Russula nigrovirens sp. Nov. (Russulaceae) from southwestern China

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Russula nigrovirens sp. nov. (Russulaceae) from southwestern China

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Abstract

A new species, Russula nigrovirens, with phenotypic similarities to R. virescens is proposed based on morphological and molecular data. Morphologically, R. nigrovirens is characterized by the combination of non-striate pileus with dull green patches, incurved margin, non-discolouring context, globose to subellipsoid basidiospores with bluntly conical to subcylindrical warts isolated or connected with irregular lines or ridges, and large, clavate basidia. Russula nigrovirens is placed in subgenus Heterophyllidia, subsection Cyanoxanthinae.

Key words: New taxon·Phylogeny·Russulales·Taxonomy

Introduction

The genus Russula Pers. (Russulaceae, Russulales, Basidiomycota) is a widely distributed genus containing about 750 species (Kirk et al. 2008) worldwide including 160 species in China (Song et al. 2007).

The Hengduan Mountains, situated in southwestern China, is one of the twenty five hotspots for biodiversity (Myers et al. 2000), and proves to be an area highly rich in macrofungi (Yang 2005). More than 70 species of Russula have been recorded from the region (Song et al. 2007, Wang et al. 2009). During a previous investigation of fungal resources of western Yunnan (Zhang et al. 2010), it was noticed that four particular collections were frequently mislabeled as R. virescens (Schaeff.) Fr. which was described and illustrated as a new species. To get an insight into the phylogenetic position of these morphologically similar species, sequences of the internal transcribed spacer (ITS) were analyzed jointly with sequences of closely related taxa within Russula subgenus Heterophyllidia Romagnesi emend. Sarnari.

Materials & methods

Sampling
Collections were obtained and photographed in the field during 2008–2010. Notes and photographs were taken for macro-morphological features and specimens were dried at 50 °C. Materials examined were deposited in the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (HKAS).

Morphological studies
Macromorphological characters were determined based on detailed field notes and photographs of fresh basidiomata. Descriptive terminology followed Vellinga (1988). Color designations were from Kornerup & Wanscher (1981). Ten percent FeSO₄ solution was used to test for chemical reactions on fresh specimens. For microscopic observations
and measurements (except for basidiospores), small sections were pretreated in 5% KOH and then stained with 1% aqueous Congo red solution. Observations and measurements of the basidiospores and ornamentation were made in Melzer’s reagent. All elements of the basidiocarps were also examined for the presence of ortho- or meta- chromatic contents or incrustations in cresyl blue (Buyck 1989). Sulfovanillin (SV) was used to test for reactions of cystidia. Micromorphological features including the pileipellis, basidia, basidiospores, pleuro- and/or cheilo- cystidia, and stipitipellis, were obtained using an Olympus BX41 microscope (Olympus Optical Co., Ltd., Tokyo) equipped with an MShot Digital Imaging System. The abbreviation [n/m/p] indicates that measurements were made on n basidiospores in m basidiomata from p collections. Dimensions of basidiospores are given by using a notation of the form (a) b–c (d). The range b–c contains 95% of the measured values. Extreme values (a and d) are given in parentheses. Q indicates length/width ratio of basidiospores measured in side view with Q av denoting the average Q of all basidiospores ± standard deviation.

For observation of basidiopores under a scanning electron microscope (SEM), tiny pieces of hymenophoral fragments from dried specimens were mounted on aluminum stubs with double-sided adhesive tape. The samples were coated with gold palladium (thickness 10 nm) and an 8600 nA current flow at 30s was applied to make the test sample conductive. After coating, the samples were placed into SEM (JEOL JSM-6510) for observing and image collection.

