU, and the F81 model for the 2nd codon of *ATP6*. Bayesian posterior probabilities (PP) were approximated by the Bayesian posterior probabilities of 100000 trees obtained before reaching convergence were discarded as a burn-in, and the remaining trees were used to calculate a 50 % majority consensus topology and to determine PP values for individual branches.
The same combined datasets were also analysed by the maximum likelihood (ML) method with raxmlGUI 1.3 (Silvestro & Michalak 2012), which includes executable files of RAxML 7.4.2 (Stamatakis 2006). The best-fit ML tree was inferred under the GTR+I+G model. The datasets were partitioned in the same way as in the Bayesian analysis so that different substitution parameters, GTR rates, and empirical base frequencies could be assigned to each partition. To check statistical support for the resultant tree topology, the rapid bootstrap (BS) option was used under the automatically assigned GTR+CAT model, setting the number of replicates to 1 000.

Species tree analyses
Since several topological conflicts were found among individual single-locus nuclear phylogenies, we conducted species tree estimation from gene trees using *BEAST program (Heled & Drummond 2010) in the software package BEAST 2 v. 2.1.3 (Bouckaert et al. 2014) to see whether the multi-locus combined phylogeny conflicts with the species phylogeny based on a multispecies coalescent model. *BEAST estimates a Bayesian species tree from the simultaneously generated, individual gene trees, and reconstructs a species phylogeny that reflects the effect of incomplete lineage sorting (ILS) or deep coalescence (Heled & Drummond 2010). In the *BEAST analysis we excluded species with any missing loci from the analysis because they could negatively effect the MCMC convergence (Castillo-Ramírez et al. 2010). Thus, we were not able to include Austroboletus cf. novae-zelandiae, Buchwaldoboletus lignicola, Chalciporus piperatus, Leccinum ‘albellellum’, Leccinum sp., L. versipelle, Octaviania arbusculans, O. asteroeperna, O. zelleri, Retiboletus griseus, ‘Turmalinea chryscarpa’, and Tylopius virens. For the other species we included specimens with sequences of all the three loci. Consequently, the concatenated nuclear datasets consisted of 78 sequences, which were grouped into 43 species according to the results of the ITS, nLSU, and EF-1α analyses. The dataset was partitioned into ITS, nLSU, and EF-1α regions.

XML command files for executing *BEAST analysis was created using BEAUİ in the BEAST 2 package. Substitution models for each locus were estimated with MrModeltest: the HKY+I+G model was assigned to the non-partitioned EF-1α dataset whereas the GTR+I+G model was used for the ITS and nLSU datasets. The number of rate categories for the discrete gamma model was set to four according to Yang (1994), and ‘piece-wise linear and constant root’ was applied to the species tree population size model. In the Bayesian MCMC prior settings, gamma distribution was applied to mutation rates and clock rates for each dataset. Both the analyses were run for 200M generations, storing an inferred tree in every 10 000 generations. The other settings were left at defaults. The runs were monitored using Tracer v. 1.6 to verify the convergence of the MCMC by checking that the ESS of each resulting statistic was sufficiently large (> 100). The output data were summarized using TreeAnnotator in the BEAST 2 package. The resulting trees were viewed with FigTree v. 1.4. (http://tree.bio.ed.ac.uk/software/figtree/).

Detection and characterization of insertion sequences within ITS2
The full-length ITS dataset (3’ end of SSU-ITS1-5.8S-ITS2-5’ end of LSU) of the Rosseebevera and Turmalinea species consisted of 51 sequences. The dataset was not properly aligned by the automatic alignment with ClustalX v. 1.83 or with Q-INS-I option in MAFFT v. 7 (Katoh & Standley 2013) due to highly divergent sequences with indels and unusually long insertions within ITS. We therefore aligned the dataset manually with SeaView. The dataset is deposited in TreeBASE (accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S15097). Internal, discontinuous repeats within the ITS region were detected with the aid of dot plot matrices generated with the BLASTN search using the default settings (Altschul et al. 1997). The search of more-or-less regular, continuous, ITS tandem repeats was implemented with Tandem Repeats Finder software v. 4.04 (Benson 1999) under the following parameter settings: match = 2, mismatch = 3, indel = 5, minimum alignment score to report repeat = 50, and maximum period size = 300. In one case the analysis detected both a single nucleotide motif and the repeated pattern that consisted of the same core motif and its successive duplication as ‘consensus patterns’ (i.e., both ‘TTTGCA’ and ‘TTTGCATTTGCA’). In that case, we counted only the single nucleotide motif as a detected consensus pattern (i.e., ‘TTTGCA’ in the above example). After careful alignment of the dataset, the boundaries of the insertions were determined based on the results of BLASTN searches and Tandem Repeats Finder analyses.

To assess the usefulness of the ITS2 insertion for taxonomic identification, we implemented barcode gap analysis using the web version of the ABGD (Automatic Barcode Gap Discovery) software, which was primarily designed to delimit species from unexplored groups of metazoans (Puillandre et al. 2012). ABGD detects gaps between the infraspecific and interspecific divergence based on a matrix of pairwise distances and groups the input sequences into several hypothetical species. The largest gap is initially detected with various prior limits of maximal distance of infraspecific divergence (P), so that the dataset is partitioned according to the gap. The gap detection is iterated within each recursive partition until ‘large gaps’ are no longer found under the parameter P and X, where X is a proxy for minimal gap width (Puillandre et al. 2012). The analysis was performed using the default settings (i.e., \( P_{\text{min}} = 0.001, P_{\text{max}} = 0.1 \), \( X = 1.5 \)) except the number of steps for \( P \) and for a distance-distribution histogram were set to 100 and 60, respectively. The web version of ABGD provides three distance models for the analysis: Jukes-Cantor, the Kimura’s two-parameter, and simple distances. Accordingly, we chose the Kimura’s two-parameter model (Kimura 1980; \( Ti/Tv = 0.9059 \) in the ITS2 insertion dataset; 1.2955 in the residual ITS dataset excluded the insertion sequences), which had a higher likelihood score than the Jukes-Cantor model based on model estimations in MrModeltest 2.3. In addition, ML analysis of the ITS2 insertion dataset was implemented using RAxML under the GTR+I model, which was chosen as the best model in MrModeltest 2.3, and rapid bootstrap analysis was carried out in the same settings as those of combined datasets described above.

RESULTS
Topological incongruence among different single-locus datasets
Prior to multi-locus analyses, we first examined topological congruence among three nuclear-locus trees (ITS, nLSU, and EF-1α) and among two mitochondrial-locus trees (ATP6 and mSSU) separately. The resulting tree topology in the ITS, nLSU, and EF-1α datasets showed conflicts in the branching order within the Rossbeeveera clade (Fig. 1; entire trees not shown). Maddison & Knowles (2006) suggest that including more individuals and more loci reduces the effect of incomplete lineage sorting and contributes to reconstructing a more accurate species tree. Since all of the species in this clade contain multiple individuals, we concatenated the datasets for the following analyses and carefully compared the resultant multi-locus phylogeny with a species tree inferred from the three single-locus Bayesian phylogenies by using *BEAST. In contrast to the nuclear dataset, there was no obvious conflict
between the mitochondrial ATP6 and mtSSU phylogenies, partly due to poor resolution of the mtSSU phylogeny. Although most of the species represented by multiple individuals formed monophyletic clades in the three nuclear datasets, we found topological discordance in the relationship in Octaviania subg. Paracea, which resulted in collapse of the monophyly of the species-level clades. That is, in the nLSU phylogeny the O. nonae sequences showed paraphyly with O. celatifilia. This topology is documented in Orihara et al. (2012b) and has only a minor influence on the phylogenetic relationships of Rossbeevera and its relatives, which are the focus of this study. Thus, we retained the sequences in the combined dataset.

Species tree and multi-locus combined tree based on the nuclear datasets

Since topological discordance among the three nuclear phylogenies was observed in the Rossbeevera clade (Fig. 1), we first inferred a species tree from Bayesian ITS, nLSU, and EF-1α ‘gene’ trees using *BEAST and subsequently compared the topology with the multi-gene tree based on the concatenated dataset. In the *BEAST analysis, all the parameters reached plateau before 5M generations. We therefore discarded the first 500 species trees as burn-in and summarized the remaining 19,501 trees for the PP calculation. ESS values of all the parameters became sufficiently large (> 100) after 200M generations. The average Log Likelihood (ln L) of the sampled species trees was 19,907.124.

The combined dataset of three nuclear loci (i.e., ITS and LSU rDNA, and EF-1α) consisted of 110 individuals and 2,586 base pairs. In the Bayesian inference, the ASDSF of the two parallel MCMC runs dropped constantly below 0.01 after c. 2.15M generations. Thus, the first 2,150 trees in each run were discarded as burn-in according to the ASDSF value, and the remaining 15,702 trees (sampled from 7,85M generations) were used to calculate a 50 % majority consensus tree and determine PPs. ESSs of all the parameters were sufficiently large. Total arithmetic mean and harmonic mean of Likelihoods (ln L) were –23 076.11 and –23 171.84, respectively. The maximum likelihood analysis resulted in one ML tree (ln L = –22 947.590387). Overall tree topologies between the Bayesian and ML trees were almost identical, and no topological conflict was observed. The resultant topology recovered a strongly supported leccinoid clade (BS = 100 %, PP = 1.00) that included Chamonixia, Leccinum, Leccinellum, Octaviania and its three subgenera, Rossbeevera, and a novel sequestrate lineage sister to Rossbeevera (Fig. 3) (i.e., Turmalinea).

Although there were some taxa omitted in the *BEAST species tree, overall topologies of the species tree and three-locus combined tree were similar (Fig. 2, 3). There was no strong topological conflict found between the two phylogenies. Both phylogenies showed that the novel Turmalinea clade was monophyletic and sister to Rossbeevera spp. In the *BEAST species tree the new lineage ‘R. cryptocyanea’ was shown to be a sister to R. eucyanea with strong statistical support despite the topological inconsistency among the single nuclear gene trees. However, the other infrageneric conflicting topologies among the gene trees were not resolved in the species tree analysis although they were resolved in the multigene combined analyses with relatively high statistical support. We therefore refrain from highlighting these infrageneric relationships here and will discuss it in more detail below.

