Two new tropical *Russula* species associated with *Quercus* show evidence of diversification across the Isthmus of Panama

Michelle Vera Castellanos

Directora

Adriana Corrales PhD.

Co-director

Slavomír Adamčík

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Michelle Vera,[1] Slavomír Adamčík[2], Katarína Adamčíková[3], Felix Hampe[4], Miroslav Caboň[2], Cathrin Manz[5], Clark Ovrebo[6], Meike Piepenbring[5], Adriana Corrales[1]

[1]Department of Biology, Faculty of Natural Sciences and Mathematics, Universidad del Rosario. Carrera 24 # 63C-69, Bogota D.C., 111221, Colombia.
[2]Institute of Botany, Plant Science and Biodiversity Center, Slovak Academy of Sciences, Du´bravska´ cesta 9, 845 23 Bratislava, Slovakia
[3]Department of Plant Pathology and Mycology, Institute of Forest Ecology, Slovak Academy of Sciences Zvolen, Akademicka´ 2, 949 01 Nitra, Slovakia
[4]Wetzlarer Str. 1, 35510 Butzbach
[5]Department of Mycology, Goethe University Frankfurt am Main, Biologicum, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany
[6]Department of Biology, University of Central Oklahoma. Edmond, OK. USA

Abstract

Russula floriformis and *R. symphoniae* are described as two new sister species of *Russula* subsection *Substriatinae* from montane forest of Colombia and Panama and associated with *Quercus* and *Oreomunnea* trees. Very similar field environmental conditions and an ITS sequence similarity higher than 99% with only 3 different positions indicate that these species are closely related and nearly cryptic. Detailed observations of microscopic structures and analyses of more DNA loci revealed more morphological and molecular characters distinguishing collections of *R. floriformis* from Colombia and *R. symphoniae* from Panama. Spatial distribution and phylogenetic proximity of *Russula* species and their ectomycorrhizal host *Quercus* tree suggests their speciation as a result of migration, adaptation and climatic isolation across the Panama Isthmus of their host tree during the Pliocene and Pleistocene events. Then we hypothesize that this could be evidence of coevolution between *Russula* and *Quercus*. Analysis of publicly available ITS sequence data suggests that there are more locally adapted species of this lineage in Central and North America.

Key words: cryptic species, coevolution, Diversity, Ectomycorrhiza, Tropical mountain forest, America, Fagaceae, Juglandaceae

INTRODUCTION

Russula is an ectomycorrhizal (ECM) fungal genus with worldwide distribution (Looney et al. 2018). This genus has more than 2000 species, but the distribution of individual species is usually limited depending on climate and geographic barriers (Adamčík et al. 2019). This genus is considered the second most taxonomically diverse among the ECM fungi, and its taxonomic classification is challenging due to the detailed morphological information required for a valid identification (Looney *et al.* 2018; Manz 2019). Estimates of the known fungal

diversity in the Americas report 400 *Russula* species from the USA (Adamčík *et al.* 2018) and 77 from Central and South America (Supplementary material 1).

Russula forms mycorrhizal associations with plants in all biomes and *Quercus* is a frequent host tree in temperate, mediterranean and subtropical habitats (Hynes *et al.* 2010, Richard *et al.* 2005, *Wang et al.* 2012). The genus *Quercus* is an important tree group on the northern hemisphere with a high diversity and broad distribution in Europe, North Africa, Asia, and the Americas. Oak trees in the New World are distributed from southern Canada trough Central America, having the greatest diversity found on southern Mexico with approximatley 160 to 210 species and gradually decreasing trough south, with nine known species in Panama and only one species, *Quercus humboldtii*, found in Colombia (Correa *et al.* 2004; Kapelle 2006; Nixon *et al.* 2006; Hooghiemstra 2006; Morales Pacheco *et al.* 2018).

The genus *Quercus* arrived to central west Mexico during the Miocene, about 10 million years ago (MA), with an ecosystem transition from a wet evergreen forest to a semi deciduous forest and migrated to South America after the closure of the Panamanian isthmus during the Pliocene, along with other highland ECM tree species (like *Alnus*) approximately 5 and 3.5 MA (Hooghiemstra 2006). The Isthmus emergence occurred approximately 3.5 MA and the principal effects caused by its emergence along the continent were the northern hemisphere glaciation, onset of both thermohaline oceanic circulation, and the Great American Biotic Interchange between North and South America that is considered one of the largest episodes of biological migration where plants were the first species to migrate (Bacon *et al.* 2015; Jaramillo, 2018). This relative recent geological connection has been determinated as an important area of species turnover for the genus *Quercus*, becoming an important migration route for individuals from Costa Rica and Panama arriving to the Andean Cordillera of Colombia (Rodriguez-Correa *et al*. 2015). *Q. humboltii* divergence from its nearest central American relative *Q. costaricensis* is estimated to had happened around 3.5 MA time coinciding with the Panama Isthmus emergence between Miocene and Pliocene (Hipp *et al.* 2019).

The range of distribution of *Quercus* in the Neotropics has been changing constantly along the Last Glacial Maximum (LGM) period and the Last Interglacial period (LIG). In Central Mexico the lowering of glaciers to 1000-1500 m.a.s.l favored the migration of montane forests species to lower altitudes and subsequently latitudes (Hooghiemstra 2006; Ramírez-Barahona and Eguiarte 2013). During this period, the glacier descent of about 500-900 m facilitated the slope down migration of montane vegetation due to climatic and ecosystem changes and it could had enhanced the possibility for migration to lower altitudes through the Panamanian Isthmus (Hooghiemstra 2006; Ramírez-Barahona and Eguiarte, 2013). This phenomenon was widespread trough most part of Central America with pollen records from Panama showing evidence of a lower altitude distribution of montane plant taxa (Hooghiemstra 2006; Ramírez-Barahona and Eguiarte, 2013). The range expansion of montane tree species, along with other potential host species, could have been the basis of ecological and genetic expansion of ECM fungal communities from Central America into South America (Becerra and Zak 2011).

Host specifity is an important driver of *Russula* species evolution (Looney *et al.* 2016). With migration, isolation and speciation of South American *Q. humboltii*, we may expect also coevolution of symbiotic fungal partner species of the genus *Russula*. However, several studies demonstrated that the majority of *Quercus* associated ECM fungal species may also colonize roots of alternative host tree species, which could evidence that adaptation to local ecological and climatic conditions may be more important than availability of their host partner (Richard *et al*. 2004; Caboň *et al.* 2019).

Recent research and collections from the Neotropics show that tropical montane forests are rich in ECM fungi due to a higher density and abundance of ECM host trees mostly in the families Betulaceae, Fagaceae, Juglandaceae, and Pinaceae (Buyck and Halling 2004; Haug *et al.* 2004; Barroetaveña *et al.* 2007; Smith *et al.* 2011; García-Guzmán *et al*. 2017; Corrales *et al.* 2018). In Neotropical montane forest the best known ECM system corresponds to the *Quercus* dominated forests (oak forest), represented by approximately twety-four *Russula* species described from oak forests from Mexico, Costa Rica, and Colombia (Franco-Molano *et al*., 2010; Singer, 1963). Another example of an ECM host tree in Central America is the genus *Oreomunnea* (Juglandaceae). This species is distributed from Mexico to Panama at elevations between 900-2600 m.a.s.l. and forms monodominant stands along its range of distribution (Herrera *et al.* 2014, Corrales *et al*. 2016). Recent evidence suggests that there is some degree of sharing of root symbiotic fungal species between *Oreomunnea* and *Quercus spp.* (Corrales *et al*. 2016; Alfonso-Corrado *et al*. 2017, Corrales & Ovrebo *in press*).

This study focuses on the analysis of morphologically similar collections of *Russula* from the subgenus *Heterophyllidia* subsect. *Substriatinae* X.H. Wang & Buyck collected in *Quercus* and *Oreomunnea* forests in Colombia and Panama at sites located south and north of the Isthmus of Panama. Species from *Russula* subgenus *Heterophyllidia* Romagn. are recognized for a mild taste, non-amyloid suprahilar spots on spores, hyphal terminations in pileipellis typically densely septate, one-celled pileocystidia (if present) and pale-colored spore prints (Buyck and Adamčík 2011). The subsection *Substriatinae* is defined by typically strongly tuberculate-striate pileus with pink and often disconnected pileipellis, relatively large spores ornamented by large isolated warts and abundant pileocystidia variable in their size and endings. The ITS sequences in our dataset show similarity higher than 99%. We aim to evaluate if these differences in the ITS region are consistent between collections from Panama and Colombia, and if there are consistent differences also in other DNA loci that provide sufficient support to recognize them as two separated species. Here we combine sequence data, morphological, and ecological observations to determine if the collections separated by the Panamanian Isthmus are distinct taxa and what is their appropriate taxonomic rank. To our knowledge, this is the first study focusing on ECM fungal species diversification based on the disjunction at the Isthmus.

