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Research

# Comparative genomics reveals a dynamic genome evolution in the ectomycorrhizal milk-cap (*Lactarius*) mushrooms

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## Summary

- Ectomycorrhizal fungi play a key role in forests by establishing mutualistic symbioses with woody plants. Genome analyses have identified conserved symbiosis-related traits among ectomycorrhizal fungal species, but the molecular mechanisms underlying host specificity remain poorly known.
- We sequenced and compared the genomes of seven species of milk-cap fungi (*Lactarius*, Russulales) with contrasting host specificity. We also compared these genomes with those of symbiotic and saprotrophic Russulales species, aiming to identify genes involved in their ecology and host specificity.
- The size of *Lactarius* genomes is significantly larger than other Russulales species, owing to a massive accumulation of transposable elements and duplication of dispensable genes. As expected, their repertoire of genes coding for plant cell wall-degrading enzymes is restricted, but they retained a substantial set of genes involved in microbial cell wall degradation. Notably, *Lactarius* species showed a striking expansion of genes encoding proteases, such as secreted ectomycorrhiza-induced sedolisins. A high copy number of genes coding for small secreted LysM proteins and *Lactarius*-specific lectins were detected, which may be linked to host specificity.
- This study revealed a large diversity in the genome landscapes and gene repertoires within Russulaceae. The known host specificity of *Lactarius* symbionts may be related to mycorrhiza-induced species-specific genes, including secreted sedolisins.

## Introduction

Fungi perform essential ecological functions in terrestrial ecosystems, whether as saprotrophs feeding on dead organic matter or as biotrophs (parasites or symbionts) acquiring nutrients from living hosts. Soilborne ectomycorrhizal (EcM) fungi establish symbiotic relationships with 60% of tree individuals on Earth, and mediate the exchange of plant carbohydrates for soil minerals (Brundrett & Tedersoo, 2018; Steidinger *et al.*, 2019). They evolved independently, at least 80 times, from diverse saprotrophic ancestors (Tedersoo *et al.*, 2010; Martin *et al.*, 2016; Lebreton *et al.*, 2021b). These multiple emergences of EcM lineages involved lineage-specific genomic innovations, such as effector-like mycorrhiza-induced small secreted proteins (MiSSPs), but also loss of gene families, such as plant cell wall-degrading enzymes (PCWDEs). Each lineage, however, retains a unique set of PCWDEs, probably reflecting their specific evolutionary history and ecological roles (Kohler *et al.*, 2015; Miyauchi *et al.*, 2020; Lebreton *et al.*, 2021b). Species-specific changes in gene repertoires

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have also been observed within a single lineage (i.e. Amanitaceae), including expansion of clade-specific small secreted proteins (SSPs) (Hess *et al.*, 2018). The loss of PCWDE genes in a few species of saprotrophic ancestors suggests a possible preadaptation to EcM symbiosis in some lineages (Hess *et al.*, 2018; Looney *et al.*, 2022).

*Lactarius* is an EcM fungal genus belonging to Russulaceae (Russulales), a lineage that is rich in EcM species and widely distributed in temperate and subtropical forests (Looney *et al.*, 2016, 2018). The specific traits, such as host specificity and defense-related latex exudation, make this genus an ideal group to investigate the evolution of EcM fungi at the genomic level (Nuytinck *et al.*, 2007; Verbeken & Nuytinck, 2013; Looney *et al.*, 2018; Wang *et al.*, 2019). Given the contrasting patterns of host specificity between *Lactarius* and *Russula* symbionts, the latter being mostly generalists, a comparison of their gene repertoires may provide novel insights into the molecular mechanisms underlying the specific interactions between EcM fungi and their host(s). It has been suggested that lectins, carbohydrate-binding proteins that are highly specific for sugar groups, could be involved in the recognition between *Lactarius deterrimus* and spruce roots during the early stage of symbiosis (Guillot *et al.*, 1991; Giollant *et al.*, 1993), but definitive demonstration is lacking. A metatranscriptomic study of host-specific patterns of gene expression between *Pinus* species and their symbiotic EcM fungi in the genus *Suillus* revealed that the host plant and EcM fungal symbiont each expresses unique gene sets during incompatible versus compatible pairings. These genes code for proteins involved in signaling pathways, including G-protein coupled receptors (GPCRs), secretory pathways, leucine-rich repeat proteins, and pathogen resistance proteins that are similar to those associated with host–pathogen interactions (Liao *et al.*, 2016). By contrast, a large-scale comparative study of *Suillus* and other less specific EcM fungal genomes found that only terpene- and nonribosomal polyketide synthases (NRPS), but not GPCRs or SSPs, expanded in host-specific *Suillus* (Lofgren *et al.*, 2021).