In the Bayesian and ML combined phylogenies, every genus and subgenus in the leccinoid clade except Leccinellum was strongly supported as monophyletic by both BS and PP (Fig. 3). The novel Turmalinea clade contains four undescribed sequestrate species and two subspecies from Japan and China. All of the lineages were strongly supported by both BS and PP (BS ≥ 89 %, PP = 1.00). In the Rossbeevera clade, two unknown species-level lineages were nested within the clade; Rossbeevera cryptocyanea sp. nov. and R. paracyanea sp. nov., both of which are morphologically similar to R. eucyanea and are distributed in Japan. The sequestrate genus Chamonixia was recovered as the earliest diverging lineage within the leccinoid clade with moderate statistical supports (Fig. 3). The sister lineage to the leccinoid clade was not resolved in our analyses. A sequestrate species,
species tree

ITS + LSU rDNA + EF-1α combined dataset

Species tree

**Spongiforma thailandica**, that was formerly supported to form a monophyletic clade with *R. griseus* and *Leccinum* spp. (Nuhn et al. 2013), formed a strongly supported monophyletic clade with *Borofutus dhakanus* and *Porphyrellus porphyrosporus* as shown in Hosen et al. (2012). Consequently, a recently proposed subfamily *Leccinoideae*, which includes *Retiboletus*, *Borofutus*, and *Spongiforma* as well as the genera in the leccinoid clade (Wu et al. 2014), was not recovered in our nuclear multi-locus analyses.

**Mitochondrial multi-locus phylogeny of the Leccinoid clade**

The two-locus mitochondrial dataset (i.e., ATP6 and SSU mtDNA) consisted of 85 specimens and was 840 bp in length. In the Bayesian inference, the ASDAF of the two parallel MCMC runs dropped constantly below 0.01 after c. 1.77M generations. Thus, the first 1,770 trees in each run were discarded as burn-in, and the remaining 16,462 trees (sampled from 8.23M generations) were used to calculate a 50% majority consensus tree and determine PPs. ESS values of all the parameters were sufficiently large (>200). Total arithmetic mean and harmonic mean of Likelihoods (ln L) were -3,541.11 and -3,614.29, respectively. The ln L of the resultant ML tree in the RAxML analysis was -3,431,324,125. Monophyly of *Leccinum, Rossbeevera*, the *Turmalinea* clade, and the three *Octaviania* subgenera were recovered with varying statistical support (Fig. 4). However, most basal topologies, including relationships of the three *Octaviania* subgenera, were not resolved or were poorly supported.

**Topological comparison between the nuclear and mitochondrial phylogenies**

We examined topological differences between nuclear and mitochondrial phylogenies (Fig. 3, 4) and compared the resultant topologies of the *Rossbeevera-Turmalinea* lineage to determine whether there were any discrepancies in patterns of divergence (Fig. 5). The nuclear phylogeny based on the combined dataset of ITS, LSU, and EF-1α had high resolution and generally showed infraspecific divergence with strong statistical support regardless of the geographic distribution of the samples. For example, sequences of *R. griseovulitina*, *R. paracyanea* and *R. cryptocyanea* diverged into two strongly supported infraspecific lineages (lineages 1 and 2 in each species; Fig. 5). However, we did not find evidence of any obvious biogeographic disjunction within these species nor did we detect distinct mor-
Nuclear phylogeny ITS + LSU rDNA + EF-α combined dataset

 Nobeloid (pileate-stipitate)

Sequestrate (truffle-like)

Fig. 3 Bayesian 50 % majority-rule consensus tree of the nuclear three-locus dataset (ITS, nLSU, and EF-α) of Rossbeevera and allied genera (i.e., the leccinoid clade). Bayesian PP and RAxML bootstrap (BS) values (1 000 replicates) are indicated above or below branches or at nodes as PP/BS. Values of PP < 0.90 or BS < 50 % are not shown. Branches supported by both PP ≥ 0.98 and BS ≥ 70 % are depicted as thickened grey lines. Incompatible topologies between the combined phylogeny and nuclear single-locus phylogenies are indicated by red asterisks. Names of new taxa and new lineages are coloured in red. Chalciporus piperatus and Buchwaldobolletus lignicola were used as outgroups.

By both PP ≥ 0.98 and BS < 70 % are shown as thickened grey lines. Incompatible topologies between the combined phylogeny and nuclear single-locus phylogenies are indicated by red asterisks. Names of new taxa and new lineages are coloured in red. Chalciporus piperatus and Buchwaldobolletus lignicola were used as outgroups.
Mitochondrial phylogeny
ATP6 + SSU rDNA
combined dataset

- Branches supported by both PP ≥ 0.98 and MLBS ≥ 70 % are depicted as thickened grey lines.

Fig. 4 Cladogram of Bayesian 50 % majority-rule consensus phylogeny of the mitochondrial two-locus dataset (ATP6 and mtSSU). Bayesian PP and RAxML BS are indicated at nodes as PP/BS. Values of PP < 0.90 or BS < 50 % are not shown. Branches supported by both PP ≥ 0.98 and BS ≥ 70 % are depicted as thickened black lines. Branches supported by either PP ≥ 0.98 or BS ≥ 70 % are shown as thickened grey lines. Names of new taxa and new lineages are coloured in red.
Little divergence was recognized within Australasian Rossbeevera lineages, at least at the species level, despite the different morphologies and distributions of the described species. On the other hand, the mitochondrial phylogeny had relatively poor resolution and also generally reflected differences in distribution within each species shown in the nuclear phylogeny. Within the terminal Australasian Rossbeevera clade, we noted distinct divergence between the nuclear and the mitochondrial datasets. The mitochondrial topology of the clade was concordant with distributional patterns rather than morphological circumscription proposed by Lebel et al. (2012a) although some branches were not statistically supported (Fig. 5). For example, specimens that were morphologically identified as R. vittatispora were remarkably divergent in the mitochondrial dataset, but clearly reflected geographical proximity between Tasmania and Victoria. In the mitochondrial phylogeny, one individual of the ‘R. paracyanea’ lineage 1 (KPM-NC 18087) was divergent from the ‘R. paracyanea’ lineage 2, whereas another individual of the ‘R. paracyanea’ lineage 1 (i.e., KPM-NC 17847) was identical to the ‘R. paracyanea’ lineage 2. A very similar pattern was observed between ‘R. cryptocyanea’ lineages 1 and 2, although they also exhibited geographical proximity among the specimens in the mitochondrial phylogeny (i.e., Kakeroma Island is separated from Amami-oshima Island only by a narrow strait and they belong to the same group of islands, whereas Ishigaki Island is about 650 km distant from Amami-oshima).

Characterization of a minisatellite-like insertion within ITS2

We found that species of Rossbeevera and the Turmalinea clade have an unusually long insertion with indistinct tandem repeats within their ITS2 rDNA region. The inserted sequences ranged in size from 199 to 603 bp (Table 2). The Tandem Repeats Finder analysis detected one or two continuous tandem repeats in the ITS2 of 12 of 16 lineages of the clade (Table 2). Most of the detected tandem repeats contained a common GAGTGAAAGTG motif or similar derivative sequences. This core pattern and the derivatives scattered within insertions of every lineage in the group. In addition, one terminal lineage of the Turmalinea clade (i.e., ‘T. persicina’) had another highly conserved core motif, AATTATTTAATTATTGTGTGGCTTTGATTGTAAA, which is 34 bp in size and appeared three times just after the 3'-end of the GAGTGAAAGTG core pattern in the insertion. Turmalinea persicina is divided into two slightly divergent infraspecific groups (Fig. 6c). The copy number of the continuous motifs detected by the Tandem Repeats Finder analysis ranged from 1.9 to 5.9 (Table 2).
Table 2  Characterization of the minisatellite-like insertions within the ITS2 region of *Rossbeevera* and *Turmalinea* spp. Only consensus patterns with alignment score ≥ 50 are shown. Two slightly divergent infraspecific lineages of *T. persicina* were analysed independently. N.D. = not detected.

| Taxon               | Nos. of sequences examined | Insertion size (bp) | Nucleotide similarity within each lineage | Consensus pattern of tandem repeats (size (bp)) | Tandem repeats information | Entropy (0–2) |
|---------------------|---------------------------|---------------------|------------------------------------------|-----------------------------------------------|---------------------------|----------------|
| R. cryptocyanea     | 2                         | 362–366             | 94 %                                      | TAGTAAAAGGCTATGATATGGAAAGCTAGTAGGAAATGGAATGTA (46) | 2.1 84 12 158 1.76         |                |
| R. eucyanea         | 8                         | 319–324             | > 98 %                                    | TGCAACACAGCTGGAAGAGCTTAGTAAATGGAAGCTGGTACACC TGGTTAGATGAAAGCTTTAGTGAAGGATGTGTAAGGCTTAGAAAACTGGAAGATGTAAGGCTTAGATAACGGAAGCTTAGAT (160) | 1.9 80 9 465 1.88          |                |
| R. griseovelutina   | 8                         | 339–344             | > 96 %                                    | AAAAAAGCATGTAATAGGAAACTGAGTTGAAGGAAATGGAATGTA (46) | 2 79 10 130 1.79           |                |
| R. pachydermis      | 5                         | 223–224             | 100 %                                     | N.D.                                          | N.D. N.D. N.D. N.D. N.D.   |                |
| R. paracysteana-1   | 3                         | 309                 | 100 %                                     | N.D.                                          | N.D. N.D. N.D. N.D. N.D.   |                |
| R. paracysteana-2   | 2                         | 326                 | 100 %                                     | N.D.                                          | N.D. N.D. N.D. N.D. N.D.   |                |
| R. vittatispora     | 4                         | 334–340             | 98 %                                      | ATTTCTATGTGCATACGCTGGAAGAGCTTAGTAAATGGAAGCTGGTACACC TGGTTAGATGAAAGCTTTAGTGAAGGATGTGTAAGGCTTAGATAACGGAAGCTTAGAT (178) | 1.9 78 10 476 1.88          |                |
| R. aff. vittatispora| 2                         | 341                 | 99 %                                      | N.D.                                          | N.D. N.D. N.D. N.D. N.D.   |                |
| R. westraliensis    | 2                         | 335–337             | 99 %                                      | ATTTCTATGTGCATACGCTGGAAGAGCTTAGTAAATGGAAGCTGGTACACC TGGTTAGATGAAAGCTTTAGTGAAGGATGTGTAAGGCTTAGATAACGGAAGCTTAGAT (179) | 1.9 80 7 502 1.88          |                |
| R. yunnanensis      | 1                         | 199                 | N/A                                       | N.D.                                          | N.D. N.D. N.D. N.D. N.D.   |                |
| T. chrysocarpa      | 1                         | 397                 | N/A                                       | TGGTTAGAAAGCTAGATGGAAATGGAAGCTTGGTAGGAAATGGAATGTA (52) | 4 69 18 199 1.81           |                |
| T. mes. subsp. mesomorpha | 3                 | 400                 | 100 %                                     | TGAAGGCTCTAAAGCTAGATGGAAATGGAAGCTTGGTAGGAAATGGAATGTA (53) | 2.4 88 0 52 1.91           |                |
| T. mes. subsp. sordida | 2                   | 395                 | 100 %                                     | TGAAGGCTCTAAAGCTAGATGGAAATGGAAGCTTGGTAGGAAATGGAATGTA (53) | 2.4 88 0 52 1.91           |                |
| T. persicina-1      | 4                         | 600–601             | > 99 %                                    | TGTAGTAAACAGCGTGGAAAGAGCTTAGTAAATGGAAGCTGGTACACC TGGTTAGATGAAAGCTTTAGTGAAGGATGTGTAAGGCTTAGATAACGGAAGCTTAGAT (38) | 5.9 53 15 145 1.85          |                |
| T. persicina-2      | 3                         | 603                 | > 99 %                                    | TTATTTGTTGTTGGAAGCTGGTAAATGGAAGCTGGTACACC TGGTTAGATGAAAGCTTTAGTGAAGGATGTGTAAGGCTTAGATAACGGAAGCTTAGAT (25) | 2.1 78 15 69 1.86           |                |
| T. yuwanensis       | 2                         | 272–273             | > 99 %                                    | TGAAGGCTCTAAAGCTAGATGGAAATGGAAGCTTGGTAGGAAATGGAATGTA (19) | 2.2 79 12 55 1.81           |                |