MATERIALS AND METHODS

Study area

Three specimens were collected in a primary lower montane forests (1000-1400 m.a.s.l) at the Fortuna Forest Reserve in western Panama. Collections were made between April 2012 and May 2015, two on the Honda watershed (8˚45'12"N, 82˚13'08"W) and one on Zarciadero site near Bocas del Toro Road (N 8˚45.707', W 82˚15.677'). The mean annual temperature registered for the Fortuna Forest Reserve ranges from 19 to 22°C and the annual rainfall varies from ca. 5800-9000 mm (Cavelier 1996; Anderser *et al.* 2012). The sampling was done in monodominant stands of *Oreomunnea mexicana* with scattered individuals of *Quercus* cf. *lancifolia* and *Alfaroa costaricensis*.

The six Colombian collection were collected on oak forest dominated by the endemic species *Q. humboldtii* located at the Chicaque Natural Reserve (4˚36'22"N, 74˚18'17"W) on the Eastern Andean Cordillera of Colombia. The reserve includes approximately 300 ha of cloud forest between 2000 to 2700 m of altitudinal gradient. The weather is characterized by a bimodal rainfall regime, with the highest rain fall occurring between March-May and October-November and an average temperature of 15 °C year-round (Rivera Ospina and Córdoba García 1998; Ávila-de Navia and Estupiñan-Torres 2013). *Russula* collections were made between November 2018 and April 2019.

Molecular analysis

Total DNA genomic content was extracted from fresh or dried basidiocaps, stored in CTAB 2X, following Gardes and Bruns (1993) with modifications. DNA amplification was performed using 5× HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) or *taq* DNA Polymerase (New England Biolabs) following the manufacturer's instructions. In total, five loci were amplified and sequenced: (1) The internal transcribed spacer regions of ribosomal DNA (ITS), (2) partial ribosomal small subunit ribosomal DNA (mtSSU), (3) ribosomal nuclear large subunit (LSU), (4) the region between domains six and seven of the nuclear gene encoding the second largest subunit of RNA polymerase II (*rpb2*) and (5) transcription elongation factor 1-alpha (*tef-1α*). The primers and cycling protocols used are listed in the Supplementary material 2. The polymerase chain reaction (PCR) products were purified using ExoSap-IT (Thermo Fisher Scientific, Wilmington, DE, USA) and sent to Macrogen Korea (Seoul, South Korea) for sequencing.

Phylogenetic analysis

Sequences were edited in the BioEdit Sequence Alignment editor version 7.2.5 (Hall 2005) or Geneious version R10 (Kearse *et al.* 2012). Intra-individual polymorphic sites having more than one signal were marked with NC-IUPAC ambiguity codes. For phylogenetic placement of studied species we mainly used sequence data of nrLSU, *rpb2* and *tef1a* loci published by Wang et al. (2019). This dataset was supplemented by two samples of *Russula redolens* representing a lineage not recognized by the latter study. To estimate species diversity and distribution we analyzed publicly available ITS sequences retrieved by BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The final datasets were aligned by MAFFT version 7

(Katoh & Standley 2013) using the strategy E-INS-I and further manually edited. All sequences obtained in this study are listed in the Table 1.

The multi-locus dataset was analyzed using two different methods: Bayesian inference (BI) and the maximum likelihood (ML) approach. For the ML analysis, the concatenated alignment was loaded as a PHYLIP file into RAxMLGUI v. 1.2 (Silvestro and Michalak 2012) and analyzed as a partitioned dataset under the GTR + GAMMA model with 1000 bootstrap iterations (Stamatakis 2008). For the BI analysis, the dataset was divided into nine partitions: 28S, intronic region of *tef1a*, the 1st, 2nd and 3rd codon positions of *tef1a*, intronic region 7 of *rpb2*, and the 1st, 2nd and 3rd codon positions of *rpb2*. The best substitution model for each partition was computed jointly in PartitionFinder v. 1.1.1 (Lanfear *et al.* 2012). The BI was computed independently twice in MrBayes version 3.2.6 (Ronquist *et al.* 2012) with four MCMC chains for 10,000,000 iterations until the standard deviation of split frequencies fell below the 0.01 threshold. The convergence of runs was visually assessed using the Trace function in Tracer version 1.6 (Rambaut *et al.* 2013).

The nrITS dataset was computed in RAxMLGUI v. 1.2 (Silvestro and Michalak 2012) and analyzed as a partitioned dataset under the GTR + GAMMA model with 1000 bootstrap iterations (Stamatakis 2008). All analyses were computed using CIPRES Science Gateway (Miller *et al.* 2010).

Morphological analysis

Field characters associated with morphological features were described based on fresh basidiocarps following Adamčík *et al.* (2019). All samples were processed as voucher specimens for herbarium by being dehydrated in a food dryer at 30°C for 24 h. All the morphological analysis were done following Adamčík *et al.* (2019). Briefly, the dried material was sliced by hand with a razor blade. Micromorphological characteristics were observed using an Olympus CX-43, with an oil immersions lens at a magnification of 1000x. The structures were visualized and illustrated with a "camera lucida" using an Olympus U-DA drawing attachment at a projection scale of 2000x. The structures and contents of hymenial cystidia and pileocystidia were observed and measured in ammoniacal Congo Red preparations from dried material. Spores were observed on lamellae surface in Melzer's reagent. All other microscopic observations were made in ammoniacal Congo red, after a short treatment in aqueous 10% KOH solution to dissolve the gelatinous matrix and improve tissue dissociation. Additionally, all tissues were examined in Cresyl blue to notice the presence of ortho- or metachromatic reactions as explained in Buyck (1989). Trama and cystidia were observed in sulfovanillin solution (Caboň *et al.* 2017). Acid-resistant incrustation of the pileocystidia were stained with carbolfuchsin and observed in distilled water after incubation in 10% solution of HCl (Romagnesi, 1967).

RESULTS

Phylogenetic analysis

The phylogenetic tree based on LSU, *rpb2*, and *tef1*-α regions (Fig. 2) shows placement of all our collections in *Russula* subsect. *Substriatinae* in a strongly supported clade that is sister to *Russula* sect. *Heterophyllae*. Both groups are together within sect. *Ingratae*, subsect. *Cyanoxanthinae* and one residual cluster placed in the subgenus *Heterophyllidia*.

Collections from Panama and Colombia are strongly supported as sisters to Asian collections of *R. substriata* and *R. maguaensis*. American collections are clustered based on geography in two clades that are further recognized as new species *R. floriformis* sp. nova and *R. symphoniae* sp. nova.

The ITS tree (Fig. 3) also supports placement of the Colombian and Panamian collections within subsect. *Substriatinae*. Collections probably of a single species from Madagascar are placed with strong support as sister to *Substiatinae* and they may form together a group at the rank of section within the subgenus *Heterophyllidia*. This tree contains more samples from more regions than the multi loci tree and shows geography-based clustering within *Substriatinae*, but the species relationships and sometimes even the support at terminal clades are not significant. There is a support for at least four Asian and two Australian species, but only two clusters from America received support: *R. symphoniae* from Panama and a cluster of three collections from USA, probably all from the South-east coast. The ITS tree does not show support for a common ancestor of American species, but neither contradicts this hypothesis.

Russula floriformis and *R. symphoniae* are closely related species that differ by 3 positions in ITS, two in LSU, seven in *rpb2* and three in *tef1*-α region (Table 2). Also, the mtSSU region (Table 1) was sequenced but not used, because it was highly conserved among the species and didn't show any differences and because there are very few available mtSSU reference sequences from *Russula* to use it for a multi loci analysis.

Morphological analysis

The already described species of the subsection *Substriatinae* (Fig. 3) share a lot of macroscopic feautures with R. *floriformis* from Colombia and *R. symphoniae* from Panama: they have relatively small basidiomata (pileus up to 60 mm in diam.), strongly tuberculate-striate pileus margin, thin and fragile context, pileus cuticle radially cracking near the margin, and cavernate or hollow and narrow stipe (up to 10 mm wide). All species except for *R. shingbaensis* have pink to wine-red pileus cuticle and the two new American species share with *R. substriata* also pinkish tints on the stipe. *Russula floriformis* and *R. symphoniae* look very similar macroscopically being the only notorious difference that the pileus colour is usually darker wine-red to red-brown near the pileus margin in *R. floriformis* and purplish pink to grayish red in R. *symphoniae*.

There is also a lot of shared micromorphological characters specific for the subsection *Substriatinae*. We confirmed that the American species have relatively large spores ornamented by large isolated warts and a pileipellis of densely arranged hyphal terminations with abundant one-celled pileocystidia of mainly the same length, sharply delimited from thick gelatinous

subpellis and disconnecting towards the margin. The American species differ from the Asian species by relatively narrow hyphal termination in the pileipellis composed only of 1-2 unbranched cells and without shorter and more inflated subterminal cells.