**Molecular studies**

**DNA extraction and PCR amplification**

DNA was extracted from silica-gel-dried mushroom materials with the CTAB procedure by Doyle & Doyle (1987), and the ITS regions were amplified with the primers ITS5 and ITS4 (White et al. 1990). Amplifications were performed in a 50 μl reaction volume containing 5 μl of 10 × PCR reaction buffer, 5 μl dNTP mix (0.2 mmol), 2 μl each of primers (5 μmol), and 1.5 U of Taq DNA polymerase. The final volume was adjusted to 50 μl with sterile distilled H₂O (Liang et al. 2009). The thermal cycles consisted of one step of incubation at 95 °C for 10 min, 35 cycles of 95 °C for 50 s (denaturation), 50 °C for 30 s (annealing), and 72 °C for 1.5 min (elongation), and one step of extension at 72 °C for 10 min (final extension) (Du et al. 2012). PCR products were purified using the Bioteke’s Purification Kit (Bioteke Corporation, Beijing, China) and sequenced with an ABI 3730 DNA analyzer and an ABI BigDye 3.1 terminator cycle sequencing kit (Sangon Co., Ltd., Shanghai, China).

**DNA sequence alignments and phylogenetic analysis**

BLAST searches in GenBank (Altschul et al. 1990) indicate that sequences of the novel species are close to R. cyanoxantha (Schaeff.) Fr., a species in Russula subgenus Heterophyllidia subsection Cyanoxanthinae. The sequences were analyzed with species in Russula subgenus Heterophyllidia based on ‘Clade 3’ of Miller & Buyck (2002) and BLAST searches in Genbank (Altschul et al. 1990). Russula adusta (Pers.) Fr. and R. nigricans Fr. were chosen for outgroups. SeqMan 5.0 software (DNAStar SeqMan Pro, Madison, WI) was used to assemble forward and reverse sequences into contigs, inspect the ABI chromatograms and nucleotides were inspected and edited as needed. DNA sequences were aligned using G-INS-i strategy implemented in MAFFT v6 (Katoh & Toh 2008). The alignment was manually refined with BioEdit v7.0.9 (Hall 1999). To eliminate ambiguously aligned positions in the alignment as objectively as possible, the on-line program Gblocks v0.91b (Castresana 2000) was used. The program was run with settings allowing for smaller blocks, gaps within these blocks, and less strict flanking positions. Gaps in alignment were treated as missing data.

For phylogenetic analyses based on an ITS matrix, both Randomized Accelerated Maximum Likelihood (RAxML) and Bayesian inference (BI) algorithms were employed. The substitution model was chosen with the Akaike information criterion implemented in MrModeltest 2.3 (Nylander 2004). RAxML 7.2.6 (Stamatakis 2006) and MrBayes v.3.1 (Huelsenbeck and Ronquist 2005) were used in the ML and BI analyses respectively. All parameters in RAxML analysis were kept at default, statistical support values were obtained using nonparametric bootstrapping with 1000 replicates, and trees obtained prior to convergence were discarded before the consensus tree. Bayesian inference analyses were performed using the Metropolis-coupled Markov chain Monte Carlo method under the GTR + I + G model. Analyses were run with 4 chains of 2,000,000 generations, and trees were sampled every 100th generation. Bayesian posterior probabilities (BPP) values were obtained from the 50% majority-rule consensus trees and branches with BPP>95% were considered as significantly supported.
Results

Molecular studies
We analyzed a dataset of ITS sequences with 759 nucleotide sites for 30 specimens (including 26 from GenBank; Table 1). Both the RAxML and Bayesian analyses yielded similar tree topologies and only the tree inferred from ML analysis is shown (Fig. 1). Bayesian posterior probability is also displayed with the bootstrap values along the branches.

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<th>Taxon</th>
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<th>Location</th>
<th>GenBank Accession Numbers</th>
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Bayesian and RAxML phylogenetic analyses showed that four collections clustered together forming a well-supported branch with 100% bootstrap value and 1.00 posterior probability were obviously different from related sequences available in the GenBank. Phylogenetic analyses also showed that the new species grouped with *R. cyanoxantha* and *R. variata* Banning with 100% bootstrap value and 1.00 posterior probability.
**FIGURE 1.** The best RAxML likelihood tree (–ln L 4392.662321) based on the ITS dataset. Support values in bold type are RAxML likelihood bootstrap (≥70%). Values in normal type are Bayesian posterior probabilities (≥0.95). Classification followed by Sarnari (1998).

