

Russula ryukokuensis sp. nov., an Outstanding Species of the Genus *Russula* (Russulaceae) Having Minute Basidiomata from Japan

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Abstract. *Russula ryukokuensis*, a new species of *Russula* is described from mixed broad-leaved trees and coniferous forests in Japan based on macro- and microscopic features with molecular data. *Russula ryukokuensis* is characterized by small-sized, orange to reddish basidiomata, convex to plano-convex pileus with shallowly tuberculate to sulcate margin, and whitish to slightly light orange lamellae with strongly acid taste. Detailed macro- and micro-morphological descriptions of *R. ryukokuensis* are given, and its taxonomic and phylogenetic positions were confirmed using DNA sequences. Morphological observations and molecular phylogenetic analysis revealed that this species belongs to the subsection *Emeticinae* of the section *Russula* in the subgenus *Russula*.

Keywords: Agaricomycetes, ITS and LSU sequences, mycobiota, phylogeny, Russulales, taxonomy.

Introduction

Species of the genus *Russula* Pers. are widely distributed in diverse forests from the tropical to the frigid zones of all continents except Antarctica and are well-known as ectomycorrhizal fungi (Kirk *et al.*, 2008). Currently, ca. 100 species of the genus *Russula* are recorded in Japan (Imazeki and Hongo, 1989; Ikeda, 2013). Recently, numbers of molecular studies have been conducted using internal transcribed spacer (ITS) region and nuclear large subunit (LSU) gene of ribosomal DNA sequences to determine taxonomic and phylogenetic positions of *Russula* species

(Atri *et al.*, 1993, 1997; Buyck and Horak, 1999; Miller and Buyck, 2002; Chou and Wang, 2005; Das and Sharma, 2005; Buyck *et al.*, 2008, 2018; Das *et al.*, 2010, 2013a, 2013b, 2014, 2017; Shin, 2010; Dutta *et al.*, 2015; Li *et al.*, 2015; Ghosh *et al.*, 2016, 2017; Melera *et al.*, 2016; Jabeen *et al.*, 2017). However, species diversity of Japanese *Russula* inferred from molecular phylogeny is still not comprehensively studied. During recent taxonomic and phylogenetic studies on Japanese *Russula* (Shimono *et al.*, 2004, 2014, 2018), we collected remarkably small (4–10 mm across), orange to reddish basidiomata with strongly acid taste of the genus in central to western parts of Honshu.

The aim of this study was to clarify the taxo-

nomic and phylogenetic positions of this outstanding *Russula* species having minute basidiomata. In this paper, we describe this *Russula* species as a new to science based on morphological observations and molecular phylogenetic analysis using rDNA ITS and LSU sequences.

Materials and Methods

Preparation of specimens

We collected *Russula* specimens having orange to reddish, minute basidiomata with acid taste in Shiga, Mie, Hyogo and Hiroshima Prefectures, central to western Honshu of Japan during our fieldwork from 2009 to 2020. Additionally, for molecular phylogenetic analysis, specimens of *R. decolorans* (Fr.) Fr., *R. kansaiensis* Hongo and *R. omiensis* Hongo collected from our fieldwork in Japan were also investigated (Table 1). Specimens examined in this study were deposited at the herbaria of the National Museum of Nature and Science (TNS), Tsukuba, Japan and the Osaka Museum of Natural History (OSA), Osaka, Japan.

Macro-morphological Observations

Each specimen of *Russula* was photographed, and macroscopic observation was conducted in fresh state. Morphologies of the fruiting bodies were recorded according to Ikeda (2013), Imazeki and Hongo (1989) and Shimono *et al.* (2014, 2018). In this paper, infrageneric classification

of *Russula* followed Romagnesi (1985, 1987), with some references to Bon (1987, 1988), Singer (1986), Sarnari (1998, 2005) and Buyck *et al.* (2018). Color annotations of fresh materials were determined based on Kornerup and Wanscher (1978). Fresh basidiomata of each specimen were dried using a food dehydrator (Snackmaster Express FD-61, Nesco/American Harvest, WI, USA) under 46°C.

Micro-morphological observations

Microscopic features of specimens were observed from dried material mounted in 10% KOH and Melzer's reagent, using an Olympus BX-53 light microscope (Olympus, Tokyo, Japan) under Nomarski interference contrast. More than 20 randomly selected basidiospores were measured under a light microscope at 1000× magnification. For basidiospores, the factor Q (mean of quotient of length and width in any one spore) was also calculated to indicate spore shape. In addition, the surface features of basidiospores were observed by scanning electron microscopy (SEM). For SEM, a small portion from pileus was put onto double-sided adhesive tape on a specimen holder and coated with platinum-palladium using a JFC-1600 Ion Sputter Coater (JEOL, Tokyo, Japan). Specimens were examined with a JSM-6480LV SEM (JEOL) operating at 20 kV.