The main and most conspicuous character to distinguish *R. floriformis* and *R. symphoniae* are the apically constricted pileocystidia near the pileus centre often with small knobs or pearl-like appendages. Other significant characters distinguishing both species are more difficult to observe and require time for measurements (Supplementary Material 3). Spores of *R. floriformis* are more subglobose. *Russula symphoniae* has narrower and near the pileus margin more attenuated hyphal terminations in pileipellis. Marginal cells of the species from Panama seem to be smaller.

Taxonomy

Russula floriformis Vera and Corrales, sp. nov.

Holotype: COLOMBIA, Cundinamarca province, San Antonio del Tequendama, Eastern Andean Cordillera, Chicaque Natural Reserve, 4˚36'22"N, 74˚18'17"W, *Q. humboldtii* forest, 1 December 2018, *Corrales 943* (KUO, HUA).

Etymology: the species pileus resembles by its color and the strong radial striation a flower pattern.

Short diagnosis: Basidiomata strongly reminiscent of *R. substriata*, pileus thin fleshed, strongly tuberculate striate near the margin, cuticle disconnected to fine patches and radially cracked near the margin, wine-red, brown red and near the centre dark black-red; hymenium with rare lamellulae and occasional furcations; stipe cavernate, white with pinkish hue; spores relatively large, subglobose and with isolated large warts; pileipellis composed of densely arranged, one to two celled, usually cylindrical hyphal terminations, pileocystidia abundant, one-celled, subcylindrical, apically mainly obtuse.

Pileus small to medium sized, 24.1-48 mm, usually plane with depressed center when mature deep depressed to infundibuliform; margin tuberculate-striate at 5-19 mm, crenulate; cuticle very slimy, shiny, smooth at the center, near the margin peeling or radially cracking, color of red-wine near the margin, dark red-brown to almost black, with yellow-orange spots at the center. **Lamellae** moderately distant ca. 6-10/1 cm near the pileus margin, adnate or shortly decurrent, white, lamellulae rare, furcations occasional especially near the stipe, edge even and concolorous. **Stipe** 32–48 × 7–9 mm, cylindrical, narrowed near the base, flexuous or curved, longitudinally striate, pale pinkish purple almost white near the lamellae, cavernate, cortex 1.5 mm thick. **Context** 2.5 mm thick in half of the radius, fragile, white, unchanging, taste not observed, odour inconspicuous. **Spore print** not observed.

Spores (7.1–)7.7–8.2–8.7(–9.5) × (6.5–)7–7.4–7.9(–8.8) µm, mainly subglobose, Q=(1.04–)1.05–1.11–1.17(–1.26); ornamentation of relatively large, moderately distant [(3–)4–6(–8) in a 3 µm diam. circle] amyloid, obtuse warts, (0.6–)0.8–1.2(–1.4) µm high, mainly isolated, fused in pairs or short chains [0–2(–3) fusions in the circle], line connections absent; suprahilar spot not amyloid, covered by small and low warts and emarginated by more prominent ornamentation. **Basidia** (10–)28.5–39.1–49.5(–54.5) × (5–)9–11.5–14(–17.5) µm, mainly fusiform, occasionally clavate and pedunculate, 4-spored; basidiola first cylindrical or ellipsoid,

then clavate, ca. 5–13 µm wide. **Hymenial cystidia** widely dispersed to dispersed, ca. 290–590/mm^2, (28.5–)44–55–65.7(–76) × (7–)8–9.4–11(13.5) µm, mainly fusiform or lageniform, rarely lancelolate, mainly acute end pointed, mainly with an excentric, 1–3(–4) µm long appendage, thin-walled; contents heteroformous, crystalline or banded, sometimes optically empty near the basis, turning almost black in sulfovanillin; near the lamellae edges sometimes smaller, (37–)48–57.4–67(–78) × (6–)8–9.1–10.5(–11.5) µm, more frequently inflated in near terminal part, lancelated, clavate or narrowly fusiform, contents more dispersed, sometimes almost optically empty or limited to terminal part only. Lamellae edges with occasional basidia; **marginal cells** (14–)17–20.7–24.5(–30) × (3–)5–6.6–8.5(–11) µm, usually cylindrical and relatively narrow, some clavated and similar to basidiola, occasionally flexuous or apically constricted, obtuse. **Pileipellis** metachromatic in Cresyl Blue but not strongly, sharply delimited from the underlying context, 120–175 µm deep; suprapellis not distinctly gelatinized, 25–30 µm deep, disconnected, composed of repent or ascending hyphal terminations, faciculated in small or bigger groups forming pyramidal structures, near the center forming continuous layer; well delimited from 105-160 µm deep, strongly gelatinized subpellis of loose, often anastomosed, irregular oriented, but near the context dense and horizontally oriented, 1-4 µm width hyphae. **Acid-resistant incrustations** absent. Hyphal terminations near the pileus margin composed of one or two cells, thin walled; terminal cells (9–)14.5–19.6–24.5(–33) × (2.5–)3–3.9–4.5(–5.5) µm, cylindrical, subulate or lageniform, usually apically narrowed; subterminal cells are mainly branched, shorter and wider, up to 6 µm often with lateral projections or branches. Hyphal terminations near the pileus center similar, terminal cells (8–)14.5–19.7–25(–32) × (2.5–)3.5–4.2–5(–6) µm, more frequently apically constricted, subterminal cells more frequently unbranched and inflated-ellipsoid, occasionally anastomosed. **Pileocystidia** near the pileus margin frequent, even locally more frequent than other hyphal terminations, always 1-celled, mainly clavate, occasionally subcylindrical or fusiform, rarely lanceolate, thin-walled, (13.5–)17.5–23.5–29(–38) × (3.5–)4–4.5–5(–7) µm, apically mainly obtuse, occasionally acute and with terminal knob; contents with dispersed and partly granulose or banded, weakly greying in sulfovanillin. Pileocystidia near the pileus center similar in shape, (11–)16–23.3–30.5(–45) × (3.5–)4–4.5–5(–6) µm, more frequently apically constricted, with more abundant contents. **Cystidioid hyphae** dispersed in subpellis but more frequent near the context, sometimes similar to cystidia but longer and often apically mucronate, occasionally with lateral projections or branches, contents dense and granulose.

Additional material studied: Cundinamarca province, San Antonio del Tequendama, Eastern Andean Cordillera, Chicaque Natural Reserve, cloud forest dominated by *Q. humboldtii*, 3 Nov 2018, *Corrales 904*, *Corrales 911*; Chicaque Natural Reserve, cloud forest dominated by *Q. humboldtii*, 1 Dec 2018, *Corrales 952*; Chicaque Natural Reserve, cloud forest dominated by *Q. humboldtii*, 28 Apr 2019, *Corrales 1007, Corrales 1007a*.

Russula symphoniae, Manz, Hampe and Corrales, sp. nov.

Holotype: PANAMA, Chiriquí province, Fortuna Forest Reserve, Honda watershed, in monodominant *Oreomunnea mexicana* forest, 14 of December 2013, A. Corrales, *Corrales 591* (PMA, ARIZ).

Etymology: color the pileus resembles by the flower color of Panamanian plant species *Symphonia globuliphera*.

Short diagnosis: Basidiomata strongly reminiscent of *R. substriata* and *R. floriformis*, pileus thin fleshed, strongly tuberculate striate near the margin, cuticle disconnected to fine patches and radially cracked near the margin, purplish pink, grayish red and near the center darker; hymenium with rare lamellulae and furcations; stipe cavernate, white with pinkish hue; spores relatively large, broadly ellipsoid and with isolated large warts; pileipellis composed of densely arranged, one to two celled, near the pileus margin usually apically attenuated hyphal terminations, pileocystidia abundant, one-celled, subcylindrical, near the pileus center mainly apically constricted and often with knob or small appendage. .

Pileus small to medium sized, 18-56 mm, convex to plane with depressed center; margin tuberculate-striate at 5-20 mm, entire to eroded; cuticle dry to moist, shiny, viscid, with small patches or granulations, radially cracking near the margin, light purpulish pink to grayish red color near the margin, darker in center. **Lamellae** moderately distant ca. 7-8/1 cm near the pileus margin, adnate or shortly decurrent, white, lamellulae present and dispersed, furcations absent, edge even and concolorous yellowish. **Stipe** 24-36 × 4–8 mm, cylindrical, flexuous or curved, smooth to slightly striate, white to light yellow with superficial purple fibrilles, cottony stuffed to hollow, cortex 2 mm thick. **Context** 1 mm thick in half of the radius, very fragile, white with pink shade, unchanging, taste not observed, odor inconspicuous. **Spore print** not observed.