In order to link gene repertoires to ecological traits in Russulaceae, we sequenced and analysed the genome of seven *Lactarius* species in section *Deliciosi*. These milk-cap species were collected from various geographical regions and are known for their host specificity towards Pinaceae (Wang *et al.*, 2019; Tang *et al.*, 2021). The section *Deliciosi* contains at least 38 taxa worldwide, including many well known edible species (Nuytinck *et al.*, 2007). Most species in this section form ectomycorrhizas with *Pinus*, but they can also associate with other conifers (*Picea*, *Abies*, etc.), while a few species (i.e. *L. indigo* and *L. subindigo*) have been reported to interact with broadleaved trees, such as *Quercus* and *Castanopsis*. The host switch between Pinaceae and Fagaceae seems to have occurred a few times throughout evolution (Nuytinck *et al.*, 2007). Moreover, European species have a well-documented host specificity, for example, *L. salmonicolor* on *Abies* and *L. deterrimus* on *Picea*. We hypothesize that a comparison of the available gene repertoires of Russulaceae and *Lactarius* species would provide new information on the evolution of the symbiotic lifestyle within the Russulaceae, and the molecular mechanisms underlying host selection in a major group of EcM symbionts. By comparing genomes of saprotrophic and symbiotic Russulaceae species, we revealed the genetic basis for their contrasting lignocellulose- and protein-degrading abilities. We also identified major differences in their repertoires of dispensable genes and secreted proteases. Finally, we assessed the conservation of symbiotic-related traits in this fungal order.

## Materials and Methods

### DNA and RNA extraction for genome sequencing

Seven *Lactarius* strains belonging to the section *Deliciosi*, namely *L. akahatsu* QP, *L. deliciosus* 48, *L. hatsudake* 109, *L. hengduanensis* 84, *L. pseudohatsudake* 88, *L. sanguifluus* B21 and *L. vividus* 141, were selected for genome sequencing (Supporting Information Table S1). The dikaryotic (diploid) mycelia were originally isolated from fresh fruiting bodies. To produce adequate material for DNA and RNA extraction, mycelial pieces were cultured for 4–6 wk on solid ½ modified Melin–Norkrans + ½ potato dextrose agar media covered with cellophane membranes at 23°C in the dark (Wang *et al.*, 2019). Mycelia were harvested and snap-frozen in liquid nitrogen and kept at −80°C until DNA and RNA extractions. High-molecular-weight genomic DNA was extracted from 2 g of mycelia following the Joint Genome Institute (JGI) genomic DNA extraction protocol (http://1000.fungalgenomes.org/home/wp-content/uploads/2013/02/genomicDNAProtocol-AK0511.pdf; accessed in 2017), and purified with the AMPure XP magnetic beads (A3881; Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. The quality of genomic DNA (size > 23 kbp) was confirmed by pulsed-field gel electrophoresis. Mycelial total RNA was extracted using 100 mg of mycelium and the RNeasy Plant Mini Kit (74904; Qiagen) following the manufacturer's instructions. DNA and RNA samples were shipped to the JGI in DNAstable/RNAstable (Biomatrica, San Diego, CA, USA) for library construction and sequencing.

### Genome assembly and annotation

Genomic DNA of the *Lactarius* species was sequenced using the PacBio platform, then assembled using Falcon v.1.8.8 software (Chin *et al.*, 2016) and annotated at JGI following standard pipelines (Grigoriev *et al.*, 2014; Methods S1). This dataset was supplemented with genomes and corresponding annotations of 24 additional Russulales, one Polyporales, one Phallales and one Geastrales (the latter three being used as the outgroup) downloaded from the JGI MycoCosm database (Table S1). As the DNA was extracted from diploid mycelium, the gene annotation was 'haploidized' by using only the catalogue of primary alleles. The quality of all these genome assemblies and annotations was evaluated by Benchmarking Universal Single-Copy Orthologs (Busco v.3.0.2) (Simão *et al.*, 2015) using the Basidiomycota set (busco.ezlab.org/datasets/basidiomycota_odb9.tar.gz).