Table 2  Characterization of the minisatellite-like insertions within the ITS2 region of *Rossbeevera* and *Turmalinea* spp. Only consensus patterns with alignment score ≥ 50 are shown. Two slightly divergent infraspecific lineages of *T. persicina* were analysed independently. N.D. = not detected.
Fig. 6 Barcode gap analysis and ML phylogeny of minisatellite-like insertion within the nuclear ITS2 rDNA of Rossbeevera and the Turmalinea clade. – a. Results of the Automatic Barcode Gap Discovery (ABGD) analysis of the minisatellite-like insertion dataset. The dataset is composed of 51 sequences and is 838 nucleotides in length. Histogram of genetic distances between sequences and cumulative frequency of the distance value (ranked value; indicated as the blue line) in the distance matrix are shown. The ‘barcode gaps’ are represented as horizontal lines on the ranked value. – b. Results of the ABGD analysis using the ITS dataset excluding the minisatellite-like regions. The dataset is composed of the same individuals as ‘a’ and is 668 nucleotides in length. – c. ML phylogeny based on the same insertion dataset as ‘a’. 10 species-level groups recovered with prior infraspecific divergence 0.019 ≤ P ≤ 0.038 in ABGD are designated onto the phylogeny as [G1] – [G10]. These partitions correspond to the boundary of interspecific divergence designated in ‘a’. Two infraspecific groups of T. persicina are identical to those in Table 1. The RAxML bootstrap (BS) value (1 000 replicates) is indicated above or below branches (only BS values ≥ 50% are shown).
The minisatellite-like insertions found in every species of the Rossbeevera-Turmalinea lineage were highly conserved within each species (and infraspecific lineage) but highly divergent between species or genera (Table 2). Furthermore, the barcode gap analysis showed that the minisatellite sequence region can be used to delimit the boundary between intergeneric-level, interspecific-level, and infraspecific-level divergence more distinctly than the rest of the ITS region (Fig. 6a, b). The boundary between infra- and interspecific-level divergence is supposed to be present at 0.0025 < D < 0.003, where D is a genetic distance value of pairwise distances in a dataset (Fig. 6a). The minisatellite dataset was both primarily and recursively partitioned into the ten species-level clusters already represented in Fig. 3, 5 with 0.019 ≤ P ≤ 0.038 in the recursive partitions, where P is a prior limit of maximal distance of infraspecific divergence (Fig. 6c). As in the other nuclear analyses, Australasian Rossbeevera spp. were not very divergent and this species-complex was not partitioned at the species-level genetic boundary estimated by the barcode gap analysis. Also, there was a distinct difference in interspecific genetic distance within Rossbeevera and within Turmalinea in the minisatellite dataset, while the difference was unclear in the rest of the ITS region (Fig. 6a, b).

The ITS2 minisatellite ML phylogeny mostly recovered monophyly of the Rossbeevera and Turmalinea species represented in the other phylogenies in this study (Fig. 6c; In L = −2 615.994733). The monophyly of R. griseovelutina was not supported in the ML phylogeny of the minisatellite-like insertion within the nuclear ITS2 rDNA, probably due to the effect of the long genetic distance between the Rossbeevera and Turmalinea clades. The infraspecific divergence within ‘R. paracyanea’, ‘R. griseovelutina’, ‘T. persicina’, and ‘T. mesomorpha’ was strongly supported in the analysis. Contrary to the pattern found in species of the Rossbeevera-Turmalinea lineage, the sister taxon of this group, Leccinellum griseum (= L. pseudoascum), had only a single 111 bp insertion in the ITS2 region and Tandem Repeats Finder analysis did not detect any repeating motifs in this DNA fragment.

**Taxonomy**

The phylogenetic analyses conducted in this study consistently recovered a distinct yet previously unrecognized lineage that was sister to the monophyletic genus Rossbeevera. Morphologically, members of the two clades are readily distinguishable by differences in the basidiospores and in the sterile base of the fruiting body. The unique, minisatellite-like insertions within ITS2 nuc-rDNA were highly divergent between the two clades as well as between the different species-level lineages (Fig. 6). Based on both molecular and morphological data, we propose the new genus Turmalinea for this lineage. This group is comprised of three new Japanese species (T. persicina, T. yuwanensis, and T. mesomorpha), one subspecies (T. mesomorpha subsp. sordida) and one Chinese species (T. chrysocarpa). Within the genus Rossbeevera we propose two new species from Japan, R. paracyanea and R. cryptocyanea.

**Turmalinea** Orihara & N. Maek., gen. nov. — MycoBank MB803433

Type species. Turmalinea persicina Orihara.

Etymology. The Latinized name, Turmalinea, refers to tourmaline, the reddish or bluish coloured mineral, referring to the striking colour variety of the fruitbodies.

Fruitbodies mostly less than 20 mm diam, solitary to sparse, subglobose to depressed-globose to reniform, rubbery, sessile or rarely with a short stipe at the base, surface smooth to slightly felly, pale pink to pink, or white to brownish white, often turning blue to indigo-blue when rubbed or bruised. *Rhizomorphs* white or yellow to orange. *Gleba* initially white, then maturing to blackish brown, firm, loculate, of minute irregular chambers and thin trimal plates, chambers arranged somewhat radially near centre. *Tram* mostly pulvinate, rarely becoming a short stipe, subgelatinous. Columnella absent. *Peridium* thin, up to 450 μm in dried specimens, single- or two-layered, of interwoven to parallel, septate, filamentous hyphae. *Subhyphenum* undeveloped. *Basidia* clavate to cylindrical, 2–4-spored. *Basidiospores* statismosporic, ovoid to fusoid, inamyloid, non-dextrinoid, brick-red to dark brown at maturity, with 5–10 longitudinal, often branched, costal to irregularly broken ridges up to 3.5 μm high, with or without a hilar appendage. Clamp connections absent in all tissues.

Notes — Turmalinea is morphologically characterized by ovoid to fusoid, reddish brown to dark brown basidiospores with 5–10 irregularly furcate, longitudinal ridges, and a cushion-like, subgelatinous sterile base. Two of the four species of Turmalinea have pinkish fruitbodies with yellow to orange rhizomorphs, which are unusual in hypogeous sequestrate fungi (Fig. 7a–d). The sister genus, Rossbeevera is morphologically different from Turmalinea in having a sterile base that often forms a short, reduced stipe and paler ellipsoid or fusoid to fusiform basidiospores that have 3–5 longitudinal ridges. Turmalinea also tends to form firm, depressed-globose to reniform basidiomata, which are less common in Rossbeevera (Fig. 7). In addition, there is a considerable genetic divergence within the ITS2 minisatellite-like insertion between the two genera, although the insertions are found at the same position in the ITS2 region (Fig. 6).

Ecologically, species of Turmalinea occur in broad-leaved forests and are presumably symbiotic ectomycorrhizal associates of trees in the *Fagaceae*. Although we have not confirmed their ectomycorrhizal status, it is likely that they are mycorrhizal based on the fact that most species in *Boletaceae* form ectomycorrhizas (Nuhn et al. 2013). While species of Rossbeevera exhibit tropical to temperate distribution and often fruit in summer, Turmalinea species have not yet been found in the tropics and they generally fruit during cooler weather. This might reflect physiological difference in hyphal growth between members of the two genera and, thus, might result in their distributional difference.