Spores (7.2–)7.8–8.2–8.6(–9.4) × (6.1–)6.7–7.1–7.5(–7.9) µm, subglobose to broadly ellipsoid, Q=(1.03–)1.1–1.16–1.22(–1.32); ornamentation of relatively large, moderately distant [(3–)4–6(–7) in a 3 µm diam. circle] amyloid, obtuse warts, (0.6–)0.9–1.2(–1.4) µm high, mainly isolated, fused in pairs or short chains [0–2(–3) fusions in the circle], line connections absent; suprahilar spot not amyloid, covered by small and low warts and emarginated by more prominent ornamentation. **Basidia** (10–)26–36.5–47(–54.5) × (5–)8–9.9–12(–13) µm, mainly clavate, occasionally fusiform, pedunculate, 4-spored; basidiola first cylindrical or ellipsoid, then clavate, ca. 5.5–10 µm wide. **Hymenial cystidia** dispersed, ca. 450–650/mm^2, (28.5–)45.5–57.8–70(–90) × (6.5–)7.5–8.9–10(13) µm, mainly fusiform or lageniform, rarely lancelolate, mainly acute end pointed, usually with a short, 1–3(–6) µm long, often excentric appendage, thin-walled; contents heteromorphous, granulose, banded or sometimes also crystalline, sometimes optically empty near the basis, turning almost black in sulfovanillin; near the lamellae edges smaller, (21–)33–44.7–56.5(–73.5) × (4–)6–7.8–9.5(–11.5) µm, fusiform or lanceolate, contents more dispersed. Lamellae edges with occasional basidia; **marginal cells** (9–)14–18.3–23(–29) × (3–)4–5.5–7(–9.5) µm, usually clavate and similar to basidiola, apically obtuse and rarely slightly constricted. **Pileipellis** metachromatic in Cresyl Blue but not strongly, sharply delimited from the underlying context, 90–130 µm deep, strongly gelatinized throughout; suprapellis 30–40 µm deep, near the pileus margin disconnected, composed of loose, repent or ascending hyphal terminations, near the center forming continuous layer of

densely arranged, erect hyphal terminations; well delimited from 70–100 µm deep subpellis of loose, often anastomosed, irregularly oriented, but near the context dense and horizontally oriented, 2–4 µm wide hyphae. **Acid-resistant incrustations** absent. Hyphal terminations near the pileus margin composed of one or two cells, thin walled; terminal cells (6.5–)12.5–16.9–21(–28) × (2.5–)3–3.5–4(–5.5) µm, cylindrical, subulate or lageniform, usually apically narrowed; subterminal cells mainly branched, mainly equally wide and long, occasionally shorter or wider, up to 5 µm wide, often with lateral projections or branches. Hyphal terminations near the pileus centre similar, terminal cells (8–)11–16.7–22(–40) × (2–)3–3.8–4.5(–5) µm, more frequently apically constricted, subterminal cells more frequently unbranched. **Pileocystidia** near the pileus margin frequent, always 1-celled, mainly narrowly clavate or subcylindrical, rarely fusiform, thin-walled, (13–)15.5–23–30(–52) × (3–)3.5–4–4.5(–5) µm, apically mainly obtuse, ocassionaly acute or constricted; contents dispersed and partly granulose or banded, hardly react in sulfovanillin. Pileocystidia near the pileus centre also one celled and similar in shape, (11–)16–22–28(–36) × (3–)3.5–4.1–4.8(–5.5) µm, apically usually constricted or with small, pearl-like appendage. **Cystidioid hyphae** dispersed in subpellis, not observed in the context.

Additional material studied: PANAMA, Chiriquí province, Fortuna Forest Reserve, Honda watershed, in monodominant *Oreomunnea mexicana* forest, 23 Aug 2012, Corrales 85, Chiriquí province, Fortuna Forest Reserve, Zarciadero, off of Bocas del Toro road, 2 km NW dam, trail originating at old ANAM station, 21 of May 2015, Ovrebo 5431, FH 18-045, FH 18-136

DISCUSSION

Here we describe the first members of the *Russula* subsection *Substriatinae* reported from the Americas. The identification of new collections from a geographical studied area comes with the challenge to recognize if those specimens were previously reported or described from the same area before. Also, if some species described from adjacent areas do not extend their distribution to the studied area. Our literature research resulted in a list of 26 species and subspecies described from *Quercus* dominated forests of Central and South America. Among them, 18 species are from Costa Rica, seven from Colombia and one from Panama (Supplementary material 1). Nine of these species were classified in the subgenus *Heterophyllidia*, but five are apparently members of sect. *Ingratae* or, according to our unpublished type studies, could even been part of subgenus *Archaea*. Remaining four species do not show similarities in pileus color with the species described in our study and their pileipellis structure is typical for subsect. *Griseinae* or subsect. *Virescentinae* (Buyck 1992).

The ITS variation between *R. floriformis* and *R. symphoniae* is represented only by three different nucleotide positions (Table 2), this corresponds to a similarity higher than 99% and it resulted in a lack of support for *R. floriformis* based only on the ITS tree (Fig. 3). Is the species rank appropriate for these related taxa? Several authors used an infraspecific rank for look-alike collections from distant regions, for example Buyck et al. (2003) described *R. polyphylla* subsp. *guanacastae* from *Quercus* forests of Costa Rica and the subsp. *polyphylla* was described from eastern USA (Adamčík et al. 2018). Low dissimilarity or complete similarity of ITS is reported

for several cryptic species of Basidiomycetes (Badotti et al. 2017). Adamčík et al. (2016) recognised three sympatric species of European *Xerampelinae* with >99% ITS similarity and they accepted them at species rank based on morphological differences, ecological preferences, and results of haplotype reconstruction. Such high ITS similarity shows also European *R. aurata* and Pakistani *R. aurantioflava* distinguished only by different climatic niches and geographical ditribution (Adamčík et al. 2019). On the other hand, Bazzicalupo et al. (2017) and Caboň et al. (2019) reported long distance dispersal of hemiboreal species of the Northern hemisphere with nearly or completely identical ITS among distant populations from different continents. Here we argue that the rank of these two taxa, *R. floriformis* and *R. symphoniae*, could be recognised based on our multilocus phylogenetic analysis (Fig. 2) combined with morphological arguments and ecological and geographical data.

The habitat and field data registered for *R. floriformis* and *R. symphoniae* indicates that the habitat for both species might be resctricted to host species distribution in montane tropical forests and that probably lowland forests represent a climatic barrier for their dispersal. Distribution patterns of *Quercus* evidence a clear coincidence in divergence, speciation and migrations episodes of this tree genus with geological and climatic dinamics in Central America (Kappelle, 2006; Rodríguez-Correa *et al*. 2015). Particularly, the closure of the Panamanian Isthmus provide a geographical conection between separated continents, where plants were the first specimens to migrate (Jaramillo 2018). Acording to Rodriguez-Correa (2015), the isthmus area along with the Darien region are an important modern barrier for gene flow but also an important source for *Quercus* species turnover. Botanical records of *Q. humboldtii* from mountains on the Darien region and massifs along the Colombian Caribbean (Rangel and Avella, 2011) provide evidence of the *Quercus* migration along this region to low altitude areas due to forest expantion in glacial periods, finally arriving to the Andes mountain range forming their current distribution range (Rangel and Avella, 2011; Rodriguez-Correa *et al*. 2015).

These relatively recent migration barriers around the Isthmus of Panama and the Darien region determinated the composition of oak forests in Colombia, probably also influencing their speciation process (Rodriguez-Correa *et al*. 2015). Our phylogenetic results suggest a coevolution hypothesis of ECM trees and fungi, considering that *R. floriformis* and *R. symphoniae* are closely related species with similar habitat requirements, and both were found in forests dominated by *Quercus*. It is probable that both *R. floriformis* and *Q. humboltii* evolved in approximately the same time as the results of migration, genetic isolation, and adaptation to local condition in the Colombian Andes. However, while we know a lot about *Quercus* spatial distribution and phylogenetic timing of the species evolution in the Americas (Hipp et al. 2019, another reference about distribution), the current knowledge about *R. floriformis* and *R. symphoniae* is limited to our reports from two small areas in Colombia and Panama (Fig. 1) and there are no available data about the time when these species pair divergence. To prove the hypothesis of coevolution of *Russula* with its host tree in the same time migration event, we would need more data about their actual and past occurrence and fosil recrods, but this hyphothesis seems to be at the moment the most likely explanation of the recent speciation of the sister species described in our study. Our observations are congruent with recent studies

demonstrating that local adaptation in combination with climatic disjunction are drivers of global ECM diversity (Větrovský et al. 2019) and these paterns are proved to be important also for *Russula* species divergence (Caboň *et al*. 2019).