Table 1. List of sequences newly generated from Japanese specimens of *Russula*

Species	Sampling date	Collector	Locality	Voucher number	GenBank accession number*	
					ITS	LSU
<i>Russula decolorans</i>	2002 Aug. 24	Y. Goto	Japan: Yamanashi	OSA-MY-9216	Not obtained	LC269001
<i>R. kansaiensis</i>	2011 Nov. 9	T. Kasuya	Japan: Ibaraki	TNS-F-43739	MN989318	MN989319
<i>R. omiensis</i>	1996 Apr. 20	Y. Shimono	Japan: Kyoto	OSA-MY-1763	LC269002	LC269002
<i>R. omiensis</i>	2013 Mar. 24	Y. Shimono	Japan: Kyoto	OSA-MY-9217	LC269003	LC269003
<i>R. omiensis</i>	2013 Mar. 24	Y. Shimono	Japan: Kyoto	OSA-MY-9218	LC269004	LC269004
<i>R. omiensis</i>	2013 Mar. 24	Y. Shimono	Japan: Kyoto	OSA-MY-9219	LC269005	LC269005
<i>R. ryukokuensis</i>	2009 Oct. 11	Y. Kotera	Japan: Shiga	OSA-MY-9225	LC269011	LC269011
<i>R. ryukokuensis</i>	2013 Jul. 20	T. Ueda	Japan: Shiga	TNS-F-70424	MH037291	MH037293
<i>R. ryukokuensis</i>	2016 Sep. 19	T. Kasuya	Japan: Shiga	TNS-F-70425**	MH037292	MH037294

*Identical accession numbers for ITS and LSU indicate a single rDNA sequence containing both regions.

** Holotype

DNA extraction, PCR, and sequencing

DNA extraction, PCR and DNA sequencing were carried out according to the methods introduced by Shimono *et al.* (2004, 2007, 2014, 2018). Briefly, fungal DNA was extracted from the lamellae of fresh basidiomata using Indicating FTA Cards (Whatman International Ltd, Maidstone, UK) based on the manufacturer's protocol. PCR amplifications of the ITS (ITS1-5.8S-ITS2) region and the LSU gene of rDNA were carried out using one prepared FTA disc 2 mm diam., according to the manufacturer's instruction. The ITS1F/ITS4B (Gardes and Bruns, 1993) and the ITS1/ITS4 (White *et al.*, 1990) primer pairs for the rDNA ITS region, and the BN1/TW14 (Shimono *et al.*, 2004) and the NL1/NL4 (Mori *et al.*, 2000) primer pairs for the rDNA LSU gene were used for amplification and sequencing. PCR reactions were performed using KOD FX Neo DNA polymerase (Toyobo, Tokyo, Japan) in 25 mL reaction volumes containing 5 µl of 0.2 mM dNTP, 12.5 µl of PCR buffer, and 0.5 U KOD FX Neo. PCR conditions were 94°C for 2 min, followed by 40 cycles at 98°C for 10 s, 55°C for 30 s, 68°C for 1 min, and a final 6 min at 68°C. The DNA sequencing was performed at SolGent Co. Ltd. (Daejeon, South Korea) using an ABI 3700 automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA, USA).

Phylogenetic analyses

A total of eight ITS and nine LSU sequences were newly generated from our specimens (Table 1). Additional 44 ITS and 25 LSU sequences were retrieved from the GenBank and UNITE databases for phylogenetic analysis (Table 2). DNA sequences were initially aligned using Muscle v.3.6 (Edgar, 2004a, 2004b), followed by manual alignment in the data editor of BioEdit ver. 7.0.1 (Hall, 1999). On the basis of Akaike Information Criteria values (Akaike, 1974), we chose a general time-reversible model with gamma-distributed rate heterogeneity and a proportion of invariant sites (GTR+G+I) as the optimal substitution model for analysis of the

ITS and LSU datasets. Phylogenetic analysis was performed in MEGA 7 software (Kumar *et al.*, 2016) with the maximum likelihood (ML) method. For the ML analysis, clade robustness was assessed using a bootstrap analysis with 1000 replicates (Felsenstein, 1985). Sequences of *R. decolorans* and *R. lilacea* Quél. which were strongly supported as the sister to the subgenus *Russula* Pers. emend. Romagn. in previous studies (Shimono *et al.*, 2004, 2018), were selected for outgroups. The final alignment is available from TreeBASE (<http://www.treebase.org/>) as a NEXUS file under the accession number 25896.

Results and Discussion

Phylogenetic analyses

Among Japanese *Russula* specimens having orange to reddish, minute basidiomata with acrid taste, ITS and LSU sequences of three samples collected from Ryukoku University Forest in Shiga Prefecture were successfully generated (Table 1). The ITS dataset had an aligned length of 769 characters including gaps consisting of 49 ingroups and three outgroups. The maximum likelihood analysis of the ITS dataset resulted in one ML tree with the highest log likelihood (−4258.54). The resulting ML topology is shown in Fig. 1. From the ML analysis of the ITS dataset, 49 sequences of *Russula* were divided into two major clades A and B (Fig. 1). Species included in the major clade A are members of the section *Russula* Pers. of the subgenus *Russula*, while the major clade B contains species belonging to the subgenus *Tenellula* Romagn. (Romagnesi, 1985). Major clade A corresponded with Clade 5 of Miller and Buyck (2002), Clade A of Li *et al.* (2015) and Clade VII of Buyck *et al.* (2018). The LSU dataset had an aligned length of 610 characters including gaps consisting of 32 ingroups and two outgroups. The maximum likelihood analysis of the LSU dataset resulted in one ML tree with the highest log likelihood (−1728.29). The resulting ML topology is shown in Fig. 2. Although the number of taxa in LSU dataset is limited than that of

Table 2. List of *Russula* sequences retrieved from the GenBank and UNITE databases and used in the present phylogenetic analyses

