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Morphological and genetic diversification of *Russula floriformis*, sp. nov., along the Isthmus of Panama

Michelle Vera[a], Slavomír Adamčík[b], Katarína Adamčíková[c], Felix Hampe[d], Miroslav Caboň[b], Cathrin Manz[e], Clark Ovrebo[f], Meike Piepenbring[e], and Adriana Corrales[a]

[a]Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia; [b]Institute of Botany, Plant Science and Biodiversity Center, Slovak Academy of Sciences, Dúbravská cesta 9, 845 23 Bratislava, Slovakia; [c]Department of Plant Pathology and Mycology, Institute of Forest Ecology, Slovak Academy of Sciences Zvolen, Akademická 2, 949 01 Nitra, Slovakia; [d]Wetzlarer Str. 1, 35510 Butzbach, Germany; [e]Department of Mycology, Goethe University Frankfurt am Main, Biologicum, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany; [f]Department of Biology, University of Central Oklahoma, 100 N. University Drive, Edmond, Oklahoma 73034

ABSTRACT

Species of *Russula* are ubiquitous members of ectomycorrhizal fungal communities in tropical ecosystems. However, an important part of the total tropical diversity of this genus and its biogeographic patterns is unknown due to the lack of studies on *Russula* in tropical ecosystems. We combined molecular, morphological, ecological, and biogeographic data to elaborate concepts for two new subspecies of *R. floriformis* (subsection *Substriatinae*). *Russula floriformis* subsp. *floriformis* and *R. floriformis* subsp. *symphoniae* are described as new from montane forest dominated by *Quercus* and/or *Oreomunnea* (Fagales) from Colombia and Panama, respectively. Phylogenies were constructed using nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2 (ITS), D1–D2 domains of nuc 28S rDNA (28S), and partial regions of the second largest subunit of RNA polymerase II (*rpb2*) and translation elongation factor 1-alpha (*tef1*). Similar environmental conditions, similar morphology, and an ITS sequence similarity higher than 99% with only three different positions indicate that these two subspecies are closely related. Detailed observations of microscopic structures and analyses of further DNA loci, however, revealed morphological and molecular characteristics that allow distinguishing the two subspecies of *R. floriformis*. Spatial distribution and phylogenetic proximity of the two *Russula* subspecies and their ectomycorrhizal hosts, i.e., species of *Quercus*, suggest that their diversification is a result of comigration, adaptation, and geographic isolation along the Isthmus of Panama during the Pliocene and Pleistocene.

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INTRODUCTION

The closing of the Isthmus of Panama during the Pliocene and Pleistocene produced an important biotic exchange between Central and South America that resulted in the migration of many North American species to South America and vice versa (Stehli and Webb 1986; Cody et al. 2010). The biogeographic patterns associated with this migration have been studied for many species of plants and animals (Webb and Rancy 1996; Burnham and Graham 1999) but for the most part remain undocumented for microbial communities.

Species of *Quercus* (oaks) are common and widely distributed trees in 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 through Central America and express their greatest diversity in southern Mexico where approximately 160 to 210 species have been documented. Species numbers of oaks, however, decrease southward, with nine species known in Panama and only one species, *Quercus humboldtii*, found in Colombia (Correa et al. 2004; Hooghiemstra 2006; Kappelle 2006; Nixon 2006; Morales Pacheco et al. 2018).

Species of *Quercus* originated in the Northern Hemisphere and migrated to South America along the Isthmus of Panama (Hooghiemstra 2006), and it has been suggested that oaks have comigrated with root-associated symbiotic fungi (Halling 2001). *Quercus* arrived in central west Mexico during the Miocene about 10 million years ago (Ma) with an ecosystem transition from a wet evergreen forest to a semideciduous forest and migrated to South America after the closure of the Isthmus of Panama during the Pliocene, along with other highland ectomycorrhizal (ECM) tree species (e.g., *Alnus*), approximately 5 and 3.5 Ma (Hooghiemstra 2006). The Panama Isthmus emergence caused the closure of the gap between North

CONTACT Slavomír Adamčík slavomir.adamcik@savba.sk
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and South America and the onset of the Great American Biotic Interchange (Bacon et al. 2015; Jaramillo 2018). The divergence of *Q. humboldtii* from its nearest central American phylogenetic relative, *Q. costaricensis*, is estimated to have happened around 3.5 Ma, coinciding with the Panama Isthmus emergence and glaciation events during the Miocene and Pliocene (Hipp et al. 2020). The last migration of montane tree species to South America was facilitated by lower temperatures during the Last Glacial Maximum (LGM) and their subsequent raising during the Last Interglacial period (LIG) (Hooghiemstra 2006; Ramírez-Barahona and Eguiarte 2013).

Species of *Russula* form ECM associations with plants in major biomes worldwide (Looney et al. 2018), including frequent associations with *Quercus* in temperate, Mediterranean, subtropical, and tropical habitats (Richard et al. 2005; Morris et al. 2009; Hynes et al. 2010; Wang et al. 2012). *Russula* includes more than 2000 species worldwide, with distribution ranges of individual species often limited by climate and geographic barriers (Adamčík et al. 2019). *Russula* is considered the second most taxonomically diverse genus of ECM fungi after the genus *Cortinarius* (Pers.) Gray, and its taxonomic classification is challenging due to the detailed morphological information required for a valid identification (Looney et al. 2016; Manz 2019). In the Americas, more than 400 *Russula* species have been reported from the USA (Adamčík et al. 2018) and 77 from Central and South America, including eight species of *Russula* from Panama (Buyck and Ovrebo 2002; Hennicke and Piepenbring 2008; Adamčík et al. 2019) and 15 from Colombia (Wu et al. 1997; Vasco-Palacios and Franco-Molano 2013) (SUPPLEMENTARY TABLE 1).

Recent research and collections from the neotropics show that tropical montane forests are richer in ECM fungi than tropical lowland forests due to a higher density and abundance of ECM host trees, mostly in the families Betulaceae, Fagaceae, Juglandaceae, and Pinaceae (Buyck and Halling 2004; Barroetaveña et al. 2007; Smith et al. 2011; García-Guzmán et al. 2017; Corrales et al. 2018). In neotropical montane forests, the best-known ECM system corresponds to *Quercus*-dominated forests represented by approximately 24 *Russula* species described from oak forests from Mexico, Costa Rica, and Colombia (Wu et al. 1997; Franco-Molano et al. 2010; SUPPLEMENTARY TABLE 1).

Host specificity is an important driver of the evolution of species of *Russula* (Looney et al. 2016). With migration, climatic isolation, and speciation of South American *Q. humboldtii* from Latin American oak species, one might also expect allopatric diversification of symbiotic fungal partner species of the genus *Russula*. However, several studies have demonstrated that most ECM fungal species associated with *Quercus* may also colonize roots of alternative host tree species, which could be evidence that adaptation to local ecological and climatic conditions may be more important than availability of their host partner (Richard et al. 2005; Caboň et al. 2019).

A further common ECM host tree occurring in montane forests of Central America is the species *Oreomunnea mexicana* (Juglandaceae). This species is distributed from Mexico to Panama at elevations between 900 and 2600 m 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 species of *Oreomunnea* and *Quercus* (Corrales et al. 2016; Alfonso-Corrado et al. 2017; Corrales and Ovrebo 2021), but it is unknown to what extent the ECM diversity overlaps.

The present study focuses on the analysis of morphologically similar specimens of *Russula* from subgenus *Heterophyllidiae* Romagn. subsect. *Substriatinae* X.H. Wang & Buyck collected in *Quercus* and *Oreomunnea* forests in Colombia and Panama at sites located east and west of the narrowest part of the Isthmus of Panama. Species from *Russula* subgenus *Heterophyllidiae* are recognized by inamyloid suprahilar spots on basidiospores, densely septate hyphal terminations on the pileipellis, one-celled pileocystidia (if present), and pale-colored spore prints (Buyck and Adamčík 2011). Members of the subsection *Substriatinae* typically have strongly tuberculate-striate pilei with a pink and finely cracked surface, relatively large basidiospores ornamented by large isolated warts, and abundant pileocystidia variable in size and endings (Wang et al. 2019).

Recently, we collected specimens of this subsection in western Panama and Colombia. Nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2 (ITS) sequences of these collections show similarities higher than 99%. We aim to evaluate whether these differences in the ITS region corresponds to two distinct taxa separated by the Isthmus of Panama. We combine sequence data from four additional DNA regions and morphological and ecological observations to determine whether the collections separated by the Isthmus of Panama are distinct taxa and their appropriate taxonomic rank. To our knowledge, this is the first study focusing on ECM fungal species diversification incorporating population samples north and south of the disjunction at the Isthmus of Panama.

MATERIALS AND METHODS

Study area.—Specimens were collected in two regions of Colombia and Panama, and all represent different mycelia (FIG. 1). In Panama, five specimens were collected at