There is a recognized lack of information about the ECM fungal diversity in the Neotropics, the geographic distribution of fungal species and their associated host, resulting in an information gap in evolutionary and ecological studies (Roy *et al.,* 2016; Nilsson *et al.*, 2006, Corrales et al. 2018). Considering the variation in ecological, geographical, climatic and ECM host tree conditions in Central and South America, a high *Russula* diversity is expected for this region (del Olmo-Ruíz *et al.*, 2017; Vasco-Palacio & Franco-Molano, 2013). However the current diversity records for Panama correspond to only eight species (Buyck and Ovrebo, 2002; Adamčík *et al*. 2019; Supplementary Table 1; https://biogeodb.stri.si.edu/fungi/en/search/quick?search_key=Russula) and for Colombia, only fifteen *Russula* species have been reported so far and six of them are described as records only found in the country (Wu *et al.* 1997; Vasco-Palacio and Franco-Molano 2013). Additionally, the information collected for the fungal species often doesn't have comparable descriptions due to the fact that much of them were described many years ago, with breef and limited morphological descriptions without standarized terminology and without molecular data available (Vasco-Palacio and Franco-Molano 2013). *Russula* is the ECM genus with the highest diversification rate (Varga *et al*. 2019) and the situation of two closely related species from adjacent but isolated regions described in this study may be more common than expected. Future studies should focus on getting new information for older and recently described *Russula* species, using both morphological and genetical aproaches, to gather comprehensive information for studies in biogeography of this region.

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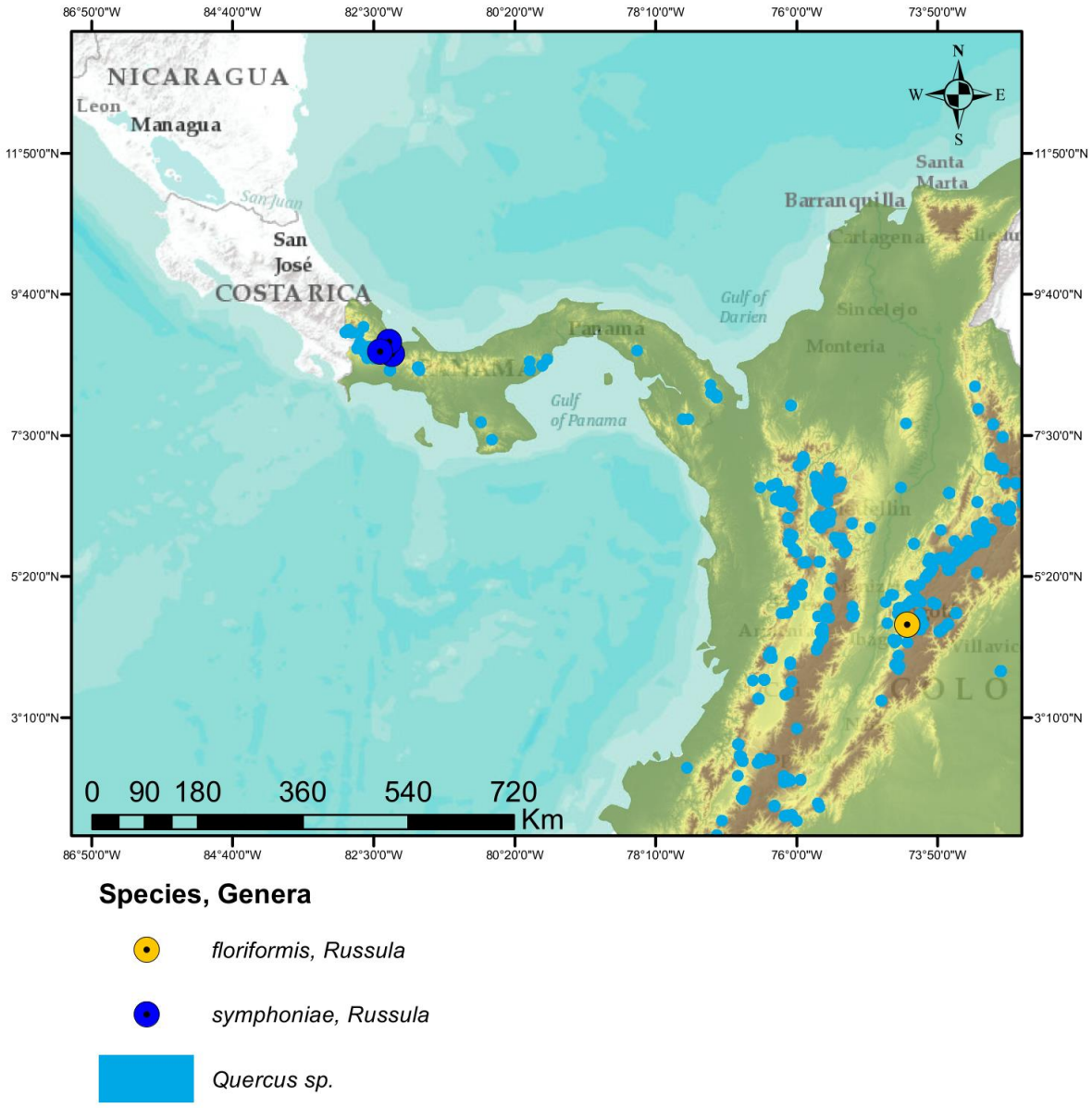

Figure 1. Origin areas of collection of *Russula* spp. analyzed for the present study and corresponding to the study areas described: In Panama were two locations, Fortuna Forest Reserve with two sample location in Fortuna Forest Reserve and in El Musgo, Jarmillo Arriba; In Colombia, all the samples were collected in Chicaque Natural Reserve. The distribution information of *Quercus* spp. was downloaded and used from the records submitted in GBIF Secretariat (2019).

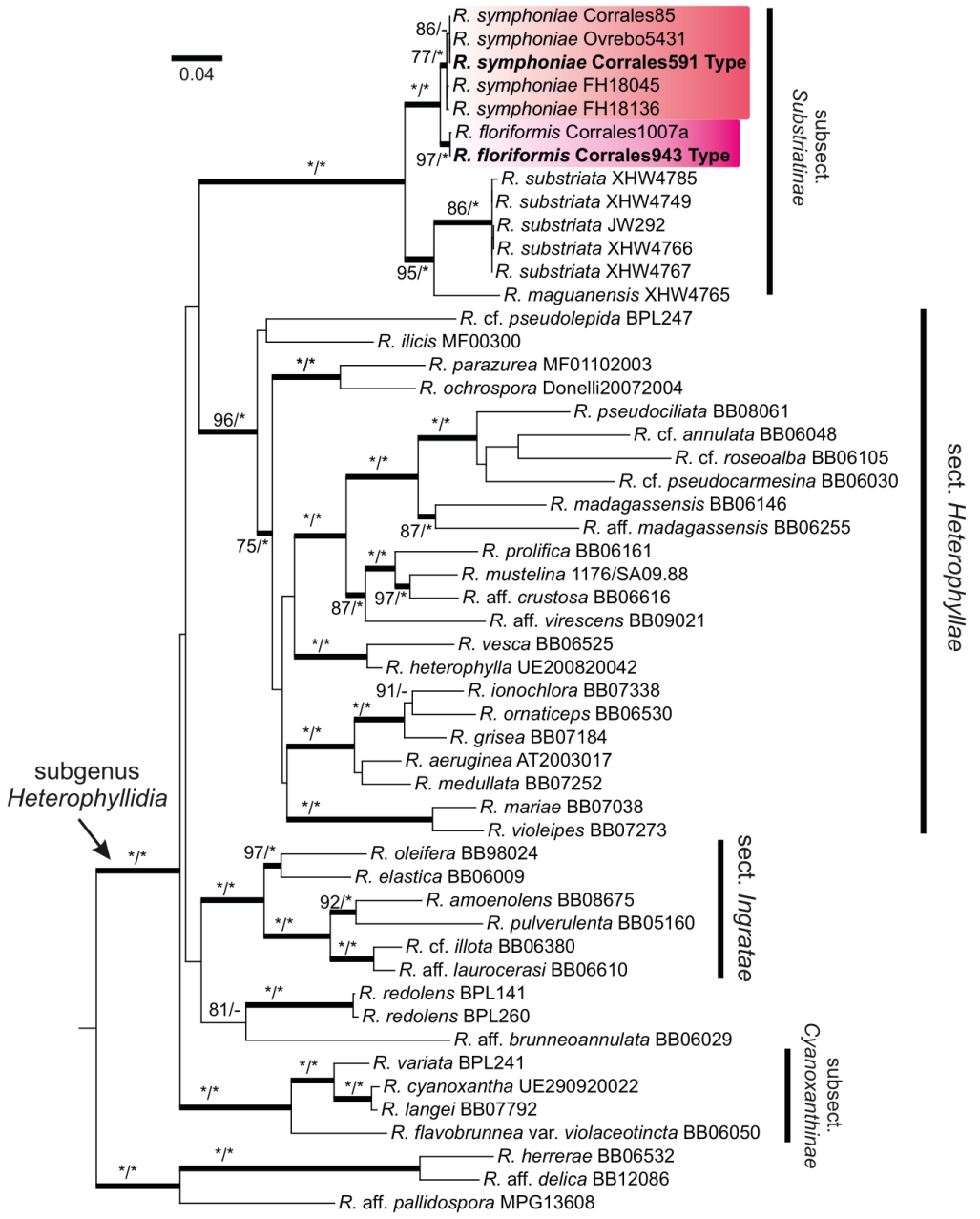

Figure 2. Phylogram generated by Maximum Likelihood (RAxML) analysis based on combined sequence data of LSU, *rpb2* and *tef1α* for showing position of studied species (highlighted by pink background) in *Russula* subsect. *Substriatinae* and the subgenus

Heterophyllidia. Branches in bold are supported by ML≥75 or BI≥0.95; asterisk (*) indicates full support. The tree is rooted with 3 collections of the subgenus *Brevipes*. Sequences obtained in this study are in the Table 1.