**Taxonomy**

*Russula nigrovirens* Q. Zhao, Y.K. Li & J.F. Liang, *sp. nov.* Fig. 2
MycoBank MB 810879

Holotype: CHINA, Yunnan Province, Yulong County, Laojunshan, in subalpine forest dominated by *Picea* sp., *Rhododendron* sp., *Sorbus* sp. and *Abies* sp., elev. 3400 m, 16 August 2008, Qi Zhao 8240 (HKAS55222! GenBank accession: KP171173).

Etymology: “nigrovirens” refers to the deep green color of the pileus.

*Pileus* 3–10 cm diameter, first hemispherical, becoming convex or plano-concave with a slightly depressed center, often subinfundibuliform with age; margin incurved, surface viscid when moist, shiny in fresh and dry conditions,
cracking and broken into small patches, patches crowded in the center, with smaller patches towards the margin; green white (26A2) to grayish green (27D5) with patches of dull green to deep green (26E4) to leaf green (29D5–7) when dry; suprapellis readily peeling even to the center, unchanging in color when bruised. Lamellae adnate, crowded, rarely forking near the stipe, with scattered lamellulae, white, cream yellow when dried, unchanging when bruised or with FeSO₄. Stipe 6–10 × 1.0–2.5 cm, cylindrical, slightly attenuate towards the base, subglabrous, smooth, dry, white to whitish, spongy inside. Context 5–8 mm thick, white to cream when dry, without color changing when bruised and treated with FeSO₄. Annulus absent. Odour indistinct. Taste mild. Spore print whitish.

**FIGURE 2.** *Russula nigrovirens* (Holotype HKAS55222!). A Basidiomata; B Basidiospores; C Basidia; D Cheilocystidia; E Pleurocystidia; F Marginal cells; G Pileipellis; H Pileocystidia; I Caulocystidia.

**Basidiospores** [100/5/5] (6) 6.5–8.5 (9.5) × (5.5) 6–8 (8.5) μm [Q = 1–1.28 (1.33), Q₉ = 1.17 ± 0.09], globose to subellipsoid; ornamentation amyloid; warts bluntly conical to subcylindrical, not exceeding 0.6 μm in height, isolated or connected with irregular lines or ridges, not forming a reticulum; suprahilar plage indistinct, not amyloid; hyaline
in 5% KOH. Basidia 45–75 × 9–14 μm, 4-spored, rarely 2-spored, sterigmata up to 8 μm long, narrowly clavate to clavate, slightly bulbous towards upper third. Lamellar trama mainly composed of nested sphaerocytes (21–38 × 19–30 μm) surrounded by connective hyphae. Cheilocystidia 46–55 × 6.5–8.5 μm, rare, narrowly clavate to clavate with rounded or mucronate apex, contents granular. Pleurocystidia 47–72 × 7–10 μm, abundant, projecting 10–20 μm beyond hymenium, slender, clavate to subfuniform, apex obtuse, bluntly acuminate or mucronate, with abundant granular contents, dark grey in SV. Marginal cells 9–27 × 3.5–5 μm, cylindrical to narrowly clavate, hyaline. Pileipellis metachromatic in cresyl blue, consisting of interwoven, ascending to repent hyphae (2–5 μm diameter), often ramifying, septate; terminal elements 15–46 × 2.5–5 μm, apices obtuse, sometimes attenuate, with a distinct granular content; pileocystidia similar to terminal cells, but only present in suprapellis, apex mucronate or subterminally constricted, always one-celled, 18–62 × 3–6.5 μm, negative in SV. Stipitipellis not well-developed, cutis composed of thin-walled, septate, cylindrical hyphae 3–5 μm diameter; caulocystidia numerous, crystalline contents, 29–96 × 3.5–6.5 μm, subcylindrical to narrow clavate. Clamp connections absent.

Habitat and distribution: gregarious or scattered in forests dominated by Picea sp., Rhododendron sp., Sorbus sp. and Abies sp. Known only from high altitude localities in southwestern China.