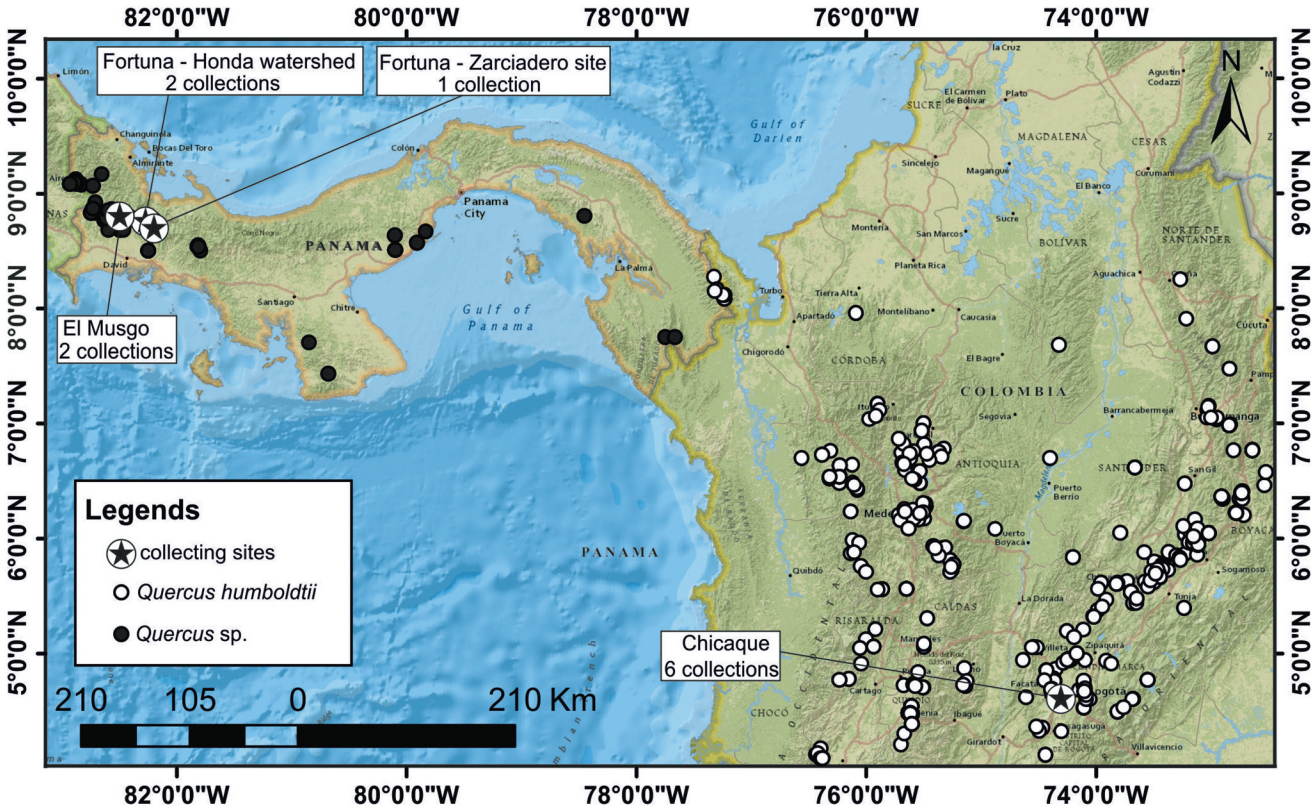


Figure 1. Collecting sites of *Russula* analyzed in the present study and distribution of *Quercus* species according to the Global Biodiversity Information Facility (www.gbif.org). All native *Quercus* records from Colombian natural habitats are labeled as *Q. humboldtii*.

three collecting sites. Two of these were located in a primary lower montane forest (alt. 1000–1400 m) at the Fortuna Forest Reserve in western Panama. Collections in this reserve were made between Apr 2012 and May 2015, two on the Honda watershed (8°45′17.16″ N, 82°14′27.12″W) and one on Zarciadero site near Bocas del Toro Road (8°56′47″N, 82°15′00.68″W). The mean annual temperature registered for the Fortuna Forest Reserve ranges from 19 to 22 C, and the annual rainfall varies from ca. 5800 to 9000 mm (Cavelier et al. 1996; Andersen et al. 2012). Sampling in Fortuna Forest Reserve was done in forests dominated by *Oreomunnea mexicana* with scattered individuals of *Quercus* cf. *lancifolia* and *Alfaroa costaricensis*. The third collecting site was El Musgo, Jaramillo Arriba, Boquete (08°47′26.9″N, 82°24′ 34.4″W). Two collections were made at El Musgo in Jun 2018 at ca. 1670 m in a moss-rich montane cloud forest dominated by several species of *Quercus*, which were the only potential ectomycorrhizal host trees present at that locality. The six Colombian specimens representing different mycelia were collected in an oak forest dominated by *Q. humboldtii* located in Chicaque Natural Park (4°36′22″N, 74°18′17″W) in the eastern part of the Andean Cordillera of Colombia during Nov 2019. The reserve includes approximately 300 ha of cloud forest between 2000 and 2700 m. The weather in this area is characterized by a bimodal rainfall regime with the highest rainfall occurring between Mar–May and Oct–Nov and an average temperature of 15 C year-round (Rivera and Cordoba 1998; Ávila de Navia and Estupiñán Torres 2013). Colombian collections were deposited at herbaria HUA and SAV and Panamanian collections at PMA, SAV, ARIZ, CSU, and UCH (herbarium codes follow those in Index herbariorum; Thiers [continuously updated]).

Molecular analysis.—Genomic DNA was extracted from fresh and dried basidiomata and stored in cetyltrimethylammonium bromide (CTAB) 2× following Gardes and Bruns (1993). DNA amplification was performed using 5× HOT FIREPol Blend Master Mix (Solis BioDyne, Tartu, Estonia) or *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts) following the manufacturers' instructions. Five loci were amplified and sequenced: (i) nuc rDNA ITS1-5.8S-ITS2 (ITS); (ii) mt 12S rDNA; (iii) D1–D2 domains of nuc 28S rDNA (28S); (iv) the region

Table 1. Samples of *Russula* spp. used in multilocus phylogenetic analyses (FIG. 2).

Russula species	Voucher no.	Country	ITS	28S	12S	*rpb2*	*tef1*
R. aeruginea	AT2003017	Sweden	—	DQ421999	—	—	—
R. aff. *brunneoannulata*	BB 06.029	Madagascar	—	KU237452	—	KU237738	KU237887
R. aff. *crustosa*	BB 06.616	Canada	—	KU237461	—	KU237747	KU237896
R. aff. *delica*	BB 12.086	Italy	—	KU237594	—	KU237879	KU238020
R. aff. *laurocerasi*	BB 06.610	Mexico	—	KU237458	—	KU237744	KU237893
R. aff. *pallidospora*	MPG13.6.08	Spain	—	KU237580	—	KU237866	KU238007
R. aff. *virescens*	BB 09.021	New Caledonia	—	KU237582	—	KU237868	KU238009
R. amoenolens	BB 08.675	Italy	—	KU237562	—	KU237848	—
R. cf. *annulata*	BB 06.048	Madagascar	—	KU237470	—	KU237756	KU237902
R. cf. *illota*	BB 06.380	Mexico	—	KU237464	—	KU237750	KU237898
R. cf. *pseudocarmesina*	BB 06.030	Madagascar	—	KU237453	—	KU237739	—
R. cf. *pseudolepida*	BPL247	USA	—	KT933821	—	KT933892	—
R. cf. *roseoalba*	BB 06.105	Madagascar	—	KU237472	—	KU237758	—
R. cyanoxantha	UE29.09.2002-2	France	—	DQ422033	—	—	—
R. elastica	BB 06.009	Madagascar	—	KU237451	—	KU237737	—
R. flavobrunnea var. *violaceotincta*	BB 06.050	Madagascar	—	KU237468	—	KU237754	KU237901
R. floriformis subsp. *floriformis*	Corrales1007a	Colombia	**MT039869**	**MT023732**	**MT039864**	**MT021754**	**MT024555**
R. floriformis subsp. *floriformis*	Corrales911	Colombia	**MT068204**	**—**	**MT075614**	**—**	**—**
R. floriformis subsp. *floriformis*	Corrales1007	Colombia	**MT068206**	**MT068212**	**MT075616**	**—**	**—**
R. floriformis subsp. *floriformis*	Corrales952	Colombia	**MT068205**	**MT068213**	**MT075615**	**—**	**—**
R. floriformis subsp. *floriformis*	Corrales904	Colombia	**MT068203**	**MT068211**	**MT075613**	**—**	**—**
R. floriformis subsp. *floriformis* HT	Corrales943	Colombia	**MT039866**	**MT023729**	**MT039861**	**MT021752**	**MT024552**
R. floriformis subsp. *symphoniae*	Ovrebo5431	Panama	**MT039868**	**—**	**MT039863**	**MT021754**	**MT024554**
R. floriformis subsp. *symphoniae*	FH-18-136	Panama	**MT039870**	**MT023733**	**—**	**MT021756**	**—**
R. floriformis subsp. *symphoniae*	Corrales85	Panama	**—**	**MT023731**	**MT039865**	**MT021758**	**—**
R. floriformis subsp. *symphoniae*	FH-18-045	Panama	**MT039871**	**MT023734**	**—**	**MT021757**	**—**
R. floriformis subsp. *symphoniae* HT	Corrales591	Panama	**MT039867**	**MT023730**	**MT039862**	**MT021753**	**MT024553**
R. grisea	BB 07.184	Slovakia	—	KU237509	—	KU237795	KU237939
R. herrerae	BB 06.532	Mexico	—	KU237486	—	KU237772	KU237915
R. heterophylla	UE20.08.2004-2	Sweden	—	DQ422006	—	—	—
R. ilicis	MF 00.300	Italy	—	KU237595	—	KU237880	KU238021
R. ionochlora	BB 07.338	France	—	KU237508	—	KU237794	KU237938
R. langei	BB 07.792	France	—	KU237510	—	KU237796	KU237940
R. madagassensis	BB 06.255	Madagascar	—	KU237475	—	KU237761	KU237906
R. madegassensis	BB 06.146	Madagascar	—	KU237456	—	KU237742	KU237891
R. maguanensis HT	XHW4765	China	—	MH714537	—	MH939989	MH939983
R. mariae	BB 07.038	USA	—	KU237538	—	KU237824	KU237968
R. medullata	BB 07.252	Slovakia	—	KU237546	—	KU237832	KU237976
R. mustelina	1176/SA 09.88	Slovakia	—	KU237596	—	KU237881	KU238022
R. ochrospora	Donelli20.07.2004	Italy	—	DQ422012	—	—	—
R. oleifera	BB 98.024	Burundi	—	KU237490	—	KU237776	KU237919
R. ornaticeps	BB 06.530	Canada	—	KU237466	—	KU237752	—
R. parazurea	MF01.10.2003	Sweden	—	DQ422007	—	—	—
R. prolifica	BB 06.161	Madagascar	—	KU237455	—	KU237741	KU237890
R. pseudociliata	BB08.061	Madagascar	—	KU237537	—	KU237823	KU237967
R. pulverulenta	BB 05.160	USA	—	KU237563	—	KU237849	—
R. redolens	BPL141	USA	—	KT933808	—	KT933879	—
R. redolens	BPL260	USA	—	KT933825	—	KT933897	—
R. substriata	XHW4749	China	—	MH714539	—	MH939991	MH939985
R. substriata	XHW4767	China	—	MH714541	—	MH939993	MH939987
R. substriata	JW292	China	—	MH939984	—	MH939990	MH939984
R. substriata	XHW4785	China	—	MH714542	—	MH939994	MH939988
R. substriata HT	XHW4766	China	—	MH714540	—	MH939992	MH939986
R. variata	BPL241	USA	—	KT933818	—	KT933889	—
R. vesca	BB 06.525	Mexico	—	KU237465	—	KU237751	KU237899
R. violeipes	BB 07.273	Slovakia	—	KU237534	—	KU237820	KU237964

Note. Sequences obtained in this study are in bold. HT denotes holotype.

between domains 6 and 7 of the nuclear gene encoding the second largest subunit of RNA polymerase II (*rpb2*); and (v) a portion of transcription elongation factor 1-alpha (*tef1*). The ITS region was amplified using the primer pair ITS1-F and ITS4 (White et al. 1990; Gardes and Brun 1993); 12S using MS1 and MS2 (White et al. 1990); 28S using LR0R and LR5 (Moncalvo et al. 2000); *rpb2* using bRPB2-6F and bRPB2-7.1R (Matheny 2005); and *tef1* using tef1F and tef1R (Morehouse et al. 2003). Polymerase chain reaction (PCR) cycling conditions for all loci were the same as described in Buyck et al. (2020). Amplicons were purified using ExoSap-IT (Thermo Fisher Scientific, Wilmington, Delaware) or QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent to Seqme Company (Dobříš, Czech Republic) and Macrogen Korea (Seoul, South Korea) for Sanger sequencing.