Species	Locality	Voucher number	Accession number*	
			ITS	LSU
<i>Russula atrorubens</i> Quél.	Canada	TU: 101718	KX579812	No data
<i>R. aureorubra</i> K.Das, A.Ghosh, Baghela & Buyck	India	KD 16-58	MF667557	No data
<i>R. aquosa</i> Laclair	Canada	NL 15.10.04.av04	KX579806	No data
<i>R. aquosa</i>	Estonia	TU: 101712	KX579811	No data
<i>R. aquosa</i>	USA	TENN: 67620	No data	KT933831
<i>R. aquosa</i>	Estonia	TU: 101708	No data	KX812873
<i>R. betularum</i> Hora	USA	AM04	GU220371	No data
<i>R. betularum</i>	USA	TENN: 67623	KT933969	No data
<i>R. betularum</i>	Estonia	TAA: 185042	AJ534937	No data
<i>R. betularum</i>	Poland	PAN: 540	KM085392	No data
<i>R. betularum</i>	Poland	sporocarp	JF834200	No data
<i>R. betularum</i>	USA	TENN: 067623	No data	KT933829
<i>R. betularum</i>	USA	r-09003	No data	JF834520
<i>R. chiui</i> G.J.Li & H.A.Wen	China	HMAS: 264832	EF225489	No data
<i>R. cremoricolor</i> Earle	Canada	UBC: F16286	EU486452	EU486452
<i>R. consobrina</i> Fr.	Estonia	TU: 118108	UDB011223	No data
<i>R. decolorans</i>	Norway	OSA-MY-7782	LC192760	No data
<i>R. decolorans</i>	Germany	GENT: FH-12-196	No data	KT933853
<i>R. decolorans</i>	Sweden	mycorrhizal roots	AY194601	No data
<i>R. cf. emetica</i> (Schaeff.) Pers.	Canada	UBC: F14306	AY228350	AY228350
<i>R. emetica</i>	Germany	TUB: lw81	AF418619	No data
<i>R. emetica</i>	Scotland	DG 18	JQ888196	No data
<i>R. emetica</i>	Canada	UBC: F20370	KC581346	KC581346
<i>R. emetica</i>	Sweden	UPS: UE05.10.2003-11	No data	DQ421997
<i>R. emetica</i>	Estonia	TU: 106402	No data	KX812896
<i>R. fellea</i> Fr.	Germany	TUB: hue218	UDB000345	No data
<i>R. fellea</i>	Germany	TUB: ue114	No data	AF325307
<i>R. fragilis</i> (Pers.) Fr.	Spain	roots	KY681458	No data
<i>R. fragilis</i>	Germany	GENT: FH-12-197	KT933993	No data
<i>R. fragilis</i>	Germany	GENT: FH-12-197	No data	KT933854
<i>R. griseascens</i> (M.Bon & Gaugué) L.Marti	Finland	TU: 101890	UDB016038	No data
<i>R. gracillima</i> Jul.Schäff.	Sweden	UPS: UE23.08.2004-14	DQ422004	DQ422004
<i>R. kansaiensis</i>	Japan	OSA-MY-1764	No data	AB154754
<i>R. laccata</i> Huijsman	Canada	UBC: F18877	HQ604844	HQ604844
<i>R. atropurpurea</i> (Krombh.) Britzelm.	Italy	3046	JF908660	No data
<i>R. atropurpurea</i>	France	Champ-22	KX449425	No data
<i>R. lilacea</i> Quél.	Slovakia	BartBuyck07.213	JN944005	No data
<i>R. luteotacta</i> Rea	Germany	GENT: FH-12-187	KT933991	No data
<i>R. odorata</i> Romagn.	Slovakia	BartBuyck07.186	JN944010	No data
<i>R. odorata</i>	Canada	FFP: 814	JQ711877	No data
<i>R. mairei</i> Singer	Germany	TUB: Lw223	AF418620	No data
<i>R. mairei</i>	Czech	PRM: 922170	MG687365	No data
<i>R. mairei</i>	Germany	GENT: FH-12-262	No data	KT933874
<i>R. persicina</i> Krombh.	Sweden	SJ98044	AF506463	AF506463
<i>R. persicina</i>	Sweden	UPS: UE21.09.2003-01	DQ422019	DQ422019
<i>R. puellaris</i> Fr.	Estonia	TU: 101839	No data	KX812887
<i>R. puellaris</i>	Slovakia	SAV: F-4224	KY582720	No data
<i>R. puellaris</i>	Russia	264	MH248054	No data
<i>R. puellaris</i>	Russia	562	KP783461	No data
<i>R. puellaris</i>	Estonia	UBC:F19703	No data	HQ604852
<i>R. puellaris</i>	Sweden	TUB: Hue83	No data	AF325315
<i>R. sanguinea</i> Fr.	Germany	GENT: FH-12-240	KT934008	No data
<i>R. sanguinea</i>	Italy	982	JF908649	No data
<i>R. sanguinea</i>	Montenegro	KK5.1	KY322542	No data
<i>R. sanguinea</i>	Germany	GENT: FH-12-240	No data	KT933869
<i>R. sanguinea</i>	Sweden	UPS: F553121	No data	KX812901
<i>R. sanguinea</i>	Switzerland	440/BB 07.319	No data	KU237503
<i>R. versicolor</i> Jul.Schäff.	Canada	FFP: 1156	JQ711937	No data
<i>R. versicolor</i>	Czech	PRM: 935921	MG687334	No data
<i>R. versicolor</i>	Germany	GENT: FH-12-259	No data	KT933873
<i>Russula</i> sp.	China	ECM183	JQ991808	No data
<i>Russula</i> sp.	Japan	C33	AB594961	No data

*Identical accession numbers for ITS and LSU indicate a single rDNA sequence containing both regions.