Another similar leccinoid sequestrate genus, Chamonixia, also has basidiospores with 6–12 longitudinal ridges, but the basidiospores are generally ellipsoid to broadly ellipsoid (Q = 1.3–1.8). Macroscopically, Chamonixia spp. form light, fragile fruitbodies whereas those in the Rossbeevera-Turmalinea lineage have a more rubbery texture. In addition, the sterile base of Chamoxia fruitbodies is never gelatinized, and often forms a persistent or branched columella. The genus *Rhodactina* is a sequestrate member of *Boletaceae* reported from India and Thailand (Pegler & Young 1969, Yang et al. 2006). The genus is similar to Turmalinea in that the fruitbodies stain reddish to purplish and the basidiospores have 5–10 longitudinal ridges (Fig. 9k, l), but is distinctive from Turmalinea in the violet brown to purplish carneous gleba and well-developed (3–5 μm high), unbranched, acute ridges of the basidiospores (Yang et al. 2006; T. Orihara pers. obs.). The ATP6 sequence from *Rhodactina incarnata* is c. 93–94 % similar to *ATP6* sequences of Turmalinea spp., suggesting that they are not members of the same lineage. We obtained an ITS1 sequence of the isolate of *R. incarnata* (GenBank KC552020), and the BLAST search suggests that it is most closely related to species of *Tyopilus*. The southern hemisphere genus, Austrogautieria, also has similar basidiospores with eight or more irregularly branched, longitudinal ridges. However, molecular phylogenetic analysis shows that Austrogautieria is a member of *Gallaceaeae* in the Hysterangiales (Hosaka et al. 2006), and therefore, is phylo-
Fig. 7  Fruitbodies of *Turmalinea* and *Rossbeevera* species. a. *Turmalinea persicina* (holotype); b. *Turmalinea yuwanensis* (holotype); c. aberrant fruitbody of *T. persicina* with a short stipe and incomplete lamellae (arrow) (KPM-NC 18008); d. *Turmalinea chrysocarpa* (holotype); e. *Turmalinea mesomorpha* subsp. *mesomorpha* (holotype); f. *Turmalinea mesomorpha* subsp. *sordida* (holotype); g. *Rossbeevera paracyanea* (holotype); h. *Rossbeevera cryptocyanea* (holotype). — Scale bars = 1 cm.
genetically distant from *Turmalinea*. Morphologically, species of *Austrogautieria* are readily distinguished from *Turmalinea* by the glutinous spore mass and the presence of a well-developed columella in the gleba.

**Turmalinea persicina** Orihara, sp. nov. — MycoBank MB803437; Fig. 7a, c, 8a–e

Holotype, JAPAN, Kyoto Pref., Sakyō-ku, Ikawara-agura-cho, Mt Amabuki, c. 200 m northwest from Jisso-in Temple, under Castanopsis sieboldii, 8 Dec. 2008, T. Orihara, Orihara952 (KPM-NC 18001; isotype TNS-F-55010).

**Etymology.** Latin, *persicina* (= peach-coloured), refers to the characteristic colour of the fruitbodies.

**Fruitbodies** up to 20 mm, subglobose to depressed-globose to reniform, rubbery, sessile or rarely with a reduced stipe at the base, surface covered with thin, smooth, pink to pinkish white peridium occasionally turning weakly blue to bluish green when bruised, becoming blackish pink at maturity due to blackish colour of the inner gleba. *Gleba* off-white, occasionally turning bluish green in youth when cut, then blackish brown at maturity, rubbery, composed of minute, irregular locules. **Sterile base** present, mostly pulvinate or rarely becoming a short, reduced stipe, translucen, subgelatinous. *Rhizomorphs* 40–325 μm thick, stramineous to yellow-brown under light microscopy. 

**Notes** — *Turmalinea persicina*, which is widely distributed throughout western Japan, is easily distinguished from other species by its pinkish white to pale pink fruitbodies with yellow to orange rhizomorphs at the base. The large, persistent, inflated basidioles are also characteristic of the species (Fig. 8b). Like other species of *Turmalinea*, its sterile base is mostly pulvinate, but we found one fruitbody with a distinct stipe and a trace of hymenophoral pores (Fig. 7c; KPM-NC 18008). However, DNA sequences from this specimen were nearly identical to other, more typical specimens collected nearby (i.e., KPM-NC 18007 and KPM-NC 18008). This is an example of the high morphological plasticity of sequestrate fungi, sometimes caused by only a few nucleotide substitutions in key functional genes, which might be expected during the evolution of sequestrate taxa from epigeous relatives (Thiers 1984, Bruns et al. 1989, Kendrick 1992, Bougher et al. 1993, Hibbett et al. 1994).

**Turmalinea yuwanensis** Orihara, sp. nov. — MycoBank MB803438; Fig. 7b, 8f–j

Holotype, JAPAN, Kagoshima Pref., Amami-oshima Island, Yamato-son, southeast foot of Mt Yuwan, 29 Nov. 2008, T. Orihara, A. Okuda, M. Ohmae, Orihara1363 (KPM-NC 18011; type TNS-F-55011); same locality, 15 Jan. 2011, Y. Sunada & T. Orihara, Orihara1364, KPM-NC 18007 (duplicate TNS-F-55010); same locality, 12 Dec. 2012, H. Inui, M. Inui & T. Orihara, KPM-NC 18004; same locality, 11 Dec. 2012, H. Inui, M. Inui & T. Orihara, KPM-NC 18006; same locality, 16 Jan. 2011, Y. Sunada & T. Orihara, Orihara1363, KPM-NC 18006; same locality, 16 Jan. 2011, Y. Sunada & T. Orihara, Orihara1364, KPM-NC 18007 (duplicate TNS-F-55011); same locality, 16 Jan. 2011, Y. Sunada & T. Orihara, Orihara1368, KPM-NC 18008; Yamaguchi Pref., Yamaguchi-shi, Tokuchi, near the Lake Ohara, under *C. cuspidata* and *O. serrata*, 28 Nov. 2014, Y. Kawaguchi, KPM-NC 23395.

**Etymology.** The epithet, *yuwanensis*, refers to the type locality of the species, Mt Yuwan-dake, which is located in Amami-oshima Island, Kagoshima Pref., Japan.

**Fruitbodies** up to 20 mm, depressed-globose to reniform, rubbery, sessile or a short, rudimentary stipe at the base, peridium surface smooth, vivid pink but white to pinkish white near the base, colour change not observed when rubbed or bruised, becoming dull pink at maturity due to blackish colour of the inner gleba. *Gleba* white in youth, then blackish brown at maturity, rubbery, composed of minute, irregular locules. **Sterile base** present, mostly pulvinate, translucent, subgelatinous, colourless when immature then becoming dull yellowish brown. *Rhizomorphs* vivid yellow, often developed. Odour somewhat sweet but unpleasant, similar to that of *Turmalinea persicina*, which is widely distributed throughout western Japan, is easily distinguished from other species by its pinkish white to pale pink fruitbodies with yellow to orange rhizomorphs at the base. The large, persistent, inflated basidioles are also characteristic of the species (Fig. 8b). Like other species of *Turmalinea*, its sterile base is mostly pulvinate, but we found one fruitbody with a distinct stipe and a trace of hymenophoral pores (Fig. 7c; KPM-NC 18008). However, DNA sequences from this specimen were nearly identical to other, more typical specimens collected nearby (i.e., KPM-NC 18007 and KPM-NC 18008). This is an example of the high morphological plasticity of sequestrate fungi, sometimes caused by only a few nucleotide substitutions in key functional genes, which might be expected during the evolution of sequestrate taxa from epigeous relatives (Thiers 1984, Bruns et al. 1989, Kendrick 1992, Bougher et al. 1993, Hibbett et al. 1994).

**Turmalinea persicina**, which is widely distributed throughout western Japan, is easily distinguished from other species by its pinkish white to pale pink fruitbodies with yellow to orange rhizomorphs at the base. The large, persistent, inflated basidioles are also characteristic of the species (Fig. 8b). Like other species of *Turmalinea*, its sterile base is mostly pulvinate, but we found one fruitbody with a distinct stipe and a trace of hymenophoral pores (Fig. 7c; KPM-NC 18008). However, DNA sequences from this specimen were nearly identical to other, more typical specimens collected nearby (i.e., KPM-NC 18007 and KPM-NC 18008). This is an example of the high morphological plasticity of sequestrate fungi, sometimes caused by only a few nucleotide substitutions in key functional genes, which might be expected during the evolution of sequestrate taxa from epigeous relatives (Thiers 1984, Bruns et al. 1989, Kendrick 1992, Bougher et al. 1993, Hibbett et al. 1994).
inner matrix inconspicuous, contains several oil drops, sterigmata 4.5–7 μm long. Basidioles persistent, 8–20 μm diam, clavulate to subspherical to vesiculate, colourless, thin-walled (< 0.8 μm thick). Trama white, colourless, of compactly interwoven, thin-walled (< 0.8 μm thick) filamentous hyphae 2–9 μm broad. Sterile base of compactly interwoven, thin-walled (< 0.8 μm broad), colourless to pale yellowish brown, septate filamentous hyphae 3–10.5 μm broad forming a cutis, walls up to 1 μm thick.

Habitat, Distribution & Season — Hypogeous to subepigeous under or on rotten wood of *Castanopsis sieboldii* subsp. *lutchuensis*. So far known from Amami-oshima Island, Okinawa and Tokashiki Islands, the Ryukyu Archipelago, Japan; late autumn (November).

**Specimens examined.** JAPAN, Kagoshima Pref., Amami-oshima Island, Naze-ki, Kinsakubaru National Forests, 19 Nov. 1988, Y. Doi, ‘Hymenogaster sp.; Yoshimi7393, TNS-F-183213; Amami-oshima Island, Yamato-son, southeast foot of Mt Yuwan, under *Castanopsis sieboldii* subsp. *lutchuensis*, 17 Nov. 2007, A. Hadano, T. Orihara, M. Ohkubo, Orihara758, KPM-NC 18010 (duplicate: TNS-F-55013); same locality, 29 Nov. 2008, T. Orihara, A. Okuda, M. Ohmae, Orihara942, KPM-NC 18011 (holotype); Okinawa Pref., Tokashiki-jima Island, Tokashik-son, Tokashiki, under *Castanopsis sieboldii* subsp. *lutchuensis*, 14 Apr. 2013, T. Orihara, KPM-NC 23377; Okinawa Island, Kunigami-son, along the path to Mt Yonaha, under *Castanopsis sieboldii* subsp. *lutchuensis*, 10 Nov. 2013, M. Moriguchi, KPM-NC 23390; same locality, 5 Feb. 2015, T. Orihara, KPM-NC 23998; same locality, 5 Feb. 2015, T. Orihara, KPM-NC 24000.

Notes — The vivid pink peridial surface and yellow rhizomorphs of *Turmalinea yuwanensis* are striking features and are easily recognized in the field. Microscopically, *T. yuwanensis* is unique in that its basidiospores have distinct but discontinuous longitudinal ridges that partially become large, irregular warts. A similar and closely related species, *T. persicina*, is distinguished from *T. yuwanensis* by the paler peridium, larger basidiospores with relatively continuous ridges, and its slender, 2-spored basidia. The Chinese species, *T. chrysocarpa* is also phylogenetically close to *T. yuwanensis*, but is readily distinguished by the yellow peridium and larger basidiospores (Fig. 8d).