Identification and annotation of transposable elements (TEs) were carried out as described by Payen *et al.* (2016) and Morin *et al.* (2019) using Rep Base v.24.02 (Bao *et al.*, 2015).

Functional annotations of Eukaryotic Orthologous Groups of Proteins (KOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO) and InterPro (IPR) domains were performed using JGI pipelines, and datasets are available on the genome portal for each species. Carbohydrate-active enzymes (CAZymes) were identified using the annotation pipeline described in Lombard *et al.* (2014) with the CAZy database (www.cazy.org) and subsequent manual curation by the CAZyme team (version of December 2020). Secreted proteins were identified using the pipeline described by Pellegrin *et al.* (2015). Lectins were detected by HMMSCAN v.3.3 using the Unilectin3D database (www.unilectin.eu, version of January 2020) (Lebreton *et al.*, 2021a). G protein-coupled receptor annotation was carried out as described by Lofgren *et al.* (2021). Candidate genes involved in the latex rubber biosynthesis were identified by BLASTP v.2.10 searches (*e*-value < 1E–5, query coverage > 50%), using the protein homologues identified in the rubber tree (*Hevea brasiliensis*) as queries (Tang *et al.*, 2016; Liu *et al.*, 2020), based on the conservation of building unit (isopentenyl diphosphate, IPP) and biosynthetic pathway (Yamashita & Takahashi, 2020).

Peptidases from the subtilase superfamily are composed of subtilisin (S8) and sedolisin (S53) families. Subtilases were initially identified in the MycoCosm gene repertoires by searching predicted proteins with one of the following annotations/keywords: S8, S53, PF00082, PF00089, PF09286, EC3.4.21.4 or EC3.4.14.9. Additional subtilases or subtilase-like proteins were further identified by BLASTP (*e*-value < 1E–3) queries against the Russulales proteomes using the 904 putative functional subtilase identified by Li *et al.* (2017), hereafter called reference subtilases. CLANS (Frickey & Lupas, 2004), a piece of software allowing the visualization of pairwise sequence similarities, was then used to remove sequences that did not cluster with the reference subtilases and to assign the remaining ones to subtilase subfamilies. In order to keep only functional subtilase candidates, amino acid sequences of each subfamily were aligned using MUSCLE in MEGAX software (Kumar *et al.*, 2018) with default parameters. According to Li *et al.* (2017), subtilase sequences lacking two of the canonical regions were discarded from any further analysis; sequences lacking only one canonical region were annotated as partial. When the three regions matched the expected conserved subtilase pattern, the subtilase candidate was annotated as containing canonical regions. If at least one of the regions lacked a perfect match to the known pattern, the subtilase sequence was annotated as containing noncanonical regions.

## Protein orthology

The orthology among the 31 Russulales proteomes was assessed using ORTHOFINDER v.2.3.3 (-M msa -S diamond -A mafft -I 1.5; Emms & Kelly, 2015). Based on this clustering, we determined the set of proteins shared by the 31 Russulales species (i.e. core genes/proteins), sets of proteins encoded in at least two genomes (i.e. dispensable genes/proteins) and sets of proteins unique to a genome (i.e. species-specific genes/proteins). For each protein set, duplicated sequences were identified. Using the same clustering, the core, dispensable and species-specific genes/proteins of the nine *Lactarius* species were also identified. In addition, orthogroups containing proteins of all *Lactarius* species sharing a similar host tree, namely pine, oak and spruce, were identified. *In silico* functional annotation was assigned to an orthogroup only if this annotation was present in at least half of the protein members of this orthogroup.