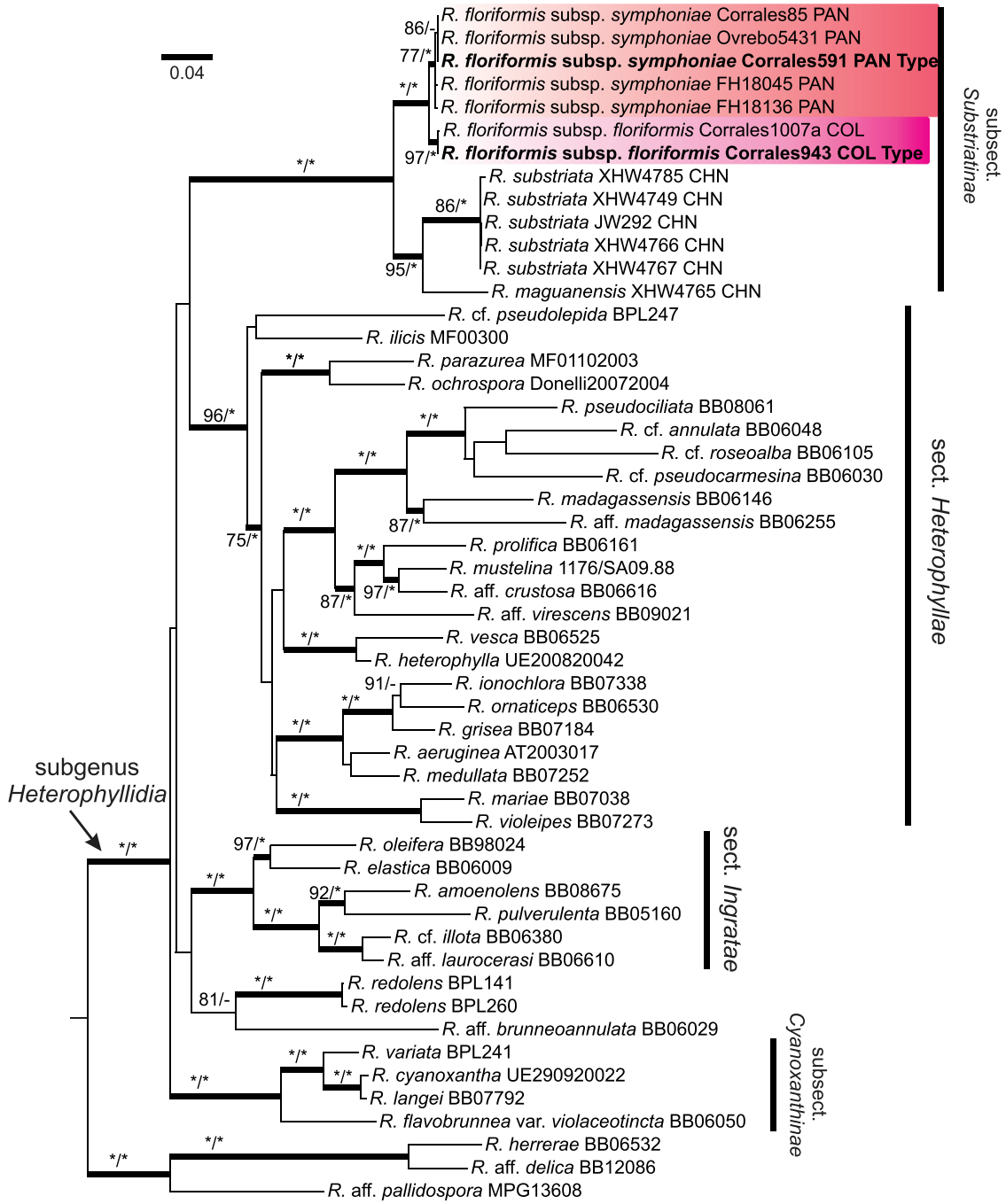


Figure 2. ML tree of *Russula* subsect. *Substriatinae* and subg. *Heterophyllidiae* based on combined sequence data of 28S, *rpb2*, and *tef1*. Studied samples are highlighted by a pink background. Branches in bold are supported by ML ≥ 75 or BI ≥ 0.95; asterisks (*) indicate full support. The tree is rooted with samples of subg. *Brevipedum*.

Phylogenetic analysis.—Sequences were edited in the BioEdit 7.2.5 sequence alignment editor (Hall 2013) and Geneious R10 (Kearse et al. 2012). Intraindividual polymorphic sites having more than one signal were marked with International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes. For phylogenetic placement of studied species, we mainly used sequence data of 28S, *rpb2*, and *tef1* published by Wang et al. (2019). This data set was supplemented with two samples of *Russula redolens* unrecognized by the latter study. We analyzed publicly available ITS sequences retrieved by BLAST searches for estimating species diversity and distribution patterns of subsection *Substriatinae*. The final data sets were aligned in MAFFT 7 (Katoh and Standley 2013) using the E-INS-i strategy and manually edited. We used complete sequences of all DNA regions. No sites were excluded. All sequences obtained in

this study and their accession numbers are listed in TABLE 1. The multilocus data set was analyzed using Bayesian inference (BI) and maximum likelihood (ML). For the BI analysis, the data set was divided into nine partitions: 28S, intronic regions of *tef1*, the 1st, 2nd, and 3rd codon positions of *tef1*, an intronic region of *rpb2*, and the 1st, 2nd, and 3rd codon positions of *rpb2*. The best substitution model for each partition was computed jointly in PartitionFinder 1.1.1 (Lanfear et al. 2012). BI runs were computed twice in MrBayes 3.2.6 (Ronquist et al. 2012) with four Markov chain Monte Carlo (MCMC) chains for 10 million iterations until the standard deviation of split frequencies fell below the 0.01 threshold. Convergence of runs was visually assessed using the trace function in Tracer 1.6 (Rambaut et al. 2013). For the ML analysis, the concatenated alignment was loaded as a fasta file to CIPRES Science Gateway (Miller et al. 2010) and analyzed using RAXML-HPC2 on XSEDE (8.2.12) as a partitioned data set (as in the BI analysis) under the GTR+GAMMA model with 1000 bootstrap iterations as recommended by the RAXML user manual (Stamatakis 2014). The ITS data set was analyzed on the CIPRES Science Gateway (Miller et al. 2010) as a partitioned data set to the ITS1, 5.8S, and ITS2 loci (as recommended by Petkovits et al. 2011) under the GTR+GAMMA model with 1000 bootstrap iterations (Stamatakis 2014). Both ITS and multilocus alignments have been deposited at TreeBASE (study no. 27076).

Morphological analysis.—Field characters associated with morphological features were described based on fresh basidiomata following Adamčík et al. (2019). All samples were processed as voucher herbarium specimens by dehydration on a food dryer at 40 C for 24 h. All micromorphological structures were observed following standards proposed by Adamčík et al. (2019). Micromorphological characteristics were observed using an Olympus CX-43 microscope with an oil immersion lens at a magnification of 1000× (Tokyo, Japan). The structures were visualized and illustrated with a camera lucida using an Olympus U-DA drawing attachment at a projection scale of 2000×. The structures and contents of hymenial cystidia and pileocystidia were observed and measured in ammoniacal Congo red preparations from dried material. Basidiospores were observed on the surface of lamellae in Melzer's reagent. All other microscopic observations were made in ammoniacal Congo red after a short treatment in an aqueous 10% KOH solution to dissolve any gelatinous matrices and improve tissue dissociation. Additionally, all tissues were examined in cresyl blue to detect the presence of ortho- or metachromatic reactions following Buyck (1989). Trama and cystidia were observed in a sulfovanillin solution (Caboň et al. 2017). To check for acid-resistant incrustations on pileipellis elements, tissues were stained with carbolfuchsin and observed in distilled water after incubation in a 10% HCl solution (Romagnesi 1967).

RESULTS

Phylogenetic analysis.—All of our collections were placed in a strongly supported clade (ML = 100, BI = 1.0) corresponding to *Russula* subsect. *Substriatinae* that is sister to *Russula* sect. *Heterophyllae* based on the combined 28S, *rpb2*, and *tef1* data set (FIG. 2). Both groups were placed in subg. *Heterophyllidiae* together with sect. *Ingratae* Quél., subsect. *Cyanoxanthinae* Singer, and one residual cluster formed by *R. redolens* Burl. and *R.* aff. *brunneoannulata* Buyck. Collections from Panama and Colombia were strongly supported (ML = 100, BI = 1.0) as the sister group to Asian collections of *R. substriata* and *R. maguanensis*. Sequences of new collections from the neotropics clustered in two clades that are recognized below as two new subspecies: *R. floriformis* subsp. *floriformis* from Colombia and *R. floriformis* subsp. *symphoniae* from Panama. The ITS tree (FIG. 3) also supports (ML = 98) the placement of the Colombian and Panamanian collections within subsect. *Substriatinae*, but only *R. floriformis* subsp. *symphoniae* received support (ML = 75). Collections of a single unclarified species from Madagascar were placed with strong support (ML = 100) as sister to subsect. *Substriatinae*. The ITS data set contained more samples from more geographic regions than the multilocus tree and showed clusters possibly reflecting geographic origins within *Substriatinae*, but clade relationships and sometimes even support at terminal clades were not significant. Two additional clusters from Latin and North America were observed based on sequences retrieved from GenBank, but only the cluster of three samples from the USA, probably all from the southeast coast, received support (ML = 100). The ITS tree did not show support for a common ancestor of all American species.