This species has been collected from only a few sites in the central Ryukyu Islands, which lie c. 300–600 km to the southwest of mainland Japan. A truffle-like fruitbody with orange rhizomorphs reported by Yoshimi & Doi (1989) from Amami-oshima island as ‘*Hymenogaster* sp.’ was confirmed as an immature *T. yuwanensis*. Given the unique phylogenetic position and rarity of *T. yuwanensis*, the conservation of its habitat is of primary importance in terms of phylogenetic diversity (Faith 1992, Faith & Baker 2006).
**Turmalinea chrysocarpa** Orihara & Z.W. Ge, sp. nov. — Myco-Bank MB803441; Fig. 7d, 9a–c

Holotype. **China**, Yunnan Prov., Manhao, Gejiu, alt. 860 m, in a broad-leaved forest, 24 Sept. 2011, Z.W. Ge 3098 (HKAS70601; isotype KPM-NC 18068).

**Etymology.** The Latin, *chryso-* (golden) and -*carpa* (fruitbody), refer to the colour of the fruitbody surface of the species, which is unusual in *Turmalinea*.

**Fruitbody** solitary, 1.5 cm diam, depressed-globose, surface smooth to slightly floccose, yellow to light orange, stiped reduced, rudimentary, with orange rhizomorphs at the base. *Gleba* chocolate brown to blackish brown at maturity, composed of minute, irregular locules. **Sterile base** present, more or less pulvinate, translucent, subgela tinous. **Odour** strong and sweet. *Basidiospores* 13.5–(13.8)–21.5–(23.5) x (6.9–)7.3–9.7–(10.8) μm, mean 17.5 x 8.5 μm (SD: 1.97 (length), 0.61 (width)), Q = 1.66–2.71, Qs = 2.06, symmetric, citriform to fusiform, dark reddish brown at maturity, with 6–9 irregularly longitudinal, often fuscate ridges 1–2.2 μm thick in water, with a distinct hilar appendage up to 6 μm long, HA/S = 0.11–0.31, HA/Sr = 0.19, spore walls 0.7–1.2 μm thick. *Basidia* evanescent, cylindrical but mostly collapsed, 20.9 x 3.2 μm, 2-spored, colourless, sterigmatum up to 7.5 μm long. **Trama** colourless, of moderately or somewhat loosely interwoven, partially sinuate, septate filamentous hyphae 2–10 μm broad, walls up to 1.2 μm thick. **Peridium** 150–330 μm thick, composed of two layers: outermost layer (i.e., peridipellis) 60–150 μm thick, of parallel to subparallel, straight, septate, colourless, thin-walled (< 0.8 μm), non-inflated filamentous hyphae 2.8–10–(13) μm broad, only the surface stained fulvous; inner layer (i.e., subpellis) 75–250 μm thick, colourless, of compactly interwoven, sinuate, septate, narrower (2.5–8 μm), thin-walled filamentous hyphae.

Notes — *Turmalinea chrysocarpa*, which is reported from Southwest China, is unique in having a yellow to light orange peridium and an inner peridial layer composed of compact, interwoven hyphae. Phylogenetic analyses support that T. *chrysocarpa* is a distinct lineage from the other *Turmalinea* spp. from Japan (Fig. 3–6). This species is readily distinguished from other members of the genus by the colour of the peridium and the dimension of basidiospores. Unfortunately, only one specimen of T. *chrysocarpa* has so far been collected. Although this may be due to the rarity of the species, it may also be due to lack of data on the diversity, phenology and ecological requirements of sequestrate fungi in China. Despite our lack of knowledge about this species, its characteristic morphology and phylogenetic distinctness are sufficient to describe it as a new *Turmalinea* species.

**Turmalinea mesomorpha** Orihara, sp. nov. — Myco-Bank MB803439; Fig. 7e, 9d–g

Holotype. **Japan**, Aomori Pref., Towada-shi, Ohoroinai, under Fagus crenata, 12 Oct. 2009, T. Sasa & T. Orihara, Orihara1080, KPM-NC 18015.

**Etymology.** The Latin, *meso-* (middle or intermediate) and -*morphe* (morphology), signifying that the species has intermediate morphology between Rossbeevera spp. and other members of *Turmalinea*.

**Subspecies autonym.** *Turmalinea mesomorpha* subsp. mesomorpha (the automatically generated name by the proposal of T. *mesomorpha* subsp. sordida, which is to be described below).

**Fruitbodies** up to 24 mm, subglobose to depressed-globose to reniform, rubbery, sessile or with a short, reduced stipe less than 5 mm long at the base, surface somewhat feltly or partially wrinkled, white to off-white, immediately turning indigo to purplish blue and finally blackish brown when touched or bruised. *Gleba* off-white to whitish yellow when immature, then chocolate brown, and finally becoming blackish brown at maturity, rubbery, composed of minute, irregular locules. **Sterile base** present, mostly pulvinate but rarely columella-like, translucent, subgelatinous, colourless to blue-grey when immature then becoming dull yellowish brown at maturity, surrounded by whitish hyphal veins irregularly radiating from the central sterile base and connected to inner portion of the peridial context at the bottom. *Rhizomorphs* white to off-white, common, showing the same pattern of discolouration as the fruitbody surface. **Odour** somewhat sweet but unpleasant. *Basidiospores* 12.5–16.4–(16.9) x (7.8–)7.9–10.4 μm, mean 14.4 x 9.1 μm (SD: 0.97 (length), 0.61 (width)), Q = 1.38–1.92, Qs = 1.58, symmetric, ovoid to citriform to fusoid, colourless at first then becoming dark reddish brown at maturity, with 5–10 irregularly longitudinal, costal or often branched ridges up to 2.6 μm high in water, a hilar appendage reduced or indistinct, occasionally with a hyaline pedicel up to 6 μm long, spore walls 0.7–1.6 μm thick. *Basidia* evanescent, cylindrical to clavate-clavate, 25.5–43.8 x 6.4–12.2 μm, mean 33.1 x 8.8 μm (n = 15), 2–4-spored, colourless or with a yellowish brown tint, sterigmatum 6–10 μm long, walls thin (< 0.8 μm thick), inner matrix somewhat granulate. *Basidioles* cylindrical to clavate-clavate, 10–18 x 6–9 μm, colourless or pigmented fulvous, walls thin (< 0.7 μm thick). **Trama** colourless or pigmented pale yellowish brown in places, of subparallel to loosely interwoven, straight, partially branched, thin-walled (< 0.8 μm thick) filamentous hyphae 2–10 μm broad. **Sterile base** of loosely interwoven, mostly sinuous, colourless, septate, thin-walled (> 0.8 μm thick) filamentous hyphae 3.5–11 μm broad, the surrounding whitish layer composed of loosely interwoven, more or less frequently branched, thin-walled (< 0.8 μm thick) filamentous hyphae 2–8 μm broad that are less sinuate than those comprising the sterile base. **Peridium** 50–150 μm thick, yellow-brown under light microscopy, composed of repent, partially branched and inflated, septate filamentous hyphae 2–11 μm broad interwoven or subparallel to surface, walls up to 1 μm thick.

Habitat, Distribution & Season — Hypogeous to subepigeous under Fagus crenata; Japan (northern Honshu); autumn (September to October).

**Specimens examined.** **Japan**, Aomori Pref., Towada-shi, Ohoroinai, under Fagus crenata (the holotype locality), 21 Sept. 2008, T. Sasa, Orihara1084, KPM-NC 18012 (duplicate: TNS-F-55014); same locality, 27 July 2009, T. Sasa & T. Orihara, Orihara1080, KPM-NC 18015 (holotype); Towada-shi, Tsuta onsen, under F. crenata, 27 July 2012, T. Muroi, KPM-NC 18059; **Iwate Pref.**, Hachimantai-shi, Appi-kogen, under F. crenata, 11 Oct. 2009, M. Taniguchi, Orihara1074, KPM-NC 18013; same locality, 12 Oct. 2009, T. Sasa & T. Orihara, Orihara1075, KPM-NC 18014.

Notes — *Turmalinea mesomorpha*, the earliest diverging species within *Turmalinea*, shares characteristics of both *Turmalinea* and *Rossbeevera*. Like most *Rossbeevera* species, the fruitbody has a whitish peridium that quickly turns indigo to purplish blue. On the other hand, most other generic level characters such as the cushion-like sterile base and the basidiospore morphology are typical of *Turmalinea*. The morphology of T. *mesomorpha* in combination with its position in the phylogeny suggests that the pinkish to yellow colours of the other *Turmalinea* species probably evolved after the divergence of T. *mesomorpha*. Compared to other *Turmalinea* species, the sterile base tends to become amorphous and surrounded with whitish hyphal veins. Interestingly, one fruitbody (KPM-NC 18059) had a columnar sterile base, which is more common in *Rossbeevera*. These peridial and sterile base characters morphologically support that the species is the earliest diverging lineage within the genus *Turmalinea*. Moreover, the aberrant sterile base morphologies found in T. *mesomorpha* and T. *pericina* suggest that these reversions have occurred somewhat frequently within the genus.

Turmalinea mesomorpha is microscopically different from T. *pericina* and T. *yuwanensis* in its larger basidia and medium-sized
basidiospores with relatively continuous longitudinal ridges. In contrast to *T. persicina* and *T. yuwanensis*, which occur in the evergreen Quercus/Castanopsis forest, *T. mesomorpha* has only been collected under the deciduous montane tree *Fagus crenata*. This suggests not only geographical but also ecological isolation from the other two Japanese *Turmalinea* species.

**Turmalinea mesomorpha** subsp. *sordida* Orihara, subsp. *nov.* — MycoBank MB803440; Fig. 7f, 9h–j

Holotype. **JAPAN**, Ehime Pref., Saijo-shi, Mt Ishizuchi, under *F. crenata*, 10 Oct. 2010, H. Ikeda, Orihara1280, KPM-NC 18016.

**Etymology.** The Latin, *sordida* (= dirty, foul), refers to the characteristic colour of the peridial surface of the subspecies.