## Phylogenomic analysis

The 934 single-copy gene orthogroups predicted with ORTHOFINDER were used for the phylogenomic analysis. Protein sequences of each orthogroup were aligned using MAFFT v.7.471 (Yamada *et al.*, 2016). After removing ambiguous regions (containing gaps and poorly aligned) with TRIMAL v.1.4.rev15 (Capella-gutiérrez *et al.*, 2009), the resulting 934 alignments were concatenated into a super-alignment. MODELTEST-NG v.0.1.6 (Darriba *et al.*, 2020) was then used to identify the best protein substitution model for each partition of this super-alignment corresponding to an orthogroup. The species tree was then reconstructed from this super-alignment using RAXML-NG v.0.9.0 (Kozlov *et al.*, 2019) with partitions and 500 bootstrap replicates. The species tree was then calibrated on a timescale with MCMCTREE available in PAML v.4.8 (Yang, 2007), using three estimated time points identified by Varga *et al.* (2019), namely the divergence between *Heterobasidion annosum* and *Stereum hirsutum* 45 million yr ago (Ma), *Auriscalpium vulgare* and *Peniophora* sp. 93 Ma and *A. vulgare* and *Lentinellus vulpinus* 135 Ma. One calibrated tree per batch of 10 single-copy genes was performed. The final tree was reconstructed based on the 50% median values obtained (mean values for branch length and extreme values for highest posterior density 95% confidence intervals). The obtained tree was plotted using MCMCTREER v.1.1 (Puttick, 2019).

## Comparison of gene families between saprotrophic and EcM fungi

The protein orthogroups with different numbers of proteins between saprotrophs and EcM species or between *Lactarius* and other EcM species were identified with a Brunner Munzel (BM) test, using the R packages BRUNNER MUNZEL v.1.4.1 (Neubert & Brunner, 2007) and STATS v.4.0.1. Figures were displayed using the R packages GGPLOT 2 and PHEATMAP v.1.0.12 (Kolde, 2019). A PCA based on CAZyme gene counts was performed with the FACTOEXTRA v.1.0.7 package (Kassambara & Mundt, 2017). For this analysis, gene families with Spearman correlation > 0.8 (CORRR v.0.4.3 package; Kuhn *et al.*, 2020) were binned together.

## Gains and losses in gene families

Expansion and contraction of *Lactarius* gene families were predicted with CAFÉ v.5 (Zenodo 10.5281/zenodo.3625141, as developed on GitHub). Singletons were removed from orthologue reconstructions. The previously identified species tree was pruned at the last common ancestor of *Lactarius* species with ITOL v.5 (Letunic & Bork, 2019).

### Gene tree reconstruction

The sedolisin gene tree was reconstructed from the protein sequences identified within Russulales (1951) and in outgroups (136; see Li *et al.*, 2017). They were aligned with MAFFT v.7.471 and trimed with TRIMAL v.1.4.rev15, which resulted in 124 sites. The best model, JTT + I + G4, identified with MODELTEST-NG (v.0.1.6), was used for phylogeny reconstruction by RAXML-NG (v.0.9.0, Kozlov *et al.*, 2019). Similarly, the GH25 family tree was reconstructed based on the alignment of 87 proteins (184 sites) using the VT model. In all, 500 bootstrap replicates were performed for the tree of GH25 genes.

### Insertion age of LTR retrotransposons

Full-length long terminal repeat (LTR) retrotransposons were identified in genome assemblies using LTRHARVEST with default parameters. This tool belongs to the GENOMETOOLS genome analysis software (v.1.5.10, Ellinghaus *et al.*, 2008). Long terminal repeats belonging to the *Gypsy* and *Copia* families were used for molecular dating of their genome invasion; selection was based on a BLASTX against REP BASE v.24.02 (Bao *et al.*, 2015). The 3′- and 5′-LTR nucleotide sequences were extracted and aligned with MAFFT v.7.471. Alignments were used to calculate Kimura's 2P distances. The insertion age was determined using the formula $T = K/2r$, with $K$ being the distance between the two LTR sequences, and $r$ being the estimated substitution rate of $1.05 \times 10^{-9}$ nucleotides $yr^{-1}$ per site for fungi (Dhillon *et al.*, 2014; Castanera *et al.*, 2016).