Russula floriformis subsp. *floriformis* and subsp. *symphoniae* were closely related taxa that differed by three positions across the ITS, two in the 28S, seven in *rpb2*, and three in *tef1* (FIG. 4). Of these 15 potential diagnostic sites, eight showed polymorphisms represented by IUPAC ambiguity codes. Sites 727 of *rpb2* and 174 of *tef1* were in intronic regions. This resulted in six sites (one in the intronic region of *rpb2*) across four loci that showed consistent differences among the two subspecies. The mt 12S rDNA region was conserved

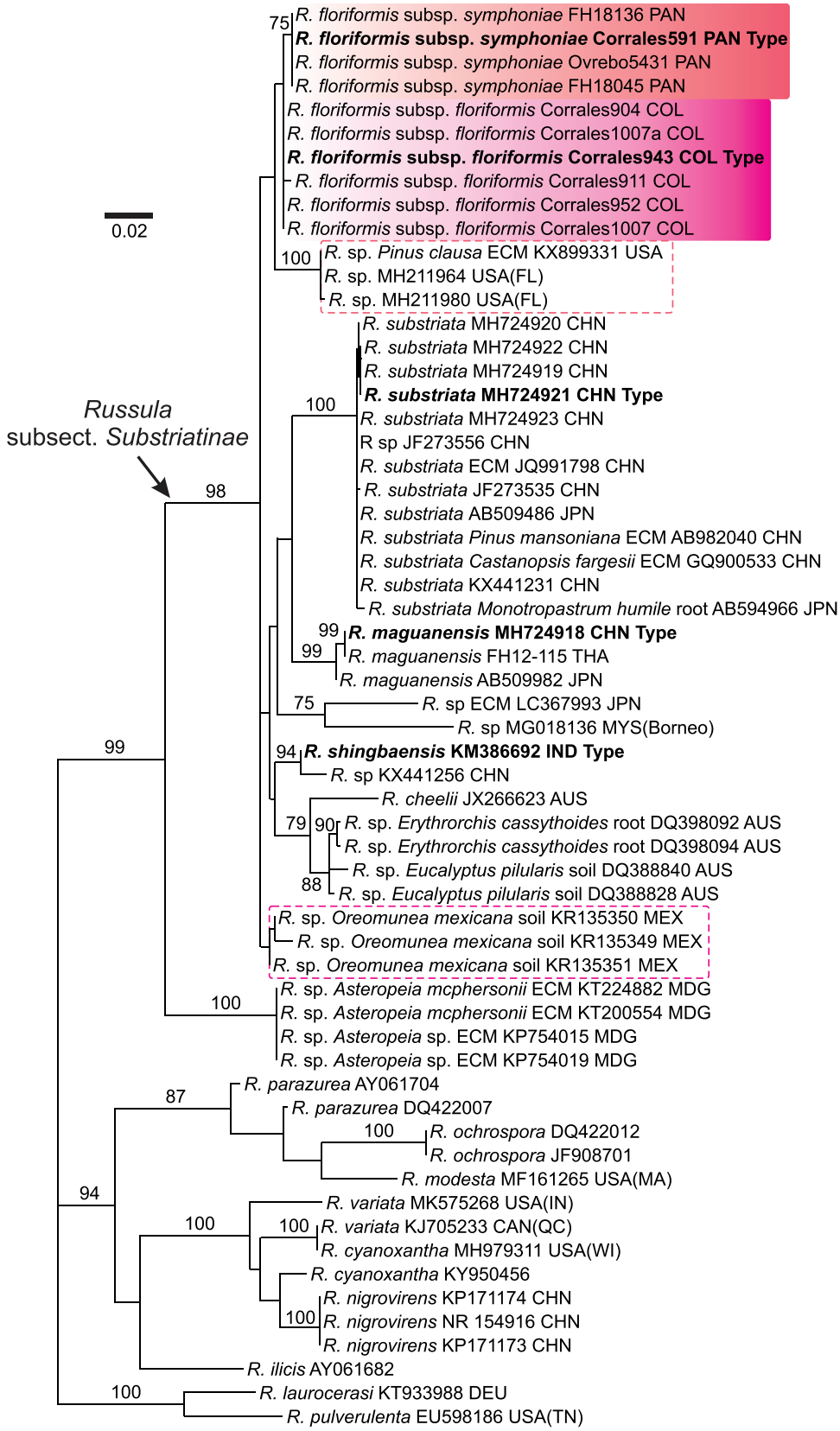


Figure 3. ML tree of the *Russula* subsect. *Substriatinae* based on sequence data of the ITS region. Names of ex-type specimens are in bold. Species studied by the authors are highlighted by a pink background. Other American *Substriatinae* sequences retrieved from GenBank are labeled by a pink dashed outline. Names of plant hosts are provided (if available) for samples from ectomycorrhizal roots (followed by "ECM"), orchid or myco-heterotrophs (followed by "root"), or soil samples (followed by "soil"). Countries are abbreviated by ISO codes (https://www.iso.org/iso-3166-country-codes.html). US states and Canadian provinces are distinguished by postal codes (http://www.icq.eps.harvard.edu/ICQpost.html).

Russula subspp. and voucher no.	ITS			28S		*rpb2*							*tef1*		
	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_FH-18-136	C	Y	G	R	A	T	T	S	Y	A	K	C	–	Y	C
Rs_FH-18-045	C	T	G	A	A	T	T	S	T	A	K	C	–	C	C
Rs_Corrales85	–	–	–	R	A	A	T	S	Y	–	–	–	–	–	–

Figure 4. Nucleotide differences of *Russula floriformis* subsp. *floriformis* (Rf) and subsp. *symphoniae* (Rs) across ITS, 28S, *rpb2*, and *tef1*. The numbers at the top indicate the positions of the polymorphic sites in each fragment. The dashes indicate the lack of data for the respective positions.

and did not reveal any differences among these two subspecies. Because very few 12S sequences of *Russula* were available, this gene region was not used in the multigene analyses.

TAXONOMY

Russula floriformis* subsp. *floriformis Vera and Corrales, sp. nov. et subsp. nov. FIGS. 5A–B, 6, 7
MycoBank MB830725

Typification: COLOMBIA. CUNDINAMARCA PROVINCE: San Antonio del Tequendama, eastern Andean Cordillera, Chicaque Natural Park, 4°36′29″N, 74°18′17″W, alt. 2131 m, *Quercus humboldtii* forest, 27 Nov 2019, *M. Vera & A. Corrales 943* (**holotype** HUA, **isotype** SAV F-20573).

Etymology: floriformis (Latin), in reference to the pileus that resembles a flower due to the color and the strong radial striation.

Diagnosis: Basidiomata strongly reminiscent of *R. substriata*, from which they differ by relatively narrow hyphal terminations in the pileipellis composed of only 1–2 unbranched cells, absence of shorter and more inflated subterminal cells, and abundant, one-celled, subcylindrical, apically mainly obtuse pileocystidia. *Russula floriformis* subsp. *floriformis* differs from subsp. *symphoniae* by a darker wine-red to red-brown color of the margin of the pilei, more rounded spores (Q < 1.15), absence of apical constrictions or appendages on pileocystidia near the pileus center, and wider (mainly >3.5 µm) and frequently cylindrical hyphal terminations in pileipellis.

Description: Pileus small to medium-sized, 24–48 mm diam, usually plane with a depressed center, becoming deeply depressed to infundibuliform at maturity; tuberculate-striate 5–19 mm from the margin, edge crenulate; surface 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 and with yellow-orange spots at the center. Lamellae moderately distant with ca. 6–10/1 cm near the pileus margin, adnate or shortly decurrent, white, lamellulae rare, furcations occasional especially near the stipe, edges even and concolorous. Stipe 32–48 × 7–9 mm, cylindrical, narrowed near the base, flexuous or curved, longitudinally striate, pale pinkish purple and almost white at the apex, cavernate, cortex 1.5 mm thick. Context 2.5 mm thick at half of the radius, fragile, white, unchanging when bruised, taste mild, odor inconspicuous. Spore print not observed.

Basidiospores (7–)7.5–*8.2*–8.5(–9.5) × (6.5–)7–*7.4*–8(–9) µm, mainly subglobose, Q = 1.04–*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 inamyloid, covered by small and low warts, outlined by more prominent ornamentation. Basidia (10–)28–*39*–50(–54) × (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–)44–*55*–66(–76) × (7–)8–*9.4*–11(–13) µm, often fusiform or lageniform, rarely lancelolate, mainly acute and pointed, usually with an eccentric, 1–3(–4) µm long appendage, thin-walled; contents heteromorphous, crystalline or banded, sometimes optically empty near the base, turning almost black in sulfovanillin; near the lamellae edges sometimes smaller, (37–)48–*57*–67(–78) × (6–)8–*9.1*–10.5(–11.5) µm, more frequently inflated near the terminal part, lanceolate, clavate or narrowly fusiform, contents more dispersed, sometimes almost optically empty or limited to the terminal part only. Lamellae edges with occasional basidia; marginal cells (14–)17–*21*–24(–30) × (3–)5–*6.6*–8(–11) µm, usually cylindrical and relatively narrow, some clavate