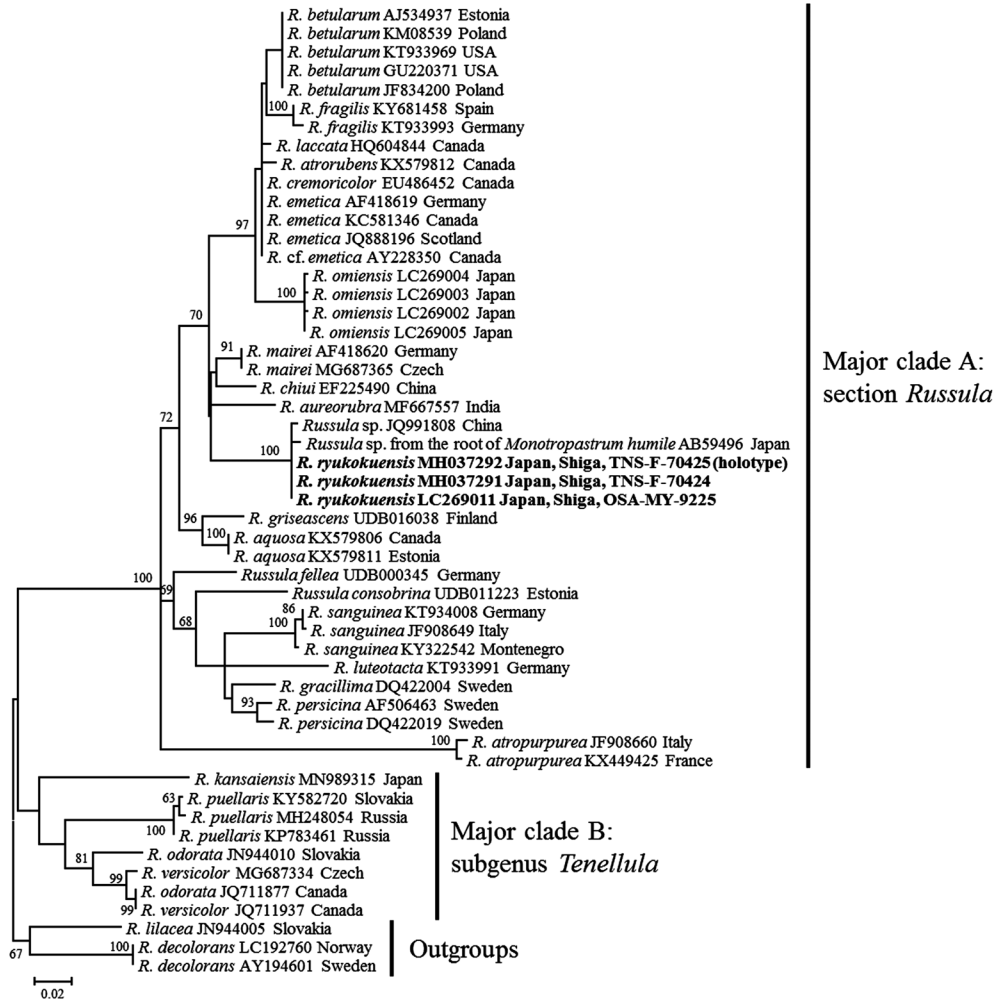


Fig. 1. A phylogenetic tree of ITS sequences of selected *Russula* species constructed by ML method, inferred by using GTR + G + I model. Bootstrap values greater than 60% are shown along the nodes in the topology. Scale bar indicates the number of substitutions per site.

ITS, the phylogenetic tree of LSU is almost identical in topology of ITS.

From the results of the present analyses of ITS and LSU datasets, sequences newly generated from the *Russula* specimens collected from Ryukoku University Forest in Shiga Prefecture, Japan, described as a new taxon here, constituted a distinct monophyletic group in a major clade A with strong bootstrap supports in both ITS (100%; Fig. 1) and LSU (99%; Fig. 2). Sequences generated from above Japanese *Russula* specimens are identical in both loci, and they were distinct from those of the other mem-

bers of the section *Russula*. In the analysis of the ITS sequences (Fig. 1), distinct monophyletic group composed of these *Russula* specimens also contains sequences of the ectomycorrhizal root tip from China (JQ991808) and the root sample of *Monotropastrum humile* (D. Don) H. Hara from Mie Prefecture, Japan (AB594961; Matsuda *et al.*, 2011). According to the results of the ITS analysis, sister group of Japanese specimens was composed of *R. mairei* Singer, and two *Russula* species having small-sized basidiomata: *R. chiui* G.J. Li & H.A. Wen and *R. aureorubra* K. Das, A. Ghosh, Baghela & Buyck (Fig. 1). *Russula*