**Diagnosis.** This subspecies is distinguished from *Turmalinea mesomorpha* subsp. *mesomorpha* by the greyish brown surface of fruitbodies that
turns deep blue to purple and finally becoming blackish brown when touched or bruised, and thicker peridium up to 450 μm thick composed of filamentous hyphae 3–15 μm broad. Basidiospores shape and dimensions not significantly different from that of T. mesomorpha subsp. mesomorpha, 11.7–(12–)15.6(–17) × (6.8–)7–9.6 μm, mean 13.7 × 8.5 μm (SD: 0.96 (length), 0.58 (width), Q₁ = 1.41–1.94, Q₃ = 1.62.

Habitat, Distribution & Season — Hypogeous to subgegeous under Fagus crenata; Japan (Shikoku); autumn (September to October).

Specimens examined. Jōnnō, Ehime Pref., Saijo-shi, Mt Ishizuchi, under F. crenata, 10 Oct. 2010, H. Ikeda, Orinaha1280, KPM-NC 18016 (holotype); Matsuyama-shi, Mt Takawan, under F. crenata, 4 Nov. 2007, F. Nagao, KPM-NC 17743.

Notes — The T. mesomorpha specimens collected from Shikoku, one of the four main islands of Japan, showed slight macroscopic differences from the other collections from the northern part of Japan, including the holotype specimen of subsp. mesomorpha. However, we did not find any significant microscopic differences between the two groups, and they share a similar ecological habitat (i.e., they are all found in association with Japanese beeches). The phylogenetic analyses conducted in this study showed slight divergence between the two lineages, suggesting their relatively recent diversification compared to the other species-level lineages in the genus. We therefore recognize the Shikoku lineage as subspecies sordida based on morphological and molecular data that are currently available.

Aoki (1978) informally reported a specimen from Tokyo that more or less fit the characters of T. mesomorpha subsp. sor-dida as ‘Gauteria sp.’ However, we were unable to locate this specimen, so we could not directly compare it to our recently collected material. Since Tokyo is geographically intermediate between the present habitats of each subspecies, the record or additional collections in that area should provide important knowledge about the biogeography of the species in the future.

Rossbeevera T. Lebel & Orihara, in Lebel et al., Fung. Diversity 52: 54 + 73. 2012, ‘Rosbeeva’.

Type species. Rossbeevera pachydermis T. Lebel, Fung. Diversity 52: 64. 2012, ‘Rosbeeva pachyderma’.

Notes — The precise description of Rossbeevera is presented in Lebel et al. (2012a) under the variant ‘Rosbeeva’. Since the correction of the type species name is omitted in the erratum by Lebel et al. (2012b), we herein specify its correct name.

Rossbeevera paracyanea Orihara, sp. nov. — MycoBank MB803442; Fig. 7g, 10a–e

Holotype. Jōnnō, Nara Pref., Nara-shi, Nara Park, under Quercus gilva, 26 Oct. 2008, M. Kawai, Orinaha908, KPM-NC 17847 (holotype);

Etyymology. The epithet (Greek, para- = para- or beside and cyanea = ultramarine blue) expressing the morphological characteristics, habitat and distribution of the species similar to those of Rossbeevera eucyanea: both of the species have strongly cyanescent fruitbodies and occur usually under Castanopsis and evergreen Quercus trees in western Japan.

Fruitingbodies solitary or in small clusters, up to 27 mm diam, sub-globose to depressed-globose, soft, peridial surface smooth, white to greyish at first, becoming dark blue-grey to dark grey and occasionally cracked at maturity to expose inner gleba and turning deep blue when touched or bruised. Gleba off-white when immature, dark reddish brown at maturity, non-gelatinous, minutely and irregularly loculate, turning indigo-blue in some portions when cut and exposed to air. Sterile base present but reduced, non-gelatinous, strongly cyanescent, often forming a short stipe. Columella absent. Odour somewhat sweet but unpleasant. Basidiospores (13.3–)14–19.3(–20.4) × (6.7–)8.9–9.2(–9.3) μm, mean 16.7 × 8 μm (SD: 1.32 (length), 0.57 (width)), Q₁ = 1.7–2.5, Q₃ = 2.1, symmetric, fusoid to fusiform, inamyloid, non-dextrinoid, colourless at first then becoming reddish brown at maturity, with 4–5 or rarely 3 smooth, longitudinal ridges up to 2.5 μm high in water, walls 0.5–1.2 μm thick, with a developed hilum appendage 2.1–4.5(–4.7) μm long (mean 3.3 μm) at the base, HA/S = 0.13–0.25, HA/S₃ = 0.2, walls at the tips thinner than on the sides. Basidia 13.8–21 × 6–11 μm (mean = 18 × 8.1 μm; n = 10), clamp to doliform, evanescent, colourless or pigmented with yellow-brown, mostly 2-spored but rarely 1-, 3- or 4-spored. Hymenium developed when immature but collapsed at maturity, colourless; basidioles clavate to clavulate to doliform almost the same size as or smaller than basidia. Subhydnum not developed, composed of single- or two-layered, colourless, isodiametric to clavulate cells 4–15 μm diam. Trama of parallel to subparallel, non-inflated, colourless, thin-walled (< 0.6 μm thick) filamentous hyphae 2.5–5 μm broad. Sterile base composed of compact, interwoven, sepalate, inflated, thin-walled (c. 1 μm thick) filamentous hyphae 2–18 μm broad. Peridium thin, composed of 2 layers: outer layer 50–160 μm thick, of compact, interwoven, sepalate, pale brown to brown filamentous hyphae 2–9.5 μm swollen in some portion to 15 μm broad, walls up to 1.2 μm thick; inner layer 15–55 μm thick, tan to reddish brown, of subparallel or tangled, sinuate, non-inflated filamentous hyphae 1.5–5 μm broad, walls 0.5–1.3 μm thick. Clamp connections absent in all tissues.

Habitat, Distribution & Season — Hypogeous to subgegeous under Quercus gilva and Castanopsis cuspidata; Japan (western Honshu); summer to early winter (July to December).

Specimens examined. Jōnnō, Nara Pref., Nara-shi, Nara Park, under Quercus gilva, 26 Oct. 2008, M. Kawai, Orinaha908, KPM-NC 17847 (holotype);

Notes — Fruitbodies of Rossbeevera paracyanea are macroscopically similar to those of another Japanese species, R. eucyanea, especially when young. Its habitat and size of basidiospores also overlap with those of R. eucyanea. However, R. paracyanea is morphologically distinguished from R. eucyana by its two-layered peridium comprised of the outer layer of partially inflated, filamentous hyphae and the thin, inner layer of somewhat pigmented, sinuate filamentous hyphae. The peridium tends to peel off at maturity due to the fact that the hyphae of the inner peridial layer are more or less parallel to the surface of the gleba. The surface of R. paracyanea fruitbodies was generally greyish or blue-grey and this helps to distinguish the species from R. eucyana, which has a whitish fruitbody. The three-locus nuclear phylogeny showed distinct divergence within R. paracyanea (i.e., lineages 1 and 2), but the mitochondrial phylogeny showed a conflicting relationship (Fig. 5). In the R. paracyanea lineage 2 specimen from Osaka Pref. (KPM-NC 17848), some of the basidia have unusually long sterigmata (up to 20 μm long) and the context of the outer layer of the peridium was almost colourless. The latter observation, however, could be due to the immature state of the specimens. Thus, we leave the species as a single taxon and treat the collections from Osaka (KPM-NC 17848 and KPM-NC 18027) as an infraspecific cryptic lineage until sufficient collections become available for additional phylogenetic and morphological comparisons.
Rossbeevera cryptocyanea Orihara — MycoBank MB811526; Fig. 7h, 10f–i

Holotype, JAPAN, Kakeroma Isl., Setouchi-machi, Akitoku, under Castanopsis sieboldii subsp. lutchuensis, 28 June 2014, T. Orihara, KPM-NC 23928.

Etymology. The epithet (Greek, crypto- = crypto- or hidden, and cyanea = ultramarine blue) expressing its close phylogenetic relationship and extreme morphological similarity to R. eucyanea.

Diagnosis. Morphologically quite similar to Rossbeevera eucyanea, but differs in smaller and narrower basidiospores with longer hilar appendices.

Fruitbodies solitary or in small clusters, up to 22 mm, subglobose to depressed-globose to reniform, soft, surface smooth to somewhat felty, white or more rarely greyish white, quickly turning deep blue when touched or exposed to air. Gleba off-white when immature, then becoming brown, non-gelatinous, minutely and irregularly loculate, quickly turning indigo-blue when cut and exposed to air. Sterile base present but reduced, non-gelatinous, cyanescent, forming a short stipe. Columella absent. Odour somewhat sweet but unpleasant. Basidiospores 13.4–(13.5–)18.3(–18.4) × 5.8(–7.2)–7.3 μm, mean 15.8 × 6.5 μm (SD: 1.2 (length), 0.36 (width)), Q = 1.9–3.0, Qm = 2.4, fusoid to fusiform, colourless at first then becoming dark brown, with 3–5(–6) longitudinal ridges up to 2 μm high in water, walls 0.5–1.2 μm thick, with large, developed hilar appendix 2.4–5.9 μm long (mean 3.7 μm) at the base, HA/S = 0.17–0.37, HA/S" = 0.23. Spore walls at the tips tend to be thinner than on the sides. Basidia 12.5–21 × 7–11.5 μm, clavulate to clavate, colourless, 2- or 4-spored. Trama composed of interwoven, septate, colourless, thin-walled, filamentous hyphae 3–8 μm broad. Peridium 50–180 μm thick in dried specimens, of somewhat loosely woven, septate, partially branched or swollen, thin-walled (< 0.5 μm) filamentous hyphae 3–15 μm, subparallel to surface, surface often stained blue. Clamp connections absent in all tissues.

Habitat, Distribution & Season — Hypogeous to subepigeous under Castanopsis sieboldii subsp. lutchuensis and Quercus glauca; Japan (Kyushu and the Ryukyu Archipelago); early summer and late autumn (June to November).