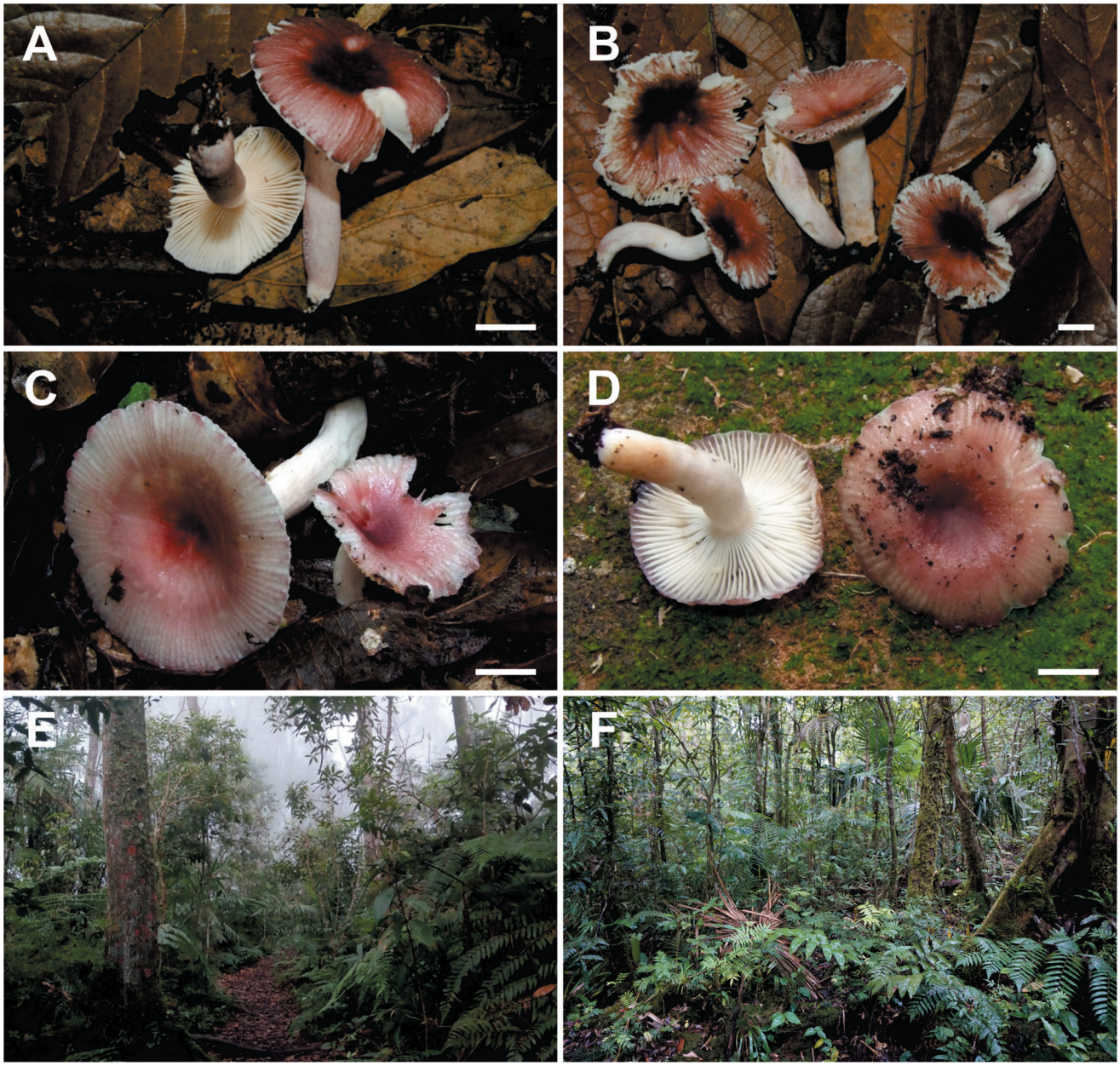


Figure 5. Basidiomata in the field and photos of typical habitat of studied *Russula* spp. A. *Russula floriformis* subsp. *floriformis* (Corrales 904). B. *R. floriformis* subsp. *floriformis* (Corrales 943). C. *Russula floriformis* subsp. *symphoniae* (FH 18-045). D. *R. floriformis* subsp. *symphoniae* (FH 18-136). E. Forest dominated by *Quercus humboldtii* in Chicaque, Colombia. F. Forest dominated by *Oreomunnea mexicana* in Fortuna Forest, Panama (photo by James Dalling). Bars = 10 mm.

and similar to basidiola, occasionally flexuous, apically constricted or obtuse. Pileipellis metachromatic in cresyl blue but not strongly so, sharply delimited from the underlying context, 120–175 µm deep; suprapellis not distinctly gelatinized, 25–30 µm deep, disconnected by gaps, composed of repent or ascending hyphal terminations, fasciculate in small or bigger groups forming pyramidal structures, near the center forming a continuous layer; suprapellis well delimited from 105–160-µm-deep, strongly gelatinized subpellis of loose, often anastomosed, irregularly oriented, but near the context dense and horizontally oriented, hyphae 1–4 µm wide. Acid-resistant incrustations absent. Hyphal terminations near the pileus margin composed of one or two cells, thin-walled; terminal cells (9–)14–*20*–24(–33) × (2.5–)3–*3.9*–4.5(–5.5) µm, cylindrical, subulate or lageniform, usually apically narrowed; subterminal cells mainly branched, shorter and wider, up to 6 µm wide, often with lateral projections or branches. Hyphal terminations near the pileus center similar; terminal cells (8–)14–*20*–25(–32) × (2.5–)3.5–*4.2*–5(–6) µm, more frequently apically constricted; subterminal cells more frequently unbranched

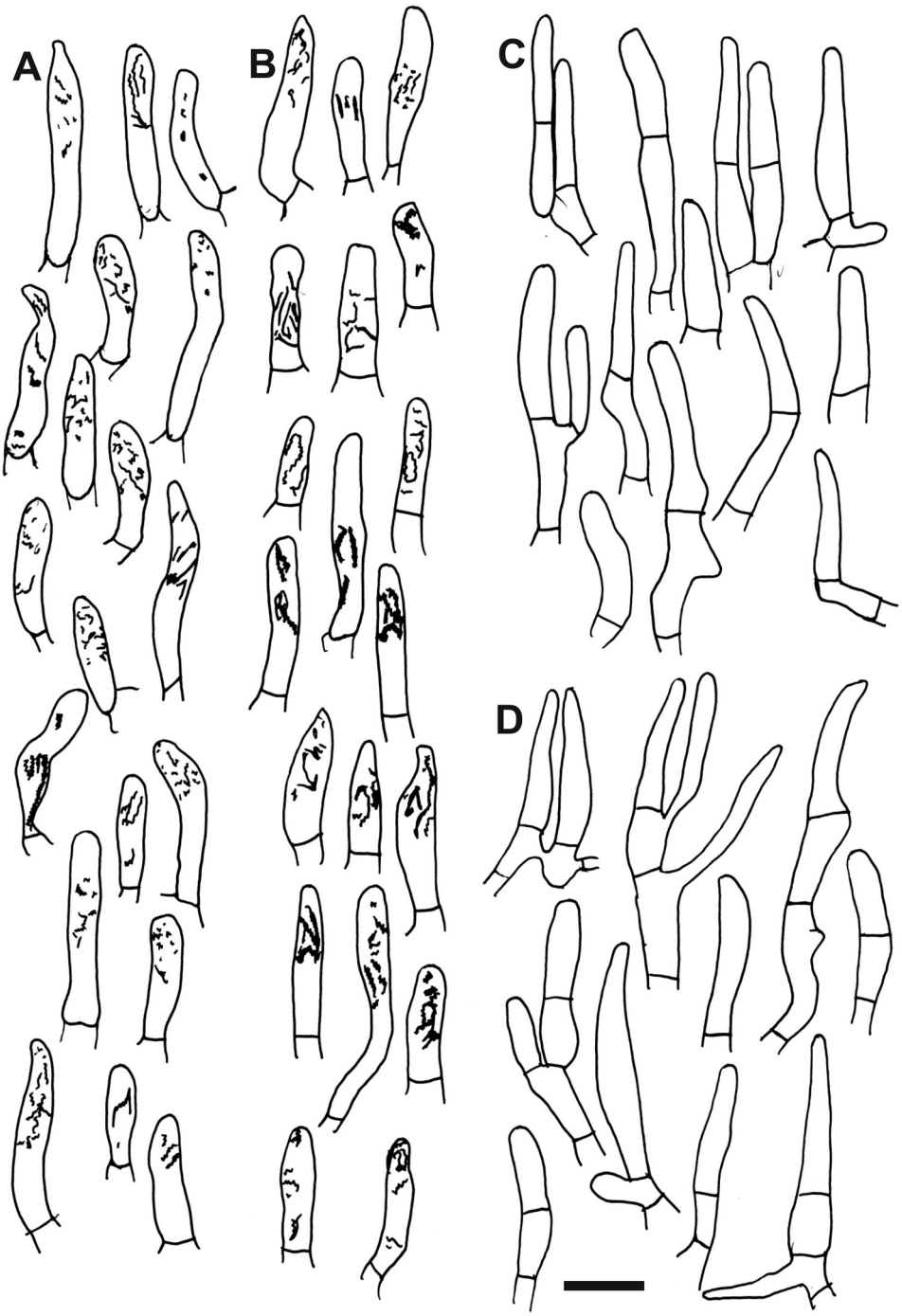


Figure 6. *Russula floriformis* subsp. *floriformis* (Corrales 943, holotype). Cellular elements of the pileipellis drawn as seen by light microscopy. A. Pileocystidia near the pileus center. B. Pileocystidia near the pileus margin. C. Hyphal terminations near the pileus center. D. Hyphal terminations near the pileus margin. Contents of cystidia as observed in Congo red. Bar = 10 µm. Drawings by Michelle Vera.

and inflated-ellipsoid, occasionally anastomosed. Pileocystidia near the pileus margin frequent, locally even more frequent than other hyphal terminations, always one-celled, mainly clavate, occasionally subcylindrical or fusiform, rarely lanceolate, thin-walled, (13.5–) 18–*24*–29(–38) × (3.5–)4–*4.5*–5(–7) µm, apically mainly obtuse, occasionally acute and with terminal knob; contents dispersed and partly granulose or banded, weakly graying in sulfovanillin; pileocystidia near the pileus center similar in shape and size, more frequently apically constricted, with more abundant contents. Cystidioid hyphae dispersed in subpellis but more frequent near