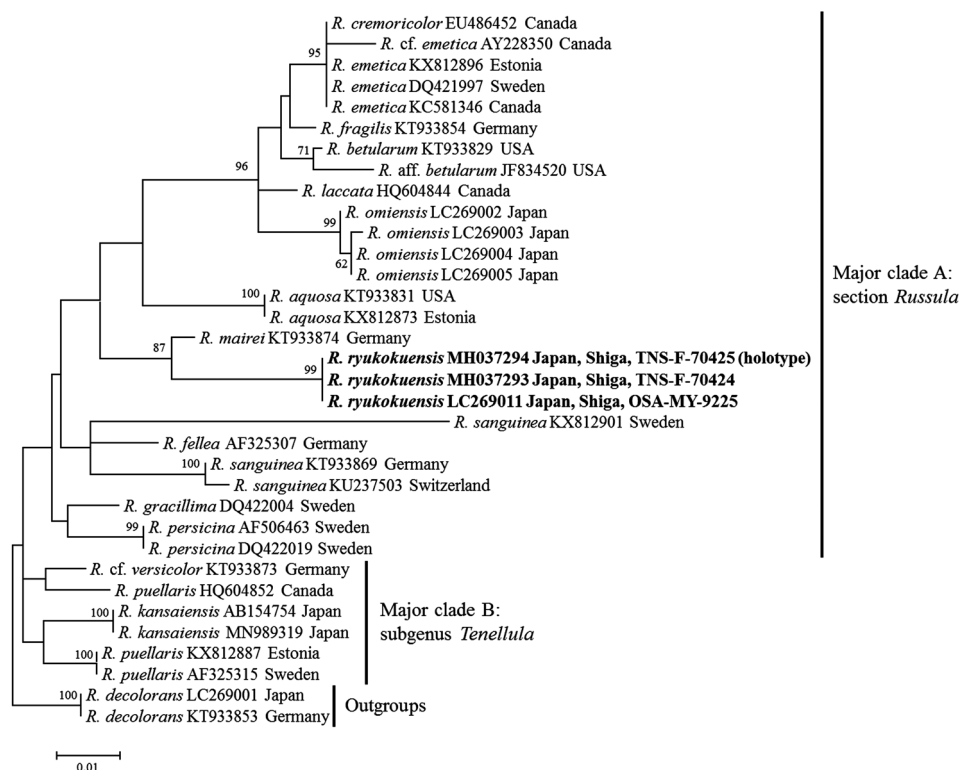


Fig. 2. A phylogenetic tree of LSU sequences of selected *Russula* species constructed by ML method, inferred by using GTR + G + I model. Bootstrap values greater than 60% are shown along the nodes in the topology. Scale bar indicates the number of substitutions per site.

mairei, *R. chiui* and *R. aureorubra* are members of the subsection *Emeticinae* Melzer & Zvára in the section *Russula*.

Morphological Observations

The combination of brightly reddish-tinged pileus, strongly acrid-tasting lamellae, white spore print, and septate pileocystidia assigned the Japanese *Russula* specimens forming orange to reddish and minute basidiomata into the subsection *Emeticinae* of the section *Russula* (Romagnesi, 1985, 1987). However, the specimens clearly differ from the other members of the subsection *Emeticinae* in their remarkably small-sized, orange red to deep orange pileus, shallowly tuberculate to sulcate pileal margin, slightly light orange lamellae, and reddish orange stipe which is translucent when moist. Therefore, these *Russula* specimens should be treated as a

new taxon inferred from both phylogenetic and morphological evidences; morphological details are presented in the taxonomy part.

Taxonomy

***Russula ryukokuensis* Shimono & T.Kasuya, sp. nov.**

(Figs. 3, 4)

MycoBank no.: MB824905.

Diagnosis: *Russula ryukokuensis* is well characterized by small-sized (4–10 mm across), orange red to deep orange, convex to plano-convex pileus with indented center and shallowly tuberculate to sulcate margin, whitish to slightly light orange lamellae with strongly acrid taste, translucent when moist, reddish orange stipe, and warty ornamentation with reticulum of basidiospore surface.