Additional specimens examined: CHINA, Yunnan Province: Yulong County, Gaomeigu, elev. 3000 m, 16 August 2008, Qi Zhao 841 (HKAS55042! GenBank accession: KP171174); elev. 3000 m, 19 August 2008, Qi Zhao 8248 (HKAS55230!); elev. 3000 m, 20 July 2008, Qi Zhao 844 (HKAS55045! GenBank accession: KP171175); Dali County, Cangshan, elev. 3600 m, 12 August 2010, Qi Zhao 826 (HKAS69567! GenBank accession: KP171176).

Discussion

Russula nigrovirens is well distinguished by the non-striate pileus with dull green patches, incurved margin, globose to subellipsoid basidiospores with bluntly conical to subcylindrical warts isolated or connected with irregular lines or ridges, white spore print, and large, clavate basidia. Considering the combination of its deep green pileus, white stipe, context negative with FeSO₄, inamyloid suprahilar plage, one-celled, SV-negative pileocystidia, white spore print, and mild taste, R. nigrovirens is placed in Russula subgenus Heterophyllidia subsection Cyanoxanthiniae (Singer 1986, Romagnesi 1987, Sarnari 1998). Bayesian and RAXML phylogenetic analyses showed that R. nigrovirens was different from known Russula species for which ITS sequences were available. It is closely related to the European species R. cyanoxantha and the North American species R. variata. However, the latter two species have variable pileus color, context that tends to change to green with FeSO₄ and shorter basidia. In addition, R. cyanoxantha differs from R. nigrovirens in its basidiospore ornamentation that is never connected with fine lines, and narrow hymenial cystidia (∼7 μm). Ecologically, R. cyanoxantha invariably grows in temperate to boreal areas (Romagnesi 1967, Phillips 1981, Bon 1988) while R. nigrovirens grows only in subalpine area. Russula variata, described in forests of oaks and other hardwoods from eastern North America, is distinguished by its smooth pileus, soft, repeatedly forked lamellae, and narrow hymenial cystidia (≤5 μm). In the field, R. nigrovirens is often confused by local residents with the European species R. virescens. However, the ITS sequences clearly distinguish the two species as they fell into two distinct clades (Fig. 1). Russula virescens differs from R. nigrovirens by its cracked pileus with tuberculate-striate margin, pale yellow lamellae, white stipe turning pale grayish yellow when injured, broadly elliptic basidiospores with the ornamentation forming a partial or complete reticulum, and smaller basidia (∼50 μm) (Romagnesi 1967, Shaffer 1970). Russula parvovirens Buyck et al. (2006), from the United States, also has green patches on the pileus but a greenish brown to metallic bluish coloured pileus with slightly striate margin, thin flesh that turns brownish orange with FeSO₄, orthochromatic pileipellis in cresyl blue, pale cream spore print, basidiospore ornamentation forming an incomplete network, and shorter basidia (∼45 μm; Buyck et al. 2006), while R. griseoviridis McNabb (1973), described from New Zealand, differs in having...
greyish green pileus with greyish red colouration, pileus margin that is often radially split, free to adnexed lamellae, basidiospore ornamentation forming an incomplete reticulum and smaller basidia (35–53 × 6.5–11 μm; McNabb 1973).

Several species in Subgenus *Heterophyllidia* with a green pileus described from the Himalayan Mountains and southwestern China are similar to *R. nigrovirens*. *Russula sikkimensis* described from India (Das *et al.* 2013), has a smooth pileus, context turning pale yellow with FeSO₄, partially reticulate basidiospore ornamentation, and smaller basidia (40–54 × 7.5–10 μm). *Russula atroaeruginea* (Li *et al.* 2013) differs from *R. nigrovirens* in a blackish green tinged pileus without patches, context turning pale yellowish with age, smaller basidia (40–48 × 9–11 μm), and absence of cheilocystidia (Li *et al.* 2013). *Russula viridella* var. *yunnanensis* Singer (1935) is distinguished by a green pileus with purple colouration without patches, acrid context, cream spore print, smaller basidia (≤50 μm), and pleurocystidia with an obtuse or acuminate tip (Singer 1935).

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