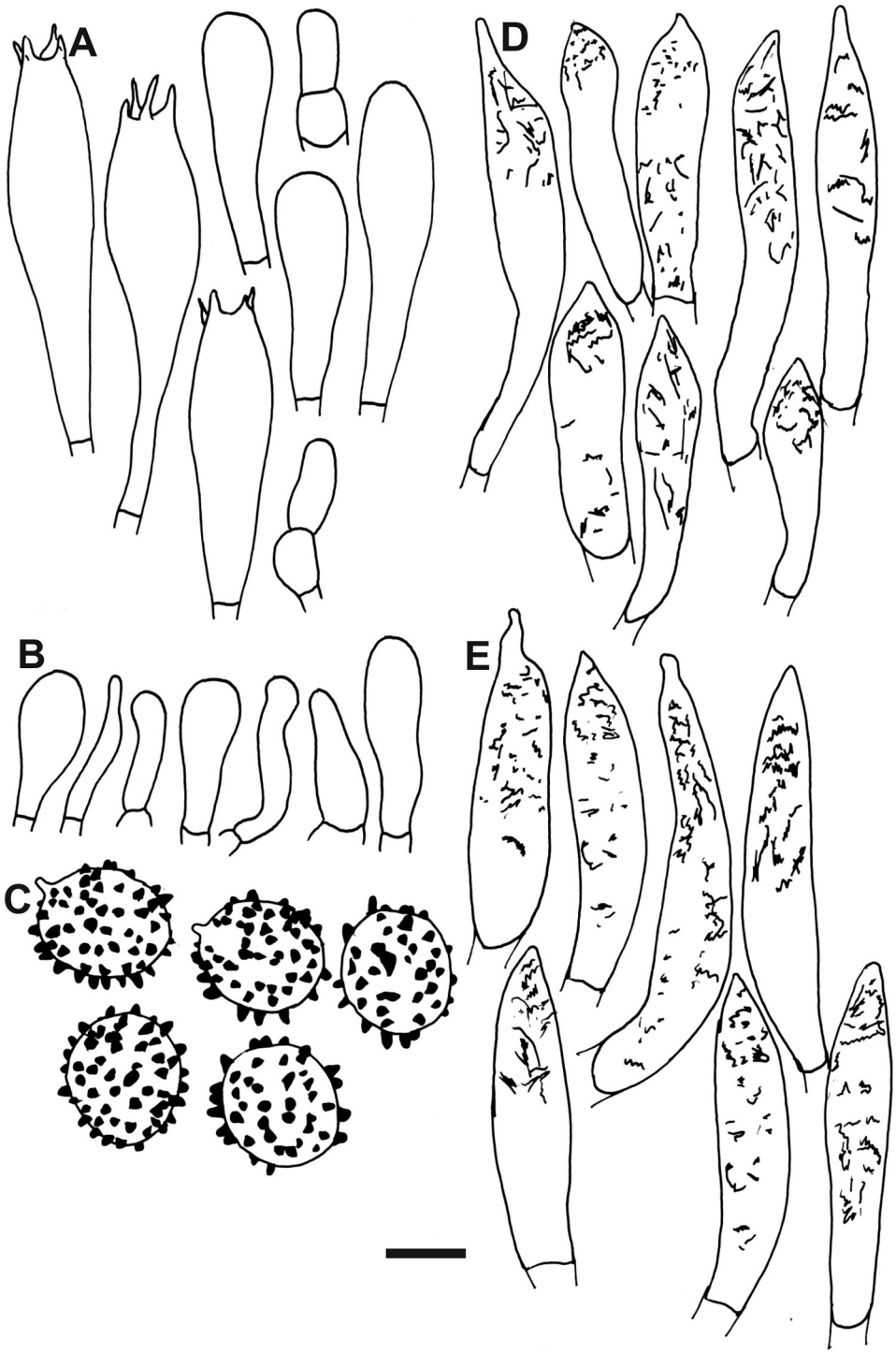


Figure 7. *Russula floriformis* subsp. *floriformis* (Corrales 943, holotype). Cellular elements of the hymenium drawn as seen by light microscopy. A. Basidia and basidiola. B. Marginal cells. C. Basidiospores in Melzer's reagent. D. Hymenial cystidia near the lamellae edges. E. Hymenial cystidia on the lamellae sides. Cystidia with contents as observed in Congo red. Bar = 5 µm for basidiospores and = 10 µm for all other elements. Drawings by M. Vera.

the pileus context, sometimes similar to cystidia but longer and often apically mucronate, occasionally with lateral projections or branches, contents dense and granulose.

Other specimens examined: COLOMBIA. CUNDINAMARCA PROVINCE: San Antonio del Tequendama, eastern Andean Cordillera, Chicaque Natural Park, 4°36′29″N, 74°18′17″W, alt. 2131 m,

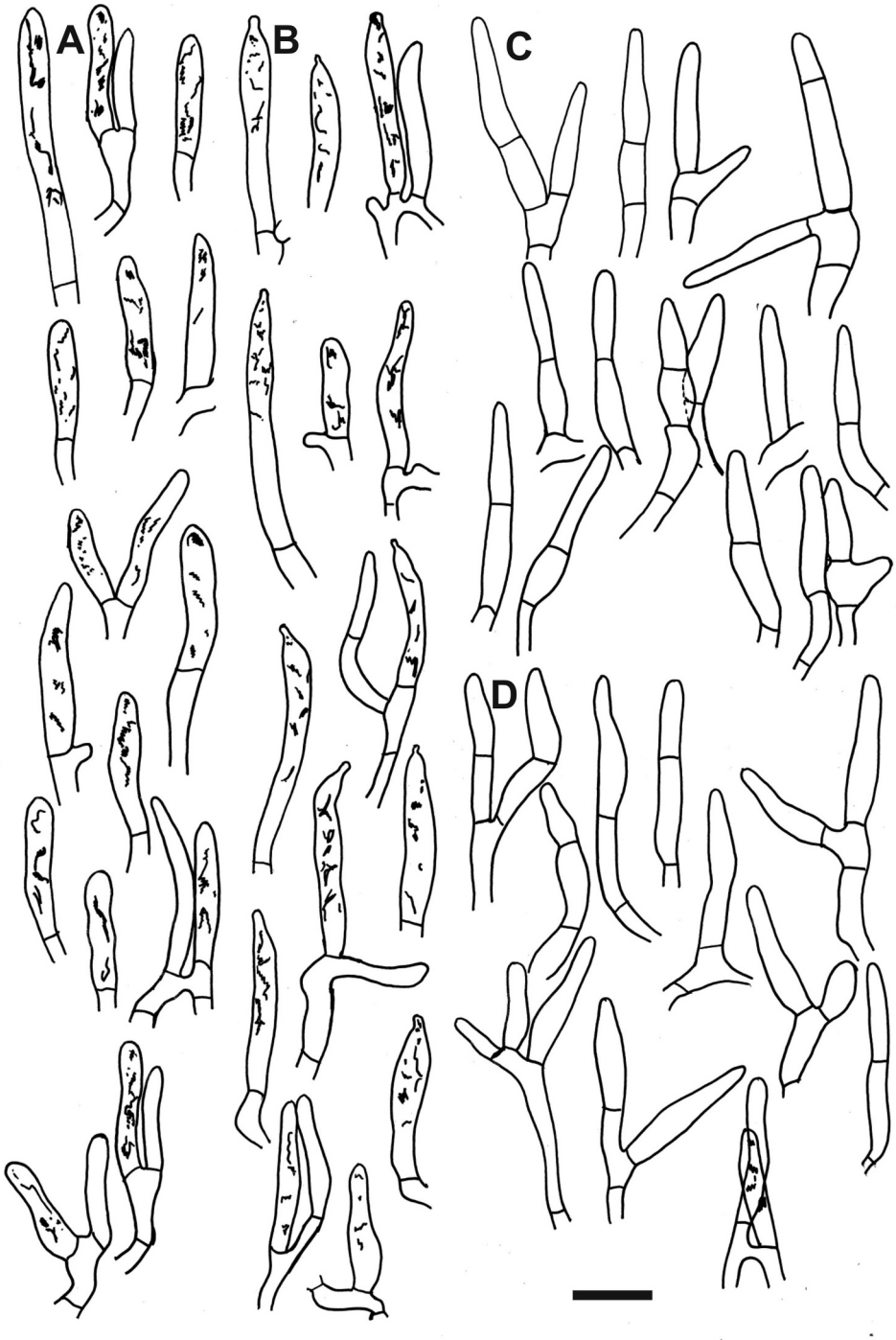


Figure 8. *Russula floriformis* subsp. *symphoniae* (Corrales 591, holotype). Cellular elements of the pileipellis drawn as seen by light microscopy. A. Pileocystidia near the pileus center. B. Pileocystidia near the pileus margin. C. Hyphal terminations near the pileus center. D. Hyphal terminations near the pileus margin. Contents of cystidia as observed in Congo red. Bar = 10 µm. Drawings by M. Vera.

cloud forest dominated by *Q. humboldtii*, 27 Nov 2019, *M. Vera & A. Corrales, Corrales 904, Corrales 911*; ibid., 30 Nov 2019, *M. Vera & A. Corrales, Corrales 952, Corrales 1007, Corrales 1007a* (SAV F-20577).

Notes: The known species of the subsection *Substriatinae* (FIG. 3) shared numerous macromorphological features with the Colombian and Panamanian samples. They had relatively small basidiomata (pileus maximum 60 mm diam), strongly tuberculate-striate pileal margins, a thin and fragile context, a radially cracked pileus surface near the margin, and cavernate or hollow and slender stipes (up to 10 mm wide). All species, except for *R. shingbaensis*, have