Type: —JAPAN, Shiga Pref., Otsu, Seta, Ryu-

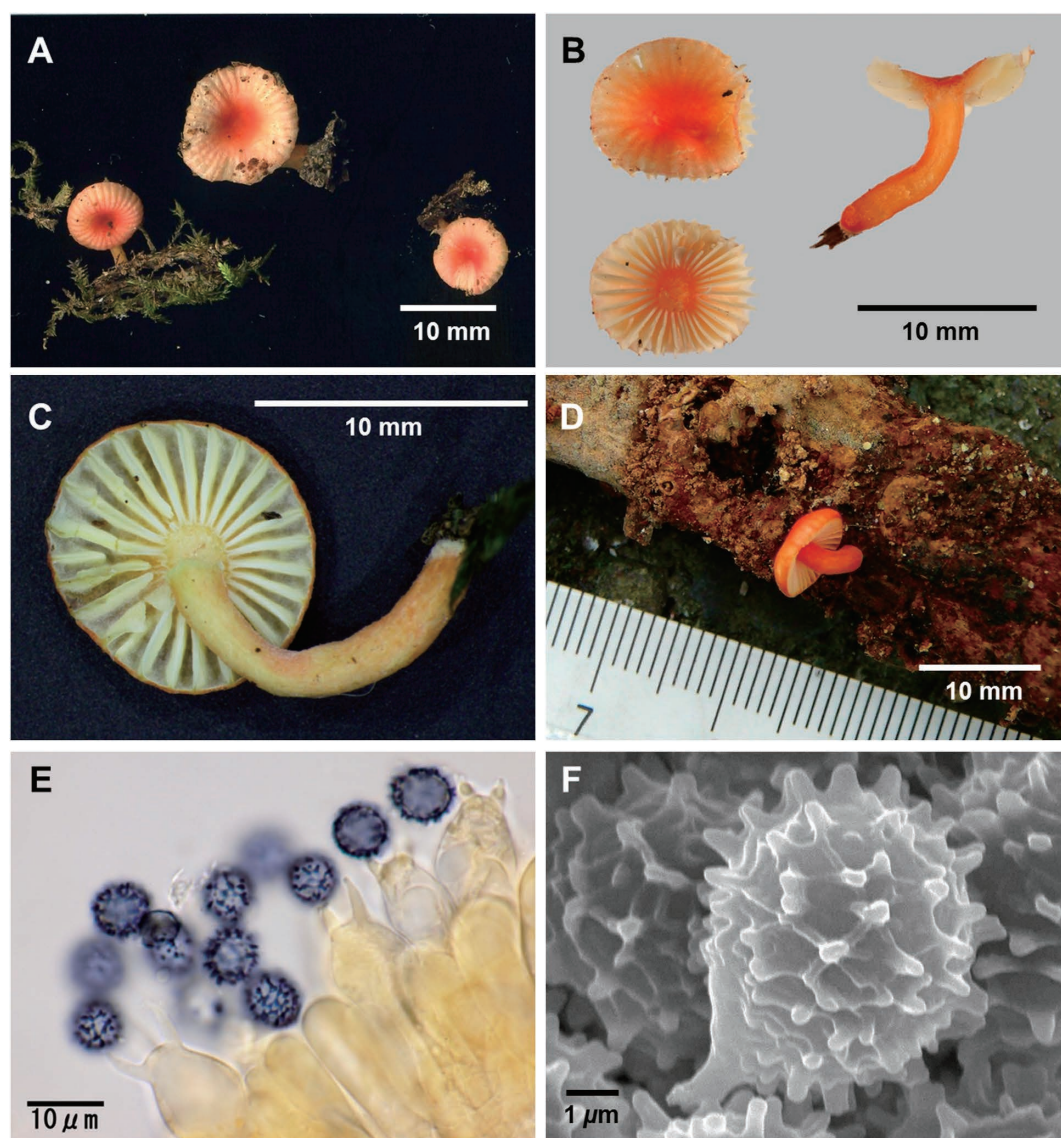


Fig. 3. Morphological features of *Russula ryukokuensis*. A. Pileal surface of basidiomata growing on the decayed wood (TNS-F-70424). B. Pileal surface, lamellae and a vertical section of basidiomata (OSA-MY-9225). C. Lamellae and a stipe of a basidioma (TNS-F-70425). D. A basidioma growing on decayed wood in the natural habitat (TNS-F-70424). E. Basidiospores in Melzer's reagent (OSA-MY-9225). F. A basidiospore under SEM (TNS-F-70425). Photographs C and F by T. Kasuya, B and E by M. Taniguchi, A and D by T. Ueda.

koku University Forest, Gen-nai Pass, ca. 160 m a.s.l., 19 September 2016, *Taiga Kasuya* (holotype TNS-F-70425).

Gene sequences ex-holotype: MH037292 (ITS), MH037294 (LSU).

Etymology: “*ryukokuensis*” refers to the type locality, Ryukoku University Forest.

Pileus (Fig. 3A–B) very small to small-sized, 4–10 mm across, parabolic when young, then convex with indented in the center, becoming plano-convex at maturity; margins smooth, incurved when young, becoming straight to uplifted and shallowly tuberculate to sulcate at maturity; surface dry, slightly matte, orange red