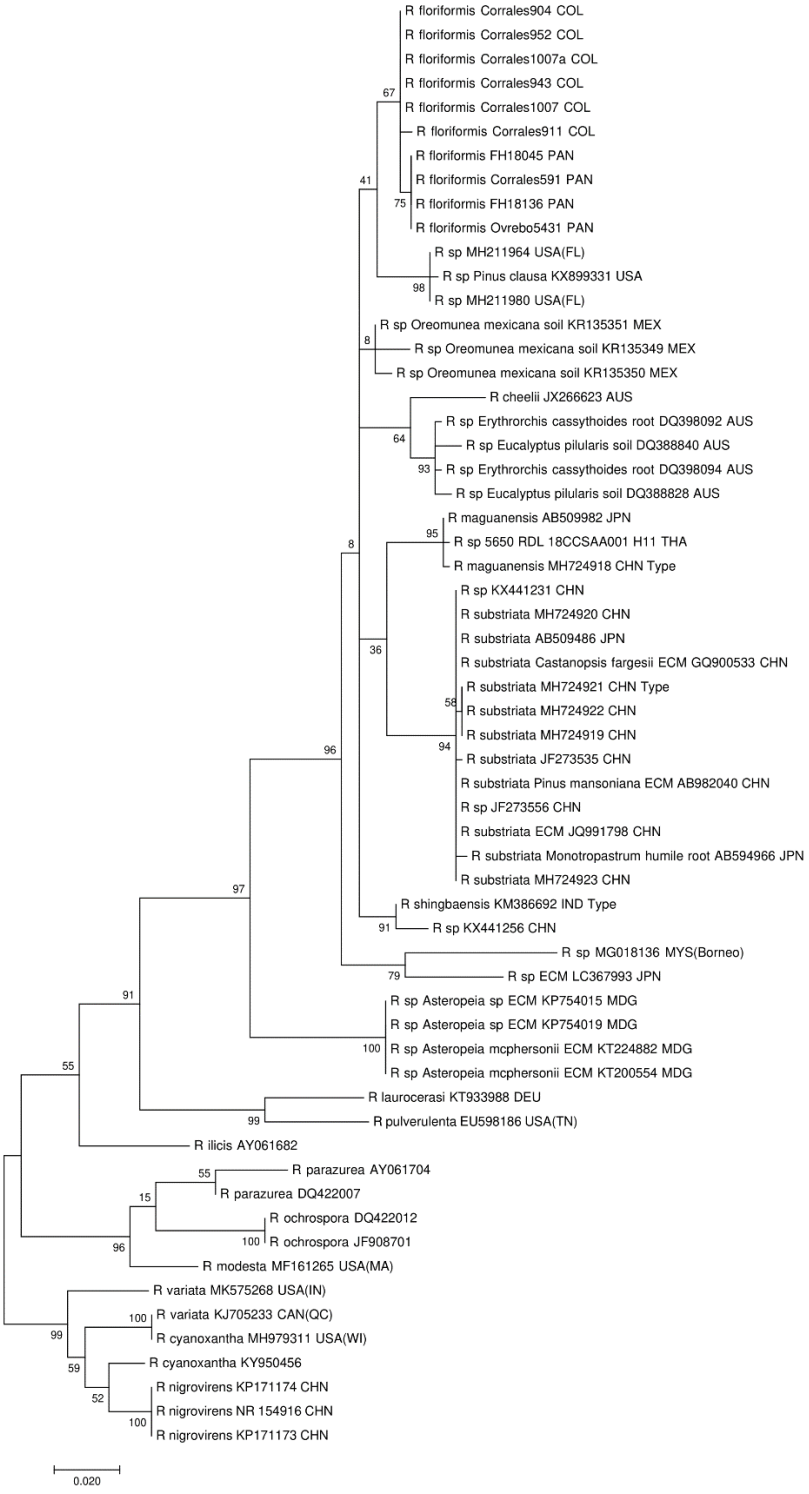

Figure 3. Phylogenetic tree of the *Russula* subsect. *Substriatinae* based on ITS region. Ex-type sequences are in bold. Studied species are highlighted by pink background, other American *Substriatinae* collections are labelled by pink outline. Plant hosts are provided (if available) for samples from ectomycorrhizal roots (followed by 'ECM'), orchid or myco-heterothrophs

(followed by ‘root’) or soil samples (followed by ‘soil’). Countries are abbreviated by ISO codes (https://www.iso.org/iso-3166-country-codes.html), in parentheses are US and Canadian states by postal codes (http://www.icq.eps.harvard.edu/ICQpost.html)

Figure 4. Basidiomata in the field. **a** *Russula floriformis* (Corrales 904). **b** *Russula floriformis* (Corrales 943). **c** *Russula symphoniae* (FH 18-045). **d** *Russula symphoniae* (FH 18-136). a-d scale bars = 10 mm. **e** Forest dominated by *Quercus humboldtii* in Chicaque, Colombia. **F** Forest dominated by *Oreomunnea mexicana* in Fortuna forest, Panama. Photo taken by James Dalling.

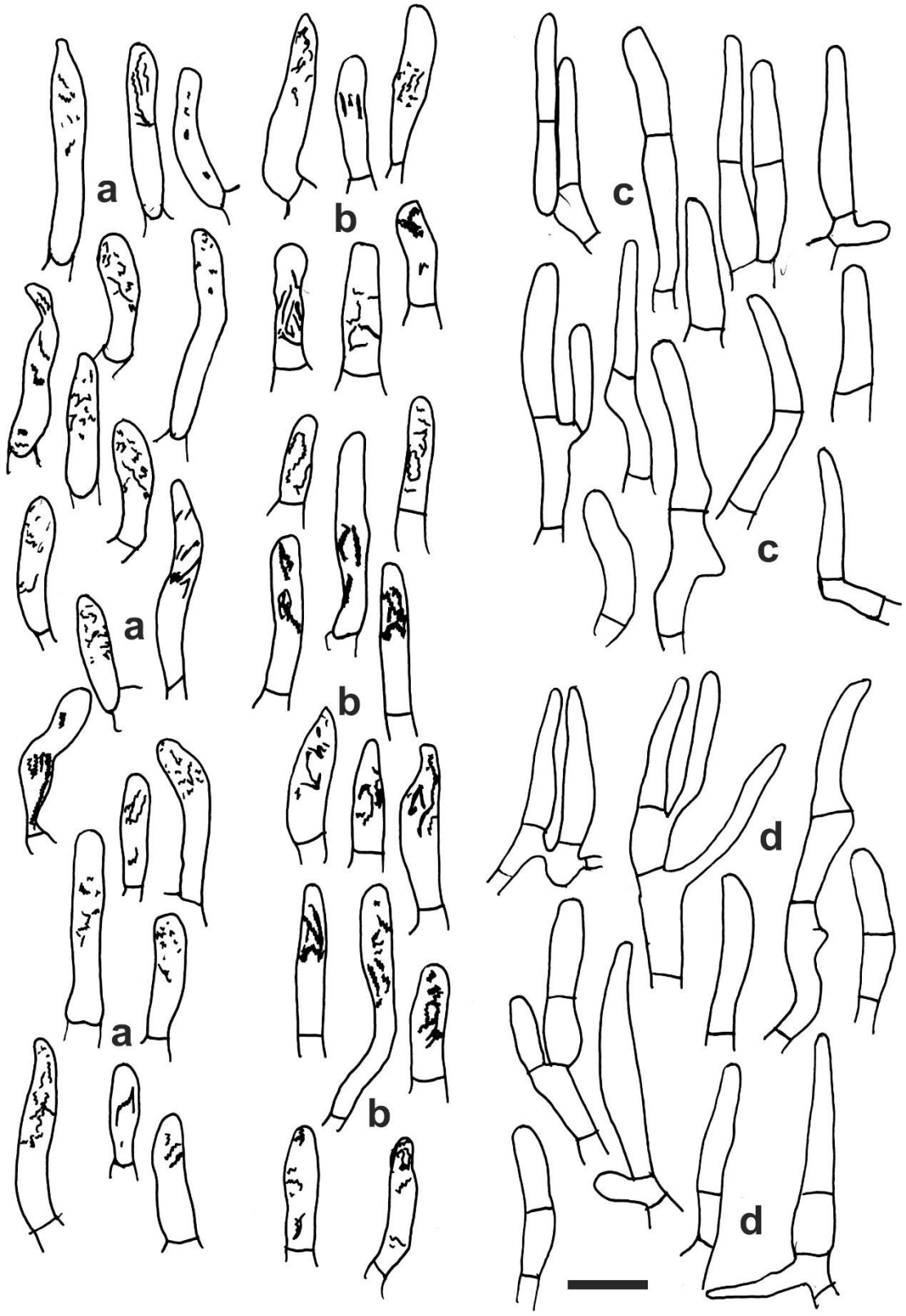
a
a
a
b
b
b
c
c
d
d

Figure 5. *Russula floriformis* (Corrales 943, holotype), pileipellis. **a.** Pileocystidia near the pileus center. **b.** Pileocystidia near the pileus margin. **c.** Hyphal terminations near the pileus center. **d.** Hyphal terminations near the pileus margin. Contents of cystidia as observed in Congo red. Scale bar = 10 μm. Drawings by Michelle Vera.