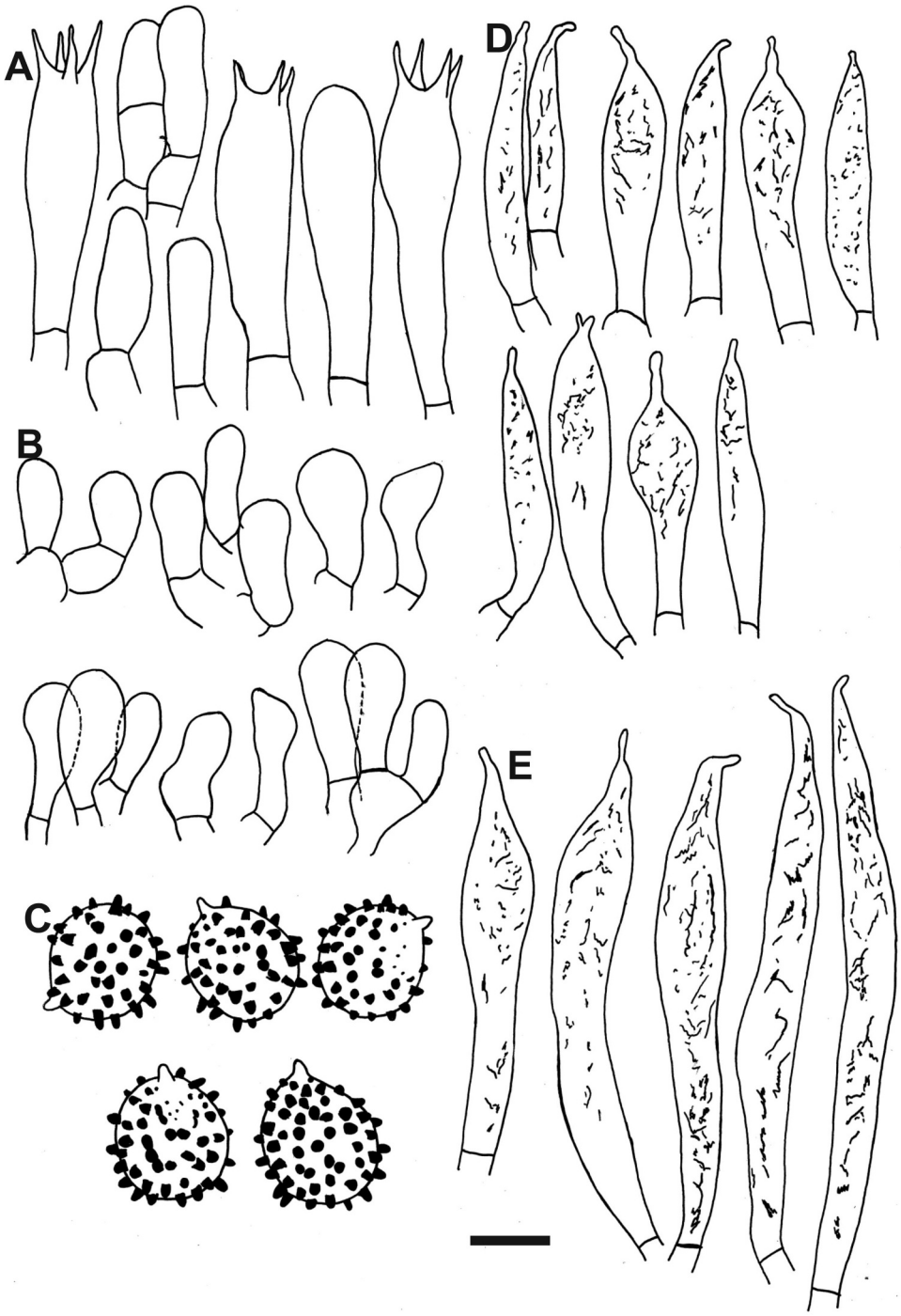


Figure 9. *Russula floriformis* subsp. *symphoniae* (Corrales 591, holotype). Cellular elements of the hymenium drawn as seen by light microscopy. A. Basidia and basidiola. B. Marginal cells. C. Basidiospores in Melzer's reagent. D. Hymenial cystidia near the lamellae edges. E. Hymenial cystidia on the lamellae sides. Cystidia with contents as observed in Congo red. Bar = 5 µm for basidiospores and = 10 µm for all other elements. Drawings by M. Vera.

a pink to wine-red pileus surface. The new American taxa shared with *R. substriata* the pinkish tints on the stipe. Our personal observations in the field confirm that the Asian species are macroscopically similar to the American species.

Several micromorphological characters specific for the subsection *Substriatinae* were observed. The American species had relatively large basidiospores ornamented by large isolated warts and a pileipellis of

densely arranged hyphal terminations with disconnected structure toward the margin and with abundant one-celled pileocystidia.

Russula floriformis* subsp. *symphoniae Manz, F. Hampe & Corrales, subsp. nov. FIGS. 5C–D, 8, 9; SUPPLEMENTARY FIG. 1

MycoBank MB834484

Typification: PANAMA. CHIRIQUÍ PROVINCE: Fortuna Forest Reserve, Honda watershed, 8°45′17.16″ N, 82°14′27.12″W, alt. 1227 m, in *Oreomunnea mexicana* forest, 14 Dec 2013, *A. Corrales 591* (**holotype** PMA, **isotype** SAV F-20574).

Etymology: symphoniae (Latin), referring to the color of the pileus that resembles the color of the flower of the Central American plant species *Symphonia globulifera*.

Diagnosis: Russula floriformis subsp. *symphoniae* differs from subsp. *floriformis* by a less bright and paler purplish pink to grayish red color of the margin of the pilei, more elongated spores (Q > 1.15), frequent apical constrictions or appendages on pileocystidia near the pileus center, and narrow (frequently <3.5 µm) and mainly attenuated hyphal terminations in the pileipellis.

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

Basidiospores (7–)8–*8.2*–8.5(–9.5) × (6–)6.5–*7.1*–7.5(–8) µm, subglobose to broadly ellipsoid, Q = (1.03–)1.10–*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 inamyloid, covered by small and low warts, and outlined by more prominent ornamentation. Basidia (10–)26–*36*–47(–54) × (5–)8–*10*–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–)46–*58*–70(–90) × (6.5–)7.5–*9*–10(–13) µm, often fusiform or lageniform, rarely lanceolate, usually acute and pointed, usually with a short, 1–3(–6) µm long, often eccentric appendage, thin-walled; contents heteromorphous, granulose, banded or sometimes also crystalline, sometimes optically empty near the base, turning almost black in sulfovanillin; near the lamellae edges smaller, (21–)33–*45*–56(–73) × (4–)6–*8*–9.5(–11.5) µm, fusiform or lanceolate, contents more dispersed. Lamellae edges with occasional basidia; marginal cells (9–)14–*18*–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 so, sharply delimited from the underlying context, 90–130 µm deep, strongly gelatinized throughout; suprapellis 30–40 µm deep, near the pileus margin disconnected by gaps, composed of loose, repent or ascending hyphal terminations, near the center forming continuous layer of densely arranged, erect hyphal terminations; suprapellis well delimited from 70–100-µm-deep subpellis of loose, often anastomosed, irregularly oriented, but near the context dense and horizontally oriented, hyphae 2–4 µm wide. Acid-resistant incrustations absent. Hyphal terminations near the pileus margin composed of one or two cells, thin-walled; terminal cells (6–)12–*17*–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 center similar; terminal cells (8–)11–*17*–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 one-celled, mainly narrowly clavate or subcylindrical, rarely fusiform, thin-walled, (13–)16–*23*–30(–52) × (3–)3.5–*4*–4.5(–5) µm, apically mainly obtuse, occasionally acute or constricted; contents dispersed and partly granulose or banded, hardly reacting in sulfovanillin; pileocystidia near the pileus center also one-celled and similar in shape and size, apically usually constricted or with a small, pearl-like appendage. Cystidioid hyphae dispersed in subpellis, not observed in the context.

Other specimens examined: PANAMA. CHIRIQUÍ PROVINCE: Fortuna Forest Reserve, Honda watershed, trail between Honda A and Honda B plots, 8°45′17.16″N, 82°14′27.12″W, alt. 1269 m, in *Oreomunnea mexicana* forest, 23 Aug 2012, *A. Corrales 85* (ARIZ; SAV F-20575); Zarciadero, off of Bocas del Toro road, 2 km NW dam, trail originating at old Autoridad Nacional del Ambiente de Panamá station, 8°45′42.42″N, 82°15′40.62″ W, alt. 1341 m, in *Oreomunnea mexicana* forest, 21 May 2015, *C. Ovrebo 5431* (CSU; SAV F-20576);

CHIRIQUÍ PROVINCE: Boquete, Jaramillo Arriba, El Musgo, 08°47′26.9″N, 82°24′34.4″W, alt. 1670 m, in moss-rich montane cloud forest dominated by *Quercus*, 15 Jun 2018, *F. Hampe & C. Manz, FH-18-045* (UCH11708); alt. 1670 m., in moss-rich montane cloud forest dominated by *Quercus*, 24 Jun 2018, *F. Hampe & C. Manz, FH-18-136* (UCH11706).

Notes: Russula floriformis subsp. *floriformis* and subsp. *symphoniae* look very similar macroscopically, with the only difference being the color of the margin of the pilei, which is usually dark wine-red to red-brown in subsp. *floriformis* but purplish pink to grayish red in subsp. *symphoniae*. The main and most conspicuous micromorphological character to distinguish *R. floriformis* subsp. *symphoniae* from subsp. *floriformis* is the presence of frequent apical constriction, small knobs or pearl-like appendages on pileocystidia near the pileus center. Other distinguishing characters are more difficult to observe (TABLE 2).

DISCUSSION

Here, we describe the first species of *Russula* subsect. *Substriatinae* from the Americas. Twenty-seven *Russula* species and infraspecific taxa are now known from *Quercus*-dominated forests of Central and South America or forests at similar altitudes with alternative host trees. Seven species are from Colombia and one from Panama (SUPPLEMENTARY TABLE 1). However, there is a serious lack of information on the diversity of ECM fungi and their host tree associations in the neotropics. This makes it difficult to study the region's complex biogeographic history (Nilsson et al. 2006; Roy et al. 2016; Corrales et al. 2018; Harrower et al. 2015). Considering the variation in ecological, geographic, and climatic conditions, and the diversity of ECM host tree species in Central and South America, high taxonomic diversity of *Russula* is expected for this region (Vasco-Palacios and Franco-Molano 2013; Del Olmo-Ruiz et al. 2017).

Previous molecular inventories of ECM fungi from Mexico, Costa Rica, and Panama, based on sequencing of fruting bodies and roots, indicated a high diversity of *Russula* (Morris et al. 2009; Desai et al. 2016; Corrales and Ovrebo 2021). This suggests that *Russula* diversity in the area is underexplored. In addition, some validly published species from the region are poorly known because the information available often includes limited morphological descriptions and lacks standarized terminology and molecular data (SUPPLEMENTARY TABLE 1; Vasco-Palacios and Franco-Molano 2013). Future studies should focus on gathering new information from described *Russula* species using both morphological and genetical approaches to improve our knowledge about the fungi and their biogeography in the region.

ITS sequences of *R. floriformis* subsp. *floriformis* and subsp. *symphoniae* differ at only three nucleotide positions, two of which include polymorphisms (FIG. 4). This corresponds to a similarity higher than 99% and results in a lack of support for the distinction of these two subspecies based on the ITS region (FIG. 3). However, a high similarity of ITS sequence data has been reported for several cryptic species of Basidiomycota, including species of *Russula*, which is well supported by morphological characeristics (Badotti et al. 2017). Tremble et al. (2020) pointed out that the barcoding signal of the ITS region may be misleading in some groups because of low gene flow within populations of a single species and insufficient homogenization through concerted evolution. In several studies of *Russula*, the ITS region shows low or contrasting support in comparison with other loci and should therefore be analyzed separately (e.g., Wang et al. 2019; Adamčík et al. 2019; Caboň et al. 2019). Three European sympatric species of subsect. *Xerampelinae* Singer, for example, are recognized as distinct species based on morphological differences, ecological preferences, and results of haplotype reconstruction analysis (Adamčík et al. 2016). They present few differences in the ITS region similar to differences found between the two subspecies of *R. floriformis*. Our study revealed no differences within the mitochondrial 12S region between the two subspecies, but two positions in 28S rDNA (one unique substitution, one polymorphism), seven in *rpb2* (three unique substitutions, four polymorphisms), and three in *tef1* (one unique substitution, two polymorphisms) (FIG. 4). High level of shared polymorphisms (nine sites across five regions studied) suggests that the populations of *R. floriformis* are in early stages of sorting of ancestral polymophism (Taylor et al. 2000). Although both subspecies are supported by strict genealogical concordance in multilocus analyses, support by individual regions differs. 12S and *tef1* did not support any grouping within *R. floriformis, rpb2* supported both subspecies, and ITS and 28S only supported the subsp. *symphoniae*. The high level of polymorphism and incongruent topologies of phylogenies based on individual loci suggest that the genetic diversification within *R. floriformis* is in an early stage of gray zone of speciation (Matute and Sepúlveda 2019). Therefore, we decided to treat the two taxa below species rank.