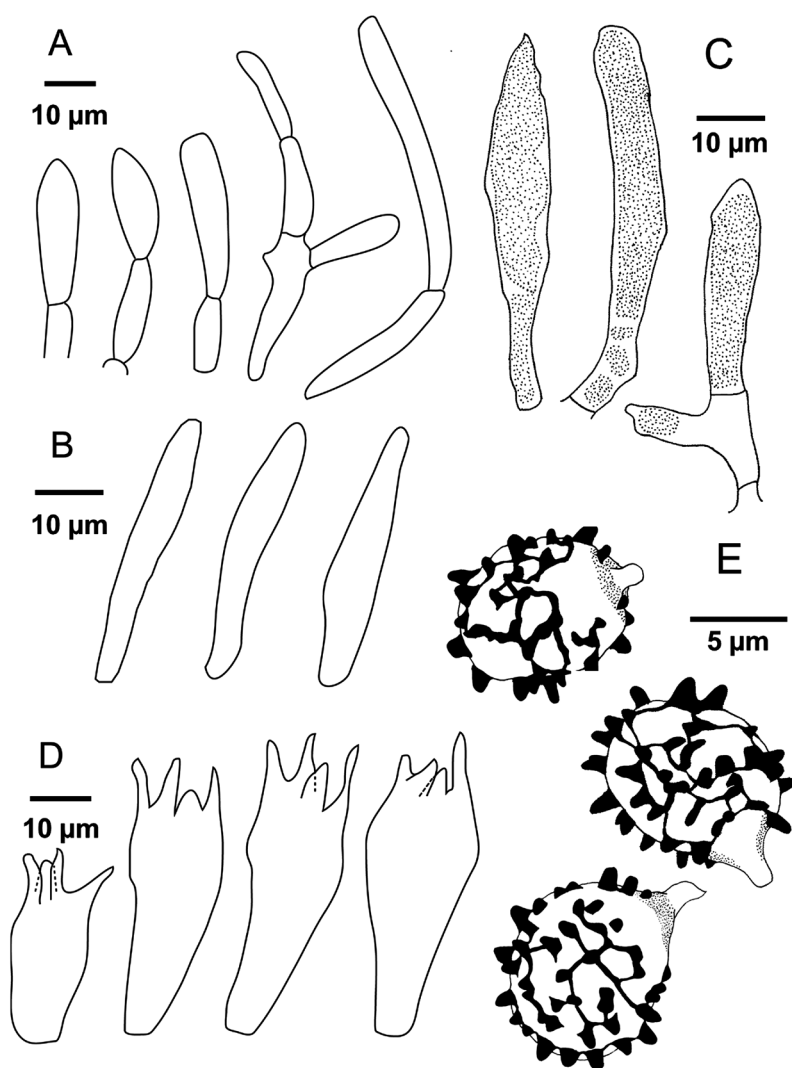


Fig. 4. Microscopic features of *Russula ryukokuensis*. A. Pileocystidia (OSA-MY-9225). B. Cheilocystidia (OSA-MY-9225). C. Pleurocystidia (TNS-F-70425). D. Basidia (OSA-MY-9225). E. Basidiospores (TNS-F-70425). Drawing by T. Kasuya.

(8A8, 7A8, 9A8) when young, then yellowish orange (8A4) in the center and deep orange (9A8) at maturity, later becoming whitish; context soft, up to 0.2 mm thick at the center, flesh light orange (6A4), odor indistinct, taste acrid. **Lamellae** (Fig. 3B–C) up to 0.2 mm broad, 21–31 (–43)/one basidioma, whitish, pale yellow to occasionally light orange (6A4), regular, adnate, distant to subdistant, edges entire, taste very acrid. **Lamellulae** absent. **Stipe** (Fig. 3C–D) 5–8 (–13) $\times$ 0.7–2 mm, central, equal,

translucent when moist, finely longitudinally striate, reddish orange (7A8, 7A5), base covered with whitish mycelium especially when young, texture soft, interior spongy to hollow.

Pileocystidia (Fig. 4A) abundant, cylindrical to clavate, 7–12 μ m across, septate, with partly branched hairs, hyaline to pale yellow in 10% KOH. **Cheilocystidia** (Fig. 4B) 53–54.5 $\times$ 7–10 μ m, Me = 53.6 $\times$ 8.5 μ m, cylindrical to fusiform, hyaline to pale yellow in 10% KOH. **Pleurocystidia** (Fig. 4C) 23–70 $\times$ 4–12 μ m, Me =

41.1 × 7.4 μm, cylindrical to fusiform, sometimes mucronate with a small appendage at apices, hyaline to pale yellow in 10% KOH. **Basidia** (Fig. 4D) 40–48 × 13–14.5 μm, Me = 44 × 13.6 μm, clavate, 4-spored, hyaline to slightly pale yellow in 10% KOH. **Basidiospores** (Fig. 3 E–F and Fig. 4E) 6.7–7.4 × 5.4–6.7 μm, Me = 6.9 × 6.1 μm, Me Q = 1.14, subglobose, surface ornamented, ornamentation consisted of prominently large, conical warts up to 1.5 μm high and reticulations connecting among warts, amyloid in Melzer's reagent, apiculus prominent, up to 2.5 μm long.

Habitat: Solitary, small groups or gregarious on damp to moist humus, on rich soil covered with mosses or on decayed wood in mixed forests of broad-leaved trees and conifers dominated by *Quercus glauca* Thunb., *Q. serrata* Murray, *Castanopsis cuspidata* (Thunb.) Schottky and *Cryptomeria japonica* (Thunb. ex L.f.). Summer (July) to early winter (December).

Additional specimens examined: JAPAN, Shiga Pref., Otsu, Seta, Ryukoku University Forest, 11 October 2009, Yuzo Kotera (OSA-MY-9225); same place, 12 September 2010, Yoshito Shimono (OSA-MY-9226); same place, 20 July 2013, Toshiho Ueda (TNS-F-70424); Mie Pref., Kameyama, Washiyama, 1 December 2016, Masahito Taniguchi (OSA-MY-9228); Hyogo Pref., Kobe, Kita-ku, Yamada-cho, Shimo-tanigami, 21 July 2019, Shohei Wada (TNS-F-70990); same place, 30 July 2020, Shohei Wada (TNS-F-70991); Hiroshima Pref., Hatsukaichi, Mominoki Forest Park, 2 August 2016, Masahito Taniguchi (OSA-MY-9227).

Known distribution: Japan, Honshu: Shiga, Mie, Hyogo and Hiroshima Prefectures.

Japanese name: *Ryukoku-hime-benitake* (Ryukoku small brittlegill, newly proposed here).