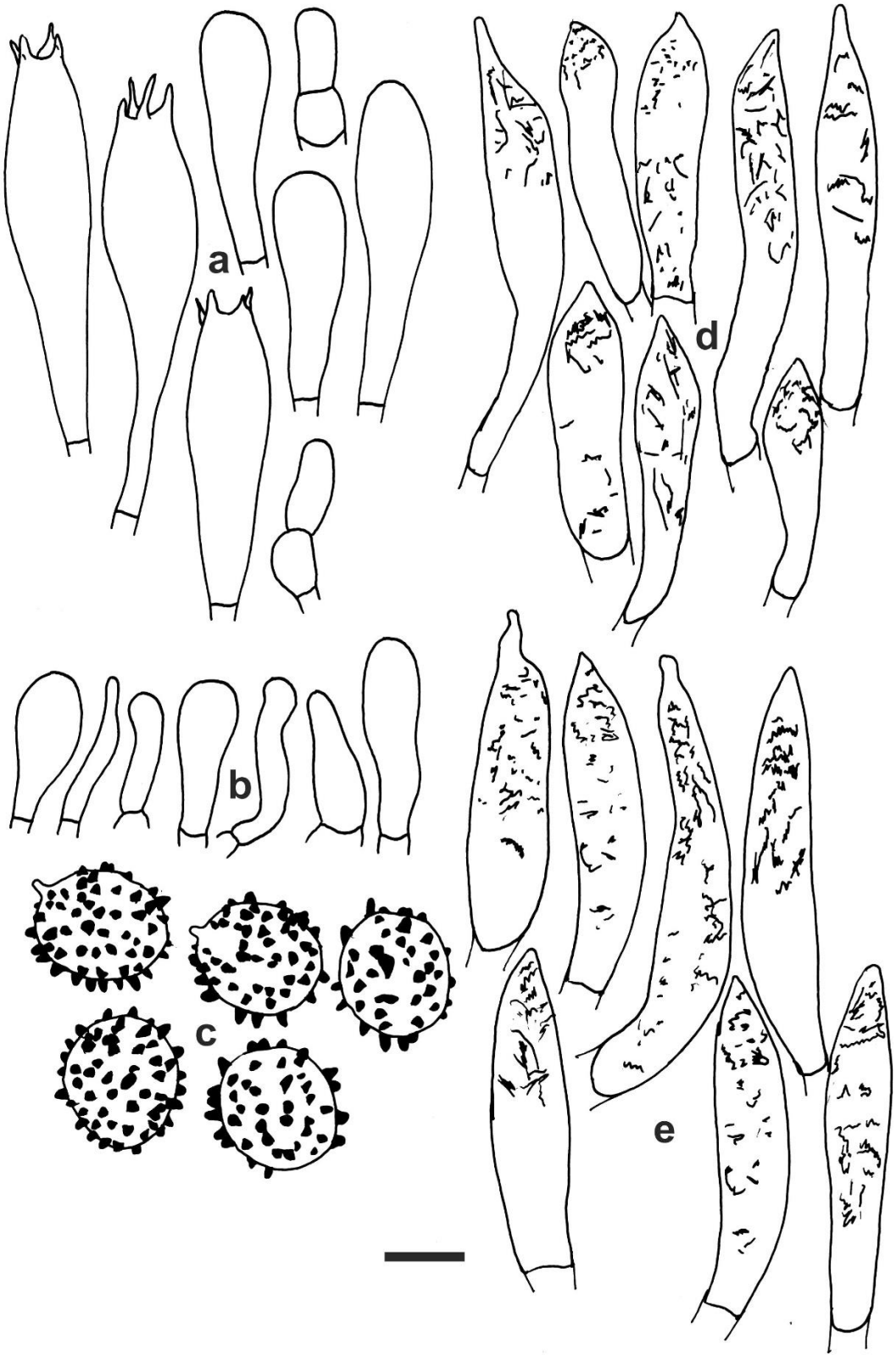

Figure 6. *Russula floriformis* (Corrales943, holotype). Microscopic features of the hymenium. **a.** Basidia and basidiola. **b.** Marginal cells. **c.** Basidiospores in Melzer's reagent. **d.** Hymenial cystidia near the lamellae edges. **e.** Hymenial cystidia on the lamellae sides. Cystidia with contents as observed in Congo Red. Scale bar = 10 μm, spores = 5 μm. Drawings by Michelle Vera.

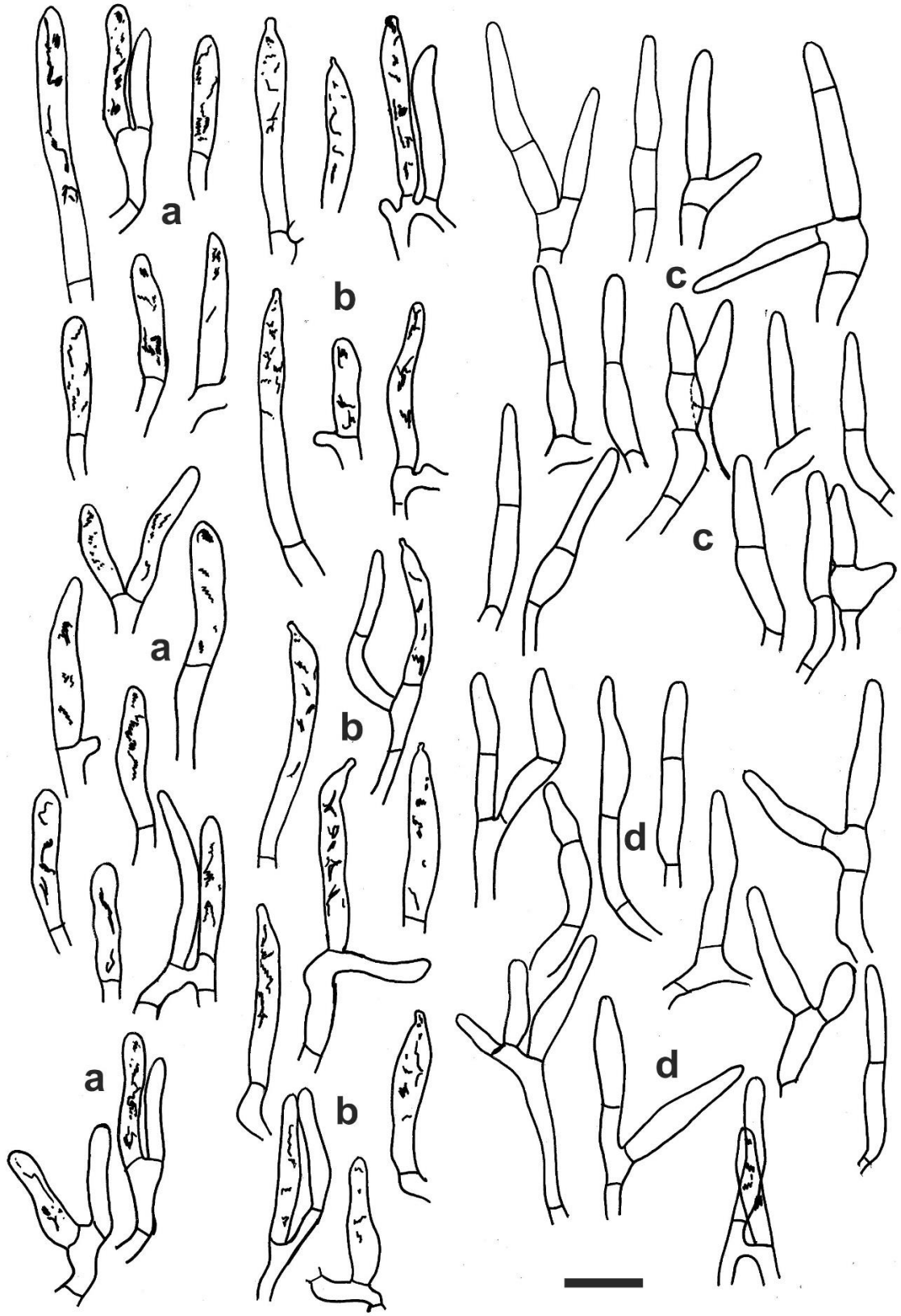

Figure 7. *Russula symphoniae* (Corrales591, holotype), pileipellis. **a.** Pileocystidia near the pileus center. **b.** Pileocystidia near the pileus margin. **c.** Hyphal terminations near the pileus center. **d.** Hyphal terminations near the pileus margin. Contents of cystidia as observed in Congo red. Scale bar = 10 μm. Drawings by Michelle Vera.