### Repeat element–gene distance analysis

We statistically measured the mean repeat–gene distances with the first 10 largest scaffolds by comparing the locations of observed genes and repeat elements and 10 000 null hypothesis genome models made by randomly reshuffling the locations of genes. The probability (*P*-value) of mean repeat–gene distances was calculated with the R package, REGIONER v.1.26.1 (Gel *et al.*, 2016). We calculated distances of all genes to the nearest repeat regions and examined significant differences among the fungi by performing Kruskal–Wallis with Dunn's test using the R package AGRICOLAE v.1.3-5 (De Mendiburu, 2021). The process was orchestrated with the visual omics pipeline, Syntey Governance Overview (SYNGO; Looney *et al.*, 2022).

### Identification of differentially expressed genes in ectomycorrhizas

Data on differential gene expression in EcM roots were obtained from Tang *et al.* (2021). In that study, RNA-sequencing datasets were produced from the free-living mycelia and EcM roots of *L. akahatsu*, *L. deliciosus*, *L. sanguifluus* and *L. vividus*. Filtered RNAseq reads were mapped onto their corresponding *Lactarius* genomes, and differentially expressed genes were identified using DESEQ2 v.1.28.1 (Love *et al.*, 2014) by comparing normalized gene expression levels in transcriptomes from ectomycorrhizas and free-living mycelia. Genes with a $\log_2$(fold-change) > 2 or < −2, and false discovery rate (FDR) *P*-value < 0.05 were considered to be differentially expressed.

## Results

### *Lactarius* genome features and species tree phylogeny of Russulales

The nuclear genomes of seven *Lactarius* strains, namely *L. akahatsu* QP, *L. deliciosus* 48, *L. hatsudake* 109, *L. hengduanensis* 84, *L. pseudohatsudake* 88, *L. sanguifluus* B21 and *L. vividus* 141, were sequenced, assembled and annotated at JGI and are available at the MycoCosm database (Grigoriev *et al.*, 2014). The quality and completeness of these genomes were confirmed by BUSCO analysis (Table S2). The size of the genome assemblies ranged from 62 to 100 Mb and contained 11 612–20 824 protein-coding genes (Fig. 1a,b). By including the published genomes from *L. quietus* (116 Mb, 18 943 genes) (Miyauchi *et al.*, 2020) and *L. psammicola* (70 Mb, 13 442 genes) (Looney *et al.*, 2022), we noticed a nearly two-fold variation in the genome size and gene content for *Lactarius* species. Ectomycorrhizal species ($n = 19$) displayed a significantly larger genome size and TE content than the saprotrophic species ($n = 12$), and among EcM fungi, *Lactarius* species ($n = 9$) presented a larger genome size and TE content than the others (*Russula*, *Lactifluus* and *Multifurca* species, $n = 10$) (Fig. 1a). The gene content of *Lactarius* species was also higher compared with other EcM species (permuted BM test, *P*-value < 0.01), but similar to saprotrophic species. Genome structural analysis (i.e. synteny) showed that no whole-genome duplication has occurred in *Lactarius*. Instead, analysis of protein orthology indicated that the higher gene/protein content in *Lactarius* species was mainly a result of duplication of dispensable genes, while conserved- and species-specific genes were less prone to this duplication event (Fig. 1b).

The species tree phylogeny of the Russulales, reconstructed from an alignment of 934 single-copy orthologous genes, confirmed the monophyletic origin of *Lactarius* sect. *Deliciosi* after the earlier divergence from *L. quietus* and *L. psammicola* (Fig. 1c). *Lactifluus* and *Multifurca*, the two other genera producing milky latex, clustered with nonmilk-cap *Russula* species, rather than with *Lactarius* species. Time calibration estimated the origin of Russulales at *c.* 260 Ma, and the common ancestor of EcM species at *c.* 70 Ma, which is consistent with the recent estimation by Looney *et al.* (2022).