Comments. From morphological observations and phylogenetic analyses, we put *R. ryukokuensis* into the subsection *Emeticinae* of the section *Russula* (Romagnesi, 1985, 1987). However, *R. ryukokuensis* is easily distinguished from the other members of this subsection by its very small-sized basidiomata, orange red to deep

orange pileus, and reddish orange, translucent stipe when moist. From above morphological characters, *R. ryukokuensis* is a unique taxon among the subsection *Emeticinae*. An Indian species, *R. aureorubra*, is also unique among the subsection *Emeticinae* because of its entirely yellow color of the basidiomata although it shares with the other species a pileus that is tinged with red (Das *et al.*, 2017). Color of *R. aureorubra* is rather similar to that of *R. ryukokuensis* because pileus of both species tinged with orange red to yellowish red. However, *R. aureorubra* has quite many lamellulae (Das *et al.*, 2017), whereas *R. ryukokuensis* completely lacks them (Fig. 3C). Sizes of basidiomata are also clearly different among both species; pileus of *R. aureorubra* is larger (28–40 mm in diameter; Das *et al.*, 2017) than *R. ryukokuensis*. Chinese species belonging to the subsection *Emeticinae*, *R. chiui* also has small-sized basidiomata with brightly red-tinged pileus (Li *et al.*, 2015). However, *R. chiui* is clearly distinguishable from *R. ryukokuensis* by its whitish to pale yellowish stipe and larger size of pileus (30–48 mm in diameter; Li *et al.*, 2015).

Morphologies of *R. ryukokuensis* share with the other Japanese species, *R. kansaiensis*, in their small-sized basidiomata, surface features of pileus, and translucent stipes when moist. However, lamellae of *R. ryukokuensis* are pale yellow to occasionally light orange and their taste is strongly acrid, whereas *R. kansaiensis* has cream to yellowish lamellae with mild taste (Hongo, 1979). Furthermore, surface structures of basidiospores of both species are also different. Surface ornamentation of basidiospores in *R. kansaiensis* is composed of only conical warts (Hongo, 1979), while those of *R. ryukokuensis* have warty ornamentation with reticulum in surfaces (Figs. 3E–F, 4E). Phylogenetic positions of these two species are also quite different. *Russula kansaiensis* is placed in the major clade B detected by the present phylogenetic analyses (Figs. 1, 2). Major clade B contains several species belonging to the subgenus *Tenellula*, which have cream to yellowish lamellae with mild tastes. Moreover, species of the subgenus *Tenel-*

lula have only warts or with few, incomplete reticulum on surfaces of basidiospores (Bon, 1987; Romagnesi, 1985). From morphological characteristics and phylogenetic position, *R. kansaiensis* is placed in the subgenus *Tenellula*, and it is clearly different species to *R. ryukokuensis*.

The family Russulaceae includes the genera *Russula* Pers., *Lactarius* Pers., *Lactifluus* (Pers.) Roussel and *Multifurca* Buyck & V.Hofst (Buyck *et al.*, 2008, 2010). Many species in these genera are believed to be mycorrhizal members forming ectomycorrhizas in fine roots of host trees from the tropical to temperate forests (Henkel *et al.*, 2000). Basidiomata of *R. ryukokuensis* were frequently collected on decayed wood (Fig. 3A and D) of broad-leaved trees such as *Quercus glauca* and *Q. serrata* in Japan. Although the lignatile nature of *R. ryukokuensis* is striking and it seems to be saprotrophic, ITS sequences of *R. ryukokuensis*, the ectomycorrhizal root tip from China (JQ991808) and the root sample from Japanese *Monotropastrum humile* (AB594961; Matsuda *et al.*, 2011) constitute a monophyletic group in the present phylogenetic analysis (Fig. 1). Several species of *Russula* produce their basidiomata in elevated positions on living trees or rotting wood. Heim (1970) noted such basidiome fruiting of *R. parasitica* (R.Heim) Buyck in Africa, also Buyck and Horak (1999) described pleurotoid *Russula* species forming their basidiomata on living trees or rotten wood from Papua New Guinea and New Zealand. In the pacific northwest of USA, *R. bicolor* Burl. commonly fruits its basidiomata at heights of 1–2 m on well-rotted stumps of large coniferous trees (Miller, pers. obs.). But according to Rayner *et al.* (1985) and Henkel *et al.* (2000), production of basidiomata in these habitats is not necessarily indicative of saprotrophic nutrition. The reason for basidiome production in elevated positions on living trees or rotting wood is unclear; possibly, there are more air turbulence and/or less moisture than at ground level which may be requirements for the basidiome production, thereby facilitating spore dispersal. Alternatively, woody substrates in temperate forests are often com-

pletely perfused with ectomycorrhizal root systems which may exploit nitrogen derived from free-living N-fixers common in decomposing wood (Weber and Sundman, 1986; Jurgensen *et al.*, 1987) and basidiomata may arise from these substrates due to the proximity of their parent mycorrhizae (Henkel *et al.*, 2000). These viewpoints support that basidiomata of *R. ryukokuensis* were frequently fruiting on decayed wood of broad-leaved trees. Therefore, we recognize *R. ryukokuensis* as a mycorrhizal species.

Additionally, although the basidiomata of *R. ryukokuensis* have never been recorded from outside Japan yet, this species presumably disperses in East Asia including China as suggested by the presence of identical ITS sequences between Japanese specimens and the mycorrhizal root tip from China (JQ991808). To clarify the mycorrhizal status and geographic distribution of *R. ryukokuensis*, further ecological and molecular studies of this species are needed.

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