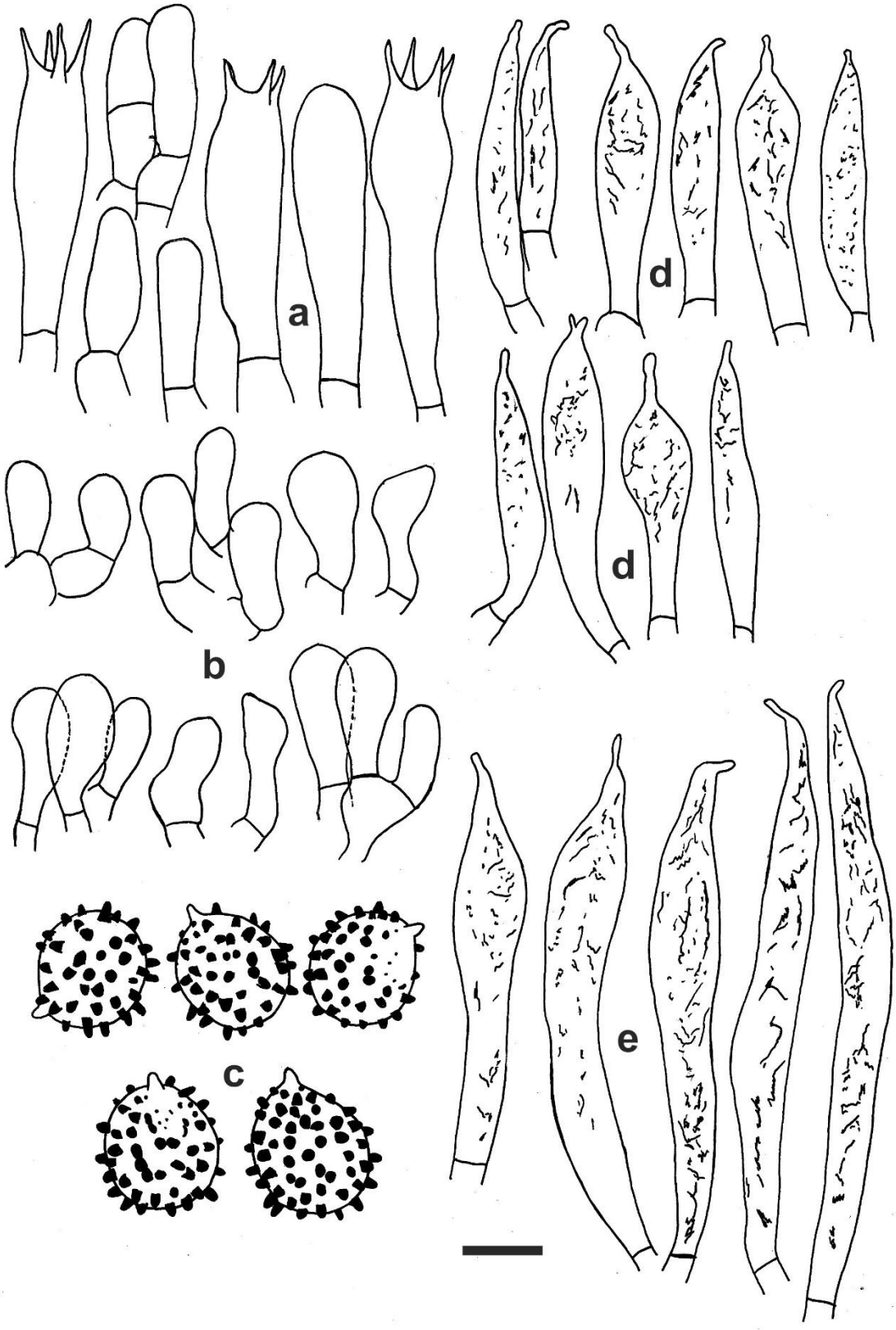

Figure 8. *Russula symphoniae* (Corrales591, holotype). Microscopic features of the hymenium. **a.** Basidia and basidiola. **b.** Marginal cells. **c.** Basidiospores in Melzer's reagent. **d.** Hymenial cystidia near the lamellae edges. **e.** Hymenial cystidia on the lamellae sides. Cystidia with contents as observed in Congo Red. Scale bar = 10 μm, spores = 5 μm. Drawings by Michelle Vera.

Table 1. List of sequences prepared during this study

	Voucher no.	*Country*	*ITS*	*LSU*	*mtSSU*	*rpb2*	*tef-1α*
R. floriformis	Corrales1007a	Colombia	xxx	xxx	xxx	xxx	xxx
R. floriformis	Corrales943	Colombia	xxx	xxx	xxx	xxx	xxx
R. floriformis	Corrales911	Colombia	xxx		xxx		
R. floriformis	Corrales1007	Colombia	xxx	xxx	xxx		
R. floriformis	Corrales952	Colombia	xxx	xxx	xxx		
R. floriformis	Corrales904	Colombia	xxx	xxx	xxx		
R. symphoniae	Corrales591	Panama	xxx	xxx	xxx	xxx	xxx
R. symphoniae	Ovrebo5431	Panama	xxx		xxx	xxx	xxx
R. symphoniae	FH18136	Panama	xxx	xxx		xxx	xxx
R. symphoniae	Corrales85	Panama		xxx	xxx	xxx	
R. symphoniae	FH18045	Panama	xxx	xxx		xxx	xxx

Table 2. Nucleotide variation according to each nuclear sides for the specimen evaluated of *R. floriformis* and *R. symphoniae*. The single line represents no data for the specimen.

	ITS			LSU		rpb2							tef1α		
	17	579	581	191	699	90	142	160	205	343	391	727	174	859	941
Rf_Corrales911	T	C	A	–	–	–	–	–	–	–	–	–	–	–	–
Rf_Corrales1007	Y	C	A	–	–	–	–	–	–	–	–	–	–	–	–
Rf_Corrales952	Y	C	A	G	G	–	–	–	–	–	–	–	–	–	–
Rf_Corrales904	C	C	A	–	G	–	–	–	–	–	–	–	–	–	–
Rf_Corrales1007a	Y	C	A	G	G	T	C	C	T	G	G	T	A	C	T
Rf_Corrales943	C	C	A	G	G	T	C	C	T	G	G	T	R	C	T
Rs_Corrales591	C	T	G	A	A	A	T	S	Y	A	T	C	G	T	C
Rs_Ovrebo5431	C	T	G	–	–	A	T	C	Y	A	T	C	G	T	C
Rs_FH18136	C	Y	G	R	A	T	T	S	Y	A	K	C	–	Y	C
Rs_FH18045	C	T	G	A	A	T	T	S	T	A	K	C	–	C	C
Rs_Corrales85	–	–	–	R	A	A	T	S	Y	–	–	–	–	–	–

Supplementary Material 2: List of molecular markers, primers and cycling protocols used in this study.

Molecular marker	**Primers**	**Cycling protocol**
Internal transcribed spacer regions of ribosomal DNA (ITS)	ITS1F+ITS4 (White et al. 1990, Gardes & Bruns 1993)	Ondrušková et al. 2017
Partial large subunit ribosomal DNA (LSU)	LROR+LR5 (Moncalvo et al. 2000)	Pastirčáková et al. 2018
Partial mitochondrial small subunit of ribosomal DNA (mtSSU)	MS1+MS2 (White et al. 1990)	Ondrušková et al. 2017
Region between domains six and seven of the nuclear gene encoding the second largest subunit of RNA polymerase II (*rpb2*)	bRPB2-6F+ bRPB2-7.1R (Matheny 2005)	Caboň et al. 2017
Transcription elongation factor 1-alpha (*tef-1α*)	*tef1*F+*tef1*R (Morehouse et al. 2003) 983F+1567R (Rehner & Buckley 2005) 526F(www.aftol.org/pdfs/EF1primer.pdf) + «GAAATRCCNGCYTCGAATTCACC» (*)	Morehouse et al. 2003