Specimens examined. JAPAN, Oita Pref., Usuki-shi, Shimonoe, under Quercus glauca, 20 Nov. 2005, Y. Sunada, Orihara1351, KPM-NC 17846; Kagoshima Pref., Amami-oshima Isl., Yamato-son, north-eastern foot of Mt Ywandake, under Castanopsis sieboldii subsp. lutchuensis, 17 Nov. 2007, A. Hadano & T. Orihara, Orihara760, KPM-NC 17843; Kakeroma Isl., Setouchi-
these results suggest that the morphological differences among the sequestrate genera and fungi fall into several distinct lineages within the leccinoid Rossbeevera including nuclear and mitochondrial multi-locus analyses, to In this study, we took several different phylogenetic approaches, Phylogeny, evolution, phylogeography and morphology of DISCUSSION

Phylogeny, evolution, phylogeography and morphology of Rossbeevera and Turmalinea

In this study, we took several different phylogenetic approaches, including nuclear and mitochondrial multi-locus analyses, to clarify evolutionary relationships of Rossbeevera and the allied taxa in the leccinoid clade. The nuclear three-locus phylogeny based on the combined dataset confirmed that sequestrate fungi fall into several distinct lineages within the leccinoid clade and this phylogeny also elucidated the placement of the new generic sequestrate lineage, Turmalinea (Fig. 3). Loss of forcible spore discharge (i.e., ballistospory) on the process of evolution of sequestrate morphology is thought to be irreversible (Hibbett 2004, Wilson et al. 2011). The major morphological characteristics of the sequestrate genera Chamonixia and Octaviania, such as basidiospores and gleba structure, are quite different from each other and from Rossbeevera and Turmalinea (Lebel et al. 2012a, Orihara et al. 2012b). The nuclear multi-locus combined phylogeny shows that the sequestrate genera in the leccinoid clade are not monophyletic (Fig. 3). In contrast, the overall morphology between the two boletoid genera, Leccinum and Leccinellum, is similar (Den Bakker & Noordeloos 2005). Unfortunately, the phylogenetic placement of Leccinellum in the leccinoid clade has not been settled and the placement of Chamonixia as the earliest diverging lineage in the clade is only moderately supported (Fig. 3). Nonetheless, given that the phylogenetic analysis is consistent with the morphological differences among the sequestrate genera and the fact that the dominant fruitbody morphology in Boletaceae is the boletoid form (Binder & Hibbett 2006, Nuhn et al. 2013), these results suggest that the Chamonixia, Octaviania, and Rossbeevera/Turmalinea lineages evolved independently from different boletoid ancestors in the leccinoid clade.

Unfortunately, the multi-locus phylogenies in this study did not clarify the sister clade to the leccinoid clade (Fig. 2, 3) so we were unable to definitively determine the sequence of morphological changes that may have occurred during the evolution of this group. Recently, Wu et al. (2014) proposed seven subfamilies across the Boletaceae based on nuclear multi-gene (nLSU, EF-1α, RPB1, and RPB2) analyses. Among the proposed subfamilies was the Leccinioideae, which includes the genera of the leccinoid clade. Our phylogenetic analyses did not recover Leccinioideae but this is likely due to the difference of loci included in the combined dataset. Instead, two genera in the Leccinioideae (i.e., Borofutus and Spongiforma) were resolved in a monophyletic clade that included Porphyrellus porphyrosporus, which was treated as a member of the Boletoideae in Wu et al. (2014). This suggests a need to re-evaluate the phylogenetic entity of the Leccinioideae based on different loci and more inclusive boletoid and sequestrate taxa. The addition of other DNA loci such as RPB1 and RPB2 will also help to resolve the generic relationships within the leccinoid clade more definitively.

The phylogenetic analyses also revealed a number of new species-level sequestrate lineages within Rossbeevera and Turmalinea with robust statistical support. Accordingly, we propose two new Rossbeevera species and four new species and one new subspecies of Turmalinea. The description of these new taxa almost doubles the number of species within the Rossbeevera-Turmalinea lineage, suggesting that this group is likely diverse and ecologically important in East Asia, Southeast Asia, and Australasia despite the fact that this lineage was only recently recognized (Lebel et al. 2012a, b). So far, Turmalinea accommodates only a few species (4 spp.) compared to Rossbeevera (10 spp.), and Octaviania (c. 30 spp.), but the long branches in the multi-gen e phylogenies (Fig. 2, 3) imply that there are more undescribed species yet to be discovered. It is also possible, however, that the long branches are the result of extinction of ancestral species. Collecting surveys of sequestrate fungi across extensive areas in East Asia will give further insight into the diversity of Turmalinea.

While Turmalinea spp. are presently known only from East Asia (e.g., China and Japan), species of Rossbeevera have also been reported from Australasia and Southeast Asia (Lebel et al. 2012a, Orihara et al. 2012a, Vernes & Lebel 2011). Orihara et al. (2012a) and this study (Fig. 2, 3) agree that the Chinese species R. yunnanensis forms the basal branch within Rossbeevera. Based on these results we postulate that the genera Turmalinea and Rossbeevera may have originated in China or SE Asia and then migrated independently into Japan (including the Ryukyu Archipelago).

Similarly, all the phylogenies shown in this study suggest that the Australasian Rossbeevera species diversified relatively recently and that they probably migrated from Asia only once. Since sequestrate fungi are thought to rely on animal mycophagy for spore dispersal (Claridge & May 1994, Fogel & Trappe 1978, Mas er 1978, Maser et al. 2008, Vernes & Dunn 2009), long-distance spore dispersal over ocean barriers would seem challenging. However, a biogeographic study by Hosaka et al. (2008) demonstrated that intercontinental, overseas dispersal has occurred separately in at least three truffle-like genera in the Hysterangiales, suggesting that overseas dispersal of truffles may be more common than previously thought. Field observations of truffle consumption by mammals indicate that Rossbeevera species are highly sought after as food. For example, Rossbeevera spores are among the most common spore types found in a diverse range of small Australian mammals (Lebel unpubl. data) and Rossbeevera spores have also been documented in mycophagous mammals from Papua New Guinea (Vernes & Lebel 2011). These observations suggest that intercontinental migration of Rossbeevera from Asia to Australasia may have been facilitated by mycophagous animals.

The sister sequestrate genera, Turmalinea and Rossbeevera, have several characteristics in common, including basidiospores with longitudinal ridges and fruitbodies that turn bluish upon bruising or exposure to air. However, Turmalinea species have firm, rubbery fruitbodies with a pulvinate sterile base and basidiospores with 5–10 longitudinal ridges whereas Rossbee vera species generally have softer fruitbodies with a reduced stipe-columella and basidiospores with 3–5 longitudinal ridges. In addition, three of the four Turmalinea species have brightly coloured, pinkish or yellow fruitbodies with yellow to orange rhizomorphs, whereas all Rossbeevera species have whitish to greyish white fruitbodies (Fig. 7). The one exceptional species...
within Turmalinea, the earliest diverging species T. mesomorpha, forms whitish fruitbodies with bluish discolouration similar to species of Rossbeevera. This parsimoniously suggests that the ancestor of the two genera had a whitish sequestate fruitbody with bluish discolouration and longitudinally ridged basidiospores as synapomorphies. Although significantly supported in the phylogenetic analyses, it is difficult to infer close evolutionary relationships between bolete genera (i.e., Leccinum and Leccinellum) and sequestate genera (i.e., Rossbeevera, Turmalinea, Octaviaria, and Chamonixia) in the leccinoid clade based on morphology. For example, basidiospores of Leccinellum and Leccinum are smooth and colourless to pale brown, whereas those of the sequestate taxa in Rossbeevera, Turmalinea, Chamonixia, and Octaviaria are distinctly ornamented and brown to blackish brown. In addition, the epigeous boletoid taxa in the leccinoid clade have cheilo- and pleurocystidia in their tissues (Den Bakker & Noordeloos 2005) while the sequestate taxa generally lack cystidia. The only distinct character that unites these genera is the reddish or bluish discolouration of fruitbodies, but this feature can also be found widely across both sequestate and epigeous boletoid taxa in the Boletaceae. Therefore, we suggest that the evolution of dark pigmentation and enhanced ornamentation of basidiospores as well as the loss of cystidia are likely features that are selected for during the transition from boletoid to sequestate fruiting form in the leccinoid clade. Future analyses using phylogenetic analysis in conjunction with molecular dating should examine the relationships between the micro- and macroscopic morphologies across the Boletales and particularly within poorly resolved groups within Boletaceae.

**Interpreting evolutionary history in light of topological discord among phylogenies**

The single-locus and combined phylogenies showed considerable topological discord at various degrees of divergence within Rossbeevera. The three single-locus nuclear trees exhibit different patterns of divergence among Japanese Rossbeevera spp. (Fig. 1). This topological incongruence can be explained by deep coalescence or incomplete lineage sorting (ILS) (Madison 1997). If this is true then sequences of ITS, nLSU, and EF-1α were divergent in the ancestral Rossbeevera species before speciation events that led to the extant Japanese and Australasian Rossbeevera spp. The *BEAST analysis estimates a Bayesian species tree that accounts for effects of ILS under the multispecies coalescent model (Heled & Drummond 2010). Most conflicting topologies in the three nuclear gene trees were not satisfactorily reconciled in the *BEAST species tree based on the three loci, but the sister relationship between R. eucyanea and R. cryptocyanea was strongly supported by the *BEAST species tree (Fig. 2). These results suggest that gene trees based on other loci may mislead interpretations of the relationship between the two species. In addition, although most species-level relationships in Rossbeevera were strongly supported in the combined phylogeny (Fig. 3), the species tree analysis suggests that these internal relationships must be viewed critically in light of the incongruence between the various datasets.

We also compared the phylogeny based on three nuclear loci (ITS, nLSU, and EF-1α) with an alternative topology based on two mitochondrial loci (ATP6 and mtSSU) (Fig. 5). The nuclear phylogeny showed no obvious phylogeographic divergence within individual taxa. In contrast, the mitochondrial tree showed molecular divergence that unexpectedly followed patterns of geography rather than morphology. The case was particularly striking within the Australasian Rossbeevera species where we detected little differentiation between species based on the three nuclear loci. In contrast, the mitochondrial genes largely exhibited a pattern of isolation by distance with geographically distant specimens also being genetically distant. Similar patterns were apparent in several species of Japanese Rossbeevera and Turmalinea (Fig. 5). Specimens of R. griseovelutina show considerable infraspecific divergence in the nuclear phylogeny. Specimens collected from western Honshu (the mainland of Japan) formed a single, homogenous clade based on the mitochondrial dataset whereas specimens from eastern Honshu and Amami-oshima Island (c. 600 km from Honshu) were genetically unique and more closely related to each other. In T. persicina, the nuclear phylogeny did not exhibit any obvious phylogeographic pattern, but the mitochondrial phylogeny suggested that specimens from Kyushu were slightly divergent from other Honshu specimens. These findings indicate that the infraspecific lineages in the genus Rossbeevera (including the Australasian species complex) may not be reproductively isolated, and that the infraspecific divergences in the mitochondrial genes are correlated to phylogeographic divergence rather than morphological and ecological differences.