Historically, the variety is a more commonly used infraspecific rank than subspecies for ECM fungi and particularly for *Russula* (Romagnesi 1967). However, we were not able to find any phylogentic study on ECM fungi, resulting in the description of taxa below species

Table 2. Characters observed on the studied material of *Russula floriformis* subsp. *floriformis* and *R. floriformis* subsp. *symphoniae*.

		R. floriformis subsp. *floriformis*			*R. floriformis* subsp. *symphoniae*				
Character		Corrales 943	Corrales 911	Corrales 952	Ovrebo 5431	Corrales 591	Corrales 85	FH-18-045	FH-18-136
Spores	Size (µm)	8.2 × 7.4	8.1 × 7.4	8.3 × 7.6	8.5 × 7.0	8.3 × 7.0	8.3 × 7.0	8.3 × 6.9	7.9 × 6.6
	Q	1.12	1.1	1.1	1.21	1.18	1.18	1.2	1.2
	Spines	5	5	4.7	4.2	4	4	4.7	4.6
	Lines	0	0	0	0	0	0	0	0
	Fusions	1.1	1.3	1.5	0.3	1.1	0.6	0.2	0.2
	Ornamentation (µm)	1.1	0.9	0.9	0.9	0.9	0.9	0.9	1.1
TC near the pileus margin	TC size (µm)	17.9 × 3.6	22.6 × 3.9	18.3 × 4.2	16.7 × 3.3	14.3 × 3.2	17.1 × 3.6	15.2 × 3.7	17.1 × 3.4
	Width of TC at the tips	2.4	2.6	2.4	2	2.2	1.8	2	2
	Difference in width of TC	1.3	1.5	1.7	1	1.1	2.1	1.7	1.5
	Attenuated TC (%)	0.5	0.5	0.4	0.8	0.5	0.7	0.9	0.9
	Cylindrical TC (%)	0.5	0.4	0.5	0.2	0.5	0.3	0.1	0.1
	Clavate TC (%)	0	0.1	0	0	0	0	0	0
TC near the pileus center	TC size (µm)	18.4 × 4.1	23.3 × 4.4	17.4 × 3.9	15.5 × 3.1	13.9 × 3.2	20.9 × 3.5	14.1 × 3.6	13.2 × 3.9
	Attenuated TC (%)	0.6	0.7	0.4	0.5	0.4	0.6	0.63	0.7
PC near the pileus margin	PC size (µm)	21.1 × 4.2	26.0 × 4.8	23.2 × 4.4	34.6 × 4.2	20.1 × 3.6	27.4 × 3.9	23.4 × 4.5	25.0 × 4.1
	Number of cells	1	1	1	1	1	1	1	1
PC near the pileus center	PC size (µm)	20.0 × 4.5	29.3 × 4.8	20.7 × 4.3	30.8 × 4.1	23.3 × 3.5	22.8 × 3.8	25.4 × 5.3	23.7 × 4.5
	Number of cells	1	1	1	1	1	1	1	1
Basidia	Size (µm)	32.8 × 9.4	42.4 × 12.8	42.2 × 12.4	30.8 × 10.3	39 × 10.2	38.1 × 10.2	40.6 × 12.3	40.3 × 10.4
Cystidia on lamellae sides	Size (µm)	49.1 × 9.0	61.3 × 9.0	54.5 × 10.6	89.1 × 9.9	64.6 × 8.6	59.2 × 8.4	81.5 × 10.9	66.6 × 9.3
	Acute cystidia (%)	0.9	0.9	0.7	1	1	1	1.0	0.85
	Length of appendage	1.6	0.9	0.4	7.3	2.6	1.6	2.3	1.9
Marginal cells	Size (µm)	21.4 × 5.4	21.0 × 7.3	19.8 × 7.1	14.8 × 5.3	15.0 × 6.5	18.7 × 5.8	16.7 × 4.4	15.1 × 4.2
Cystidia on lamellae edges	Size (µm)	56.3 × 9.2	63.1 × 8.7	52.6 × 9.4	46.9 × 7.3	36.4 × 6.8	33.6 × 6.0	54.7 × 9.4	41 × 7.9
	Acute cystidia (%)	0.9	0.8	0.9	1	1	1	1.0	0.95
	Length of appendage	1.3	1	0.6	1.9	1.8	2.3	2.9	2

Note. Values of sizes of spores and TC are averages of 30 measurements. All other values are averages of 20 measurements. Distinguishing characters are indicated by shaded boxes.

rank. The variety rank was often proposed for obscure taxa based on simple morphological characteristics (e.g., spore size, pileus color, etc.). Relatively recently, based on morphological arguments and geographic distance, Buyck et al. (2005) described *R. polyphylla* subsp. *guanacastae* Buyck from *Quercus* forests of Costa Rica, whereas the subsp. *polyphylla* was described from the eastern USA (Adamčík et al. 2018). In addition, two subspecies of a lichenised fungus, *Cora dewisanti* Moncada, A. Suárez-Corredor & Lücking, are among few fungal taxa accepted at infraspecific rank. *Cora dewisanti* subsp. *mexicana* Moncada, R.-E. Pérez & Lücking differs from C. *dewisanti* subsp. *dewisanti* by four insertions in the ITS region and occurrence in Mexico, whereas the nominal subspecies is known only from Colombia, Venezuela, and Ecuador (Moncada et al. 2019). The subspecies rank of the two taxa described in our study is justified by results of the multilocus phylogenetic analysis (FIG. 2), morphological differences, an ecological and climatic disjunction, as well as geographic distance of the studied collections. *Russula* is an ECM genus with a high diversification rate (Looney et al. 2016; Varga et al. 2019), and the case of two closely related subspecies described in this study from adjacent but climatically isolated regions of montane oak forests may be more common than expected.

The habitat and putative host species recorded for *R. floriformis* subsp. *floriformis* and subsp. *symphoniae* indicate that both subspecies are restricted to montane tropical forests. This may be due to a climatic barrier for their dispersal. Distribution patterns, divergence, and speciation of *Quercus* coincide with geological and climatic dynamics in Central America. The isthmus area in the center of Panama, along with the Darien region, presents altitudes up to 1875 m only and thereby represents climatic barriers to gene flow among *Quercus* populations (Rodríguez-Correa et al. 2015). Botanical records of *Q. humboldtii* from mountains on the Darien region and massifs along the Colombian Caribbean coast (Rangel and Avella 2011) provide evidence of *Quercus* migration to the Andes mountain range (Rangel and Avella 2011; Rodríguez-Correa et al. 2015). These relatively recent migration barriers imposed by extensive areas of lowland forest determined the composition of oak forests in Colombia and probably also influenced the speciation process (Rodríguez-Correa et al. 2015).

Russula floriformis subsp. *floriformis* and subsp. *symphoniae* are closely related subspecies with similar habitat requirements, both found in tropical montane forests dominated by species of *Quercus* or *Oreomunnea*. These ecological features, together with our phylogenetic results, support a comigration hypothesis and contemporary allopatric diversification of ECM trees and their symbiotic fungi. *Quercus humboldtii* diverged from Central American oak species as the result of migration, genetic isolation, and adaptation to local conditions in the Colombian Andes around 3.5 Ma (Hipp et al. 2020). Given that there are no known *Russula* fossils, molecular clock analyses of this lineage should use a similar calibration to the one used in the study on North American *Russula* subsect. *Roseinae* Sarnari (Looney et al. 2020). According to the latter study, *Roseinae* species pairs with sequence differences higher than those found for *R. floriformis* subsp. *floriformis* and subsp. *symphoniae* (SUPPLEMENTARY FIG. 2) split more recently than 3.5 Ma ago. The ITS substitution rate of *R. floriformis* estimated by dividing the ML branch length of 0.004 by 2T (7 million years) is 0.57E-9, which is less than the average value known for ECM fungi but similar to values calculated, for example, for *Hygrophorus* (Ryberg and Matheny 2012). This suggests that the diversification of subspecies of *R. floriformis* is younger than divergence of the host tree (probably younger than 1.75 Ma). However, although we understand the spatial distribution of *Quercus* and timing of divergence of its species in the Americas (Hooghiemstra 2006; Hipp et al. 2020), current knowledge about *R. floriformis* is limited to our reports from two areas in Colombia and Panama (FIG. 1). To test the hypothesis of diversification of *Russula* together with host trees, we need more data regarding the occurrence of the fungi along with fossil records. Our observations are congruent with recent studies demonstrating that local adaptations in combination with climatic disjunction are drivers of global ECM diversity (Větrovský et al. 2019). These patterns are also important for *Russula* divergence (Looney et al. 2016; Caboň et al. 2019).

This study draws attention to the recent diversification of two *Russula* subspecies along the Isthmus of Panama. Most probably, other ECM fungi comigrated with species of *Quercus* to Colombia relatively recently. Future studies should examine whether further ECM fungi distributed in Panama and Colombia represent the same species or diverged into distinct taxa. The Isthmus of Panama is an excellent study area to improve our understanding of the relatively recent quaternary evolutionary processes of ECM fungi.

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ORCID

Michelle Vera http://orcid.org/0000-0003-4832-2992
Slavomír Adamčík http://orcid.org/0000-0003-2156-5767
Katarína Adamčíková http://orcid.org/0000-0001-5407-5233
Felix Hampe http://orcid.org/0000-0001-7642-7930
Miroslav Caboň http://orcid.org/0000-0002-2255-3816
Cathrin Manz http://orcid.org/0000-0003-0515-0334
Meike Piepenbring http://orcid.org/0000-0002-7043-5769
Adriana Corrales http://orcid.org/0000-0001-9885-4634

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