### TE profiles and evolution within Russulales

As TE accumulation accounts for the larger size of *Lactarius* genome assemblies, we further investigated the composition and evolution of these repeated elements, keeping in mind that a substantial proportion of TEs might not have been assembled owing to the high number of repeats. In this study, we identified more TE in EcM genomes than in saprotroph genomes (BM test, Bonferroni *P*-value < 0.01, Fig. 2a). For instance, the *Harbinger* and *hAt* found in most EcM species, were absent in the Russulales

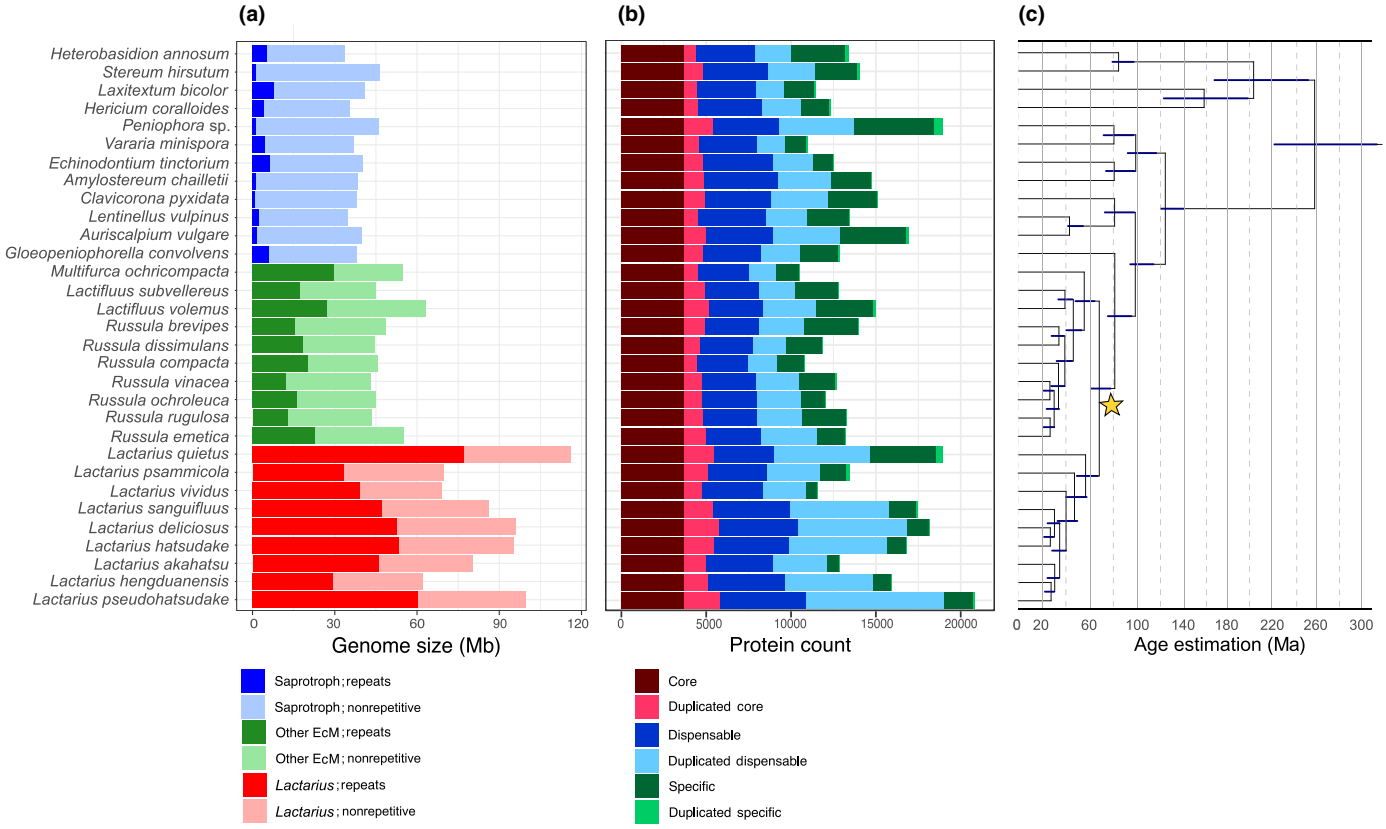


**Fig. 1** Genome and phylogeny of Russulales. (a) Size of genome assemblies and proportion of transposable elements (TEs) in the assembly in Russulales. (b) Conserved, dispensable and species-specific genes in Russulales. Counts of duplicated protein sequences are also shown. (c) Species tree phylogeny of Russulales calibrated on a timescale (million yr ago, Ma). The confidence interval is shown on the branch and the star indicates the transition from saprotrophic to ectomycorrhizal (EcM) lifestyle.

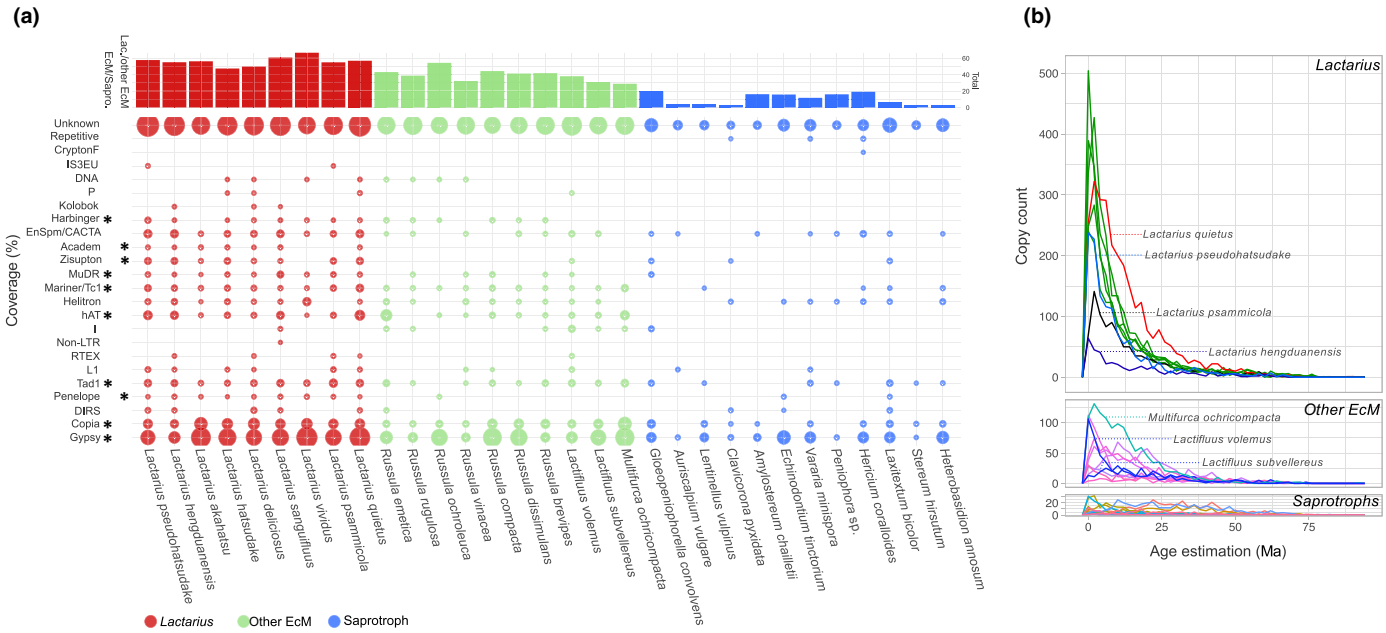


**Fig. 2** Transposable element composition and evolution in Russulales. (a) Genome coverage of transposable element (TE) categories annotated in the Russulales genomes. *, Brunner Munzel test significance, Bonferroni *P*-value < 0.01 between ectomycorrhizal (EcM) and saprotrophs or *Lactarius* spp. and other EcM species. (b) Estimated ages of Copia and Gypsy long terminal repeats. TE counts per age were binned by 2 million yr ago (Ma). LTR, long terminal repeat.

saprotrophs. *Lactarius* also contains some TE categories, such as *Academ*, *Zisupton* and *Penelope*, that were hardly found in other EcM species (Fig. 2a). Other TEs, such as *Mariner*, *Gypsy* and *Copia*, also largely expanded in EcM species (BM test, Bonferroni *P*-value < 0.01). We estimated that the accumulation of the most abundant *Gypsy* and *Copia* LTRs started at *c.* 70 Ma. The TE invasion coincided with the estimated origin of the symbiotic Russulales, while the massive LTR expansion in *Lactarius* species took place in the last 10 Ma after their speciation (Fig. 2b). We observed a striking heterogeneity in TE expansion rate among *