Disagreement between the phylogenetic signals in nuclear vs mitochondrial loci have been documented for epigeous fungal genera and families such as the saproxylic genus Sparassis (Polyporales) (Dai et al. 2006), ectomycorrhizal genus Strobilomyces (Boletales) (Sato et al. 2007, Sato & Murakami 2008), and the Clavicipitaceae, which includes fungi with diverse trophic modes (Sung et al. 2007). However, studies of these fungi did not detect any obvious biogeographic pattern in the diversification of mitochondrial loci (ATP6 and/or COX3 genes). It is possible that the unique ecological constraints imposed on sequestate fungi (e.g. belowground fruiting, lack of active spore dispersal) may be correlated with geographical divergence in the mitochondrial phylogeny, but more data and comparisons with additional sequestate taxa and related epigeal taxa are clearly needed to resolve this question.

The comparison between the nuclear and mitochondrial phylogenies also revealed divergent topological patterns within infraspecific lineages of Rossbeevera. One R. griseovelutina specimen (TNS-F-36991) is resolved in the R. griseovelutina lineage 2 based on the nuclear markers (ITS, nLSU, EF-1α) but is grouped together with the members of the R. griseovelutina lineage 1 based on the mitochondrial markers (ATP6 and mtSSU) (Fig. 5). Since the other specimens of R. griseovelutina lineage 2 in the nuclear tree showed phylogenetic affinity in the mitochondrial tree, ILS between the two lineages is not likely. This conflict could be more reasonably explained by introgression of mitochondrial genes because both R. griseovelutina lineage 1 and lineage 2 are found in western Honshu where lineage 1 occurs in evergreen Castanopsis forests and lineage 2 is found in nearby deciduous Fagus-Quercus forests. It is possible that distribution of the two lineages overlaps in some areas in western Honshu, and this could cause the introgression of mitochondrial genes. It is not obvious, however, why the two individuals of the lineages 1 and 2 collected from Amami-oshima Island and Eastern Honshu, which are geographically distant from one another (c. 1 200 km), have almost identical ATP6 and mtSSU sequences. Given that the mitochondrial sequences of Rossbeevera tend to reflect geographic distance, transoceanic spore dispersal as well as gene introgression would be necessary to explain these topologies. The precise pattern of migration cannot be concluded thus far due to insufficient samples. However, additional sampling throughout the region might help to clarify the process of transoceanic spore dispersal of truffles in the future.

Several studies on the evolutionary biology of fungi and animals have detected similar incongruence between nuclear and mitochondrial datasets. Dai et al. (2006) demonstrated that three Sparassis species distinct in the nuclear phylogeny
shared identical mitochondrial ATP6 sequences. The authors inferred that this was due to clonal inheritance within the partly overlapping populations. Similarly, Robinson et al. (2001) found remarkably low divergence in ATP6 of Agaricus bisporus isolates with different mitochondrial haplotypes but they did not mention the possibility of ILS or introgression. Peters et al. (2007) examined the mitochondrial paralogy between gadwalls and falcated ducks and rejected the hypothesis of ILS based mainly on incongruence between the divergence time of the two species estimated by nuclear introns and mtDNA genes. Similar approaches and addition of sequences of R. griseovelutina may offer further insights about evaluation of ILS and introgression in Rossbeevera.

Although ILS seems unlikely in the case of the topological conflict within R. griseovelutina, it seems probable that ILS does account for the topological incongruence in R. paracyanea (Fig. 5). Both infraspecific lineages of R. paracyanea were from the same region (western Honshu, Japan), and Fig. 5 shows the infraspecific divergence in the mitochondrial two-locus phylogeny is deeper compared to that in the nuclear three-locus phylogeny. Although we cannot conclude any historical event that affects the topological incongruence based only on the small number of R. paracyanea samples, the parsimonious explanation is incongruence caused by ILS derived from mitochondrial polymorphism within the ancestor of the extant R. paracyanea lineages. Considering these cases of putative ILS between the nuclear and mitochondrial loci and the likely ILS found among the nuclear single-locus trees, it seems that ILS is not a rare event in this group of fungi. Furthermore, the frequent polymorphism in the nuclear loci of the Australasian Rossbeevera ‘spp.’ may be another example of the ongoing process of lineage sorting (Fig. 1).

Similar topological patterns to R. paracyanea were recovered in the relationship between R. cryptocyanea lineages 1 and 2 (Fig. 5). In this case, however, both ILS and introgression are possible causes of the topological discordance because the pattern of divergence in the mitochondrial tree also reflected geographical distances among the specimens. The distance between the two localities, Kakermom and Amami-oshima Islands, is c. 15 km, whereas Ishigaki Island is c. 650 km distant from Amami-oshima Island, and the Kerama Gap, which is known as a faunal gap of many terrestrial animals, crosses between them (Ota 1998). Therefore, it is also possible that the two different infraspecific lineages of R. cryptocyanea occurred sympatrically and that this caused introgression of mitochondrial genes.

Overall, the analysis of nuclear vs mitochondrial loci revealed that genetic distances in the mitochondrial tree generally correspond to geographic distance within each species-level lineage of Rossbeevera and Turmalinea. While some topological conflicts within Rossbeevera species may be the results of recent gene introgression, other conflicts strongly suggest the possibility of incomplete lineage sorting between closely related lineages. These examples indicate that careful, multifaceted characterization using both morphological and phylogeographical methods with nuclear and mitochondrial datasets is the key to delimit boundaries between infrageneric taxa. It is also possible that these types of complex evolutionary histories may be frequently overlooked in phylogenetic studies of many other fungal taxa due to insufficient sampling of individuals and due to the lack of precise topological comparisons between phylogenies based on different loci.

**Minisatellite-like insertion in ITS2 as a DNA barcode marker**

The ITS region has recently been adopted as the official molecular barcode for fungi (Chase & Fay 2009, Schoch et al. 2012). Although most fungi in Basidionymota have an ITS region (ITS1-5.8S-ITS2) that is approximately 500–650 nucleotides, PCR amplicons of ITS in Rossbeevera and Turmalinea species are remarkably large (e.g., ITS in T. persicina is about 1 100 base pairs). The extra length of this region is due to the presence of a minisatellite-like insertion within ITS2 (Table 2). ITS minisatellites are rare in fungi, but Den Bakker et al. (2004) reported that species of Leccinum, a species-rich bolete genus in the leccinoid clade, usually contain minisatellite-like insertions in ITS1. They demonstrated that the minisatellite-like insertions were highly divergent among infraspecific lineages of Leccinum and therefore not usable as a phylogenetic marker at or above species level. Orihara et al. (2012b) briefly mentioned the presence of insertions in the ITS2 region of species in Octavania subg. Fulvoglobus, but did not characterize the DNA sequences of the insertions.

In this study we used the Automatic Barcode Gap Discovery (ABGD) software (Puillandre et al. 2012), to infer species boundaries within Rossbeevera and Turmalinea. The barcode gap analysis and ML phylogeny clearly showed that the minisatellite-like insertions of Rossbeevera and Turmalinea were highly conserved at the subspecies-, species- and genus levels and that these minisatellites have higher resolution in discriminating the boundary between intergeneric-level, infraspecific-level, and interspecific-level divergence than the rest of the ITS region (Fig. 6). The Australasian Rossbeevera spp., however, were not partitioned at the species-level boundary estimated by the analysis (Fig. 6c). This suggests that these previously described species are likely geographic variants that may still be in the process of speciation, despite the fact that several specimens from different regions of Australasia show remarkable morphological divergence (Lebel et al. 2012a).

Although the exact origin of these ITS insertions is unclear, it is likely that they originated within the leccinoid clade since no other group in the Boletales appear to have them. Although we have not characterized the structure and function of the minisatellite-like insertions, the insertion sequences of Rossbeevera and Turmalinea are highly informative and can thus be used efficiently for DNA barcoding of fruitbodies or environmental sequences. We caution, however, that the extended ITS length in Rossbeevera and Turmalinea may actually reduce the likelihood that these DNA sequences will be recovered from environmental samples since longer fragments are sometimes discriminated against in mixed pools of DNA (Huber et al. 2009, Belemmain et al. 2010).

**Conclusions**

Our finding of a new sequestrate genus, Turmalinea, reinforces the fact that the leccinoid clade is remarkably rich in sequestrate fungi. The nuclear three-locus phylogenies strongly supported the distinctness of the four new species and one new subspecies of Turmalinea as well as the two new species of Rossbeevera despite the fact that precise infrageneric relationships within Rossbeevera remain unclear. Topological comparison between nuclear and mitochondrial phylogenies documented significant infraspecific biogeographical diversification of two mitochondrial loci in the Australasian Rossbeevera species but very low differentiation in nuclear loci. Pairwise comparison among nuclear and mitochondrial trees further suggested that mtDNA introgression and ILS have occurred within multiple inter- and infraspecific lineages of Rossbeevera. Finally, we used the recently developed barcode gap analysis, ABGD, to demonstrate that the minisatellite-like insertion found in the ITS2 of Rossbeevera and Turmalinea is highly informative for identifying infra- and interspecific and intergeneric divergence. Although the ABGD approach has only rarely been used to delimit species of fungi, our results suggest that this method holds great promise for future studies of molecular identification in fungi, particularly since the ITS region has been adopted as
the official fungal barcode region (Chase & Fay 2009, Schoch et al. 2012).
In a few lineages of Rossbeevera and Turmalinea, we were not able to include multiple infraspecific OTUs in the phylogenetic analyses because these sequestrate fungi are mostly hypogeous and they are rarely collected. Moreover, we know of no DNA sequences from R. mucora or R. bispora because all of the available specimens were collected too long ago and some of them are fragmentary. We hope that in the near future comprehensive taxon sampling of both epigeous and sequestrate leccinoid taxa will reveal more precise phylogenetic and phylogeographic relationships and evolutionary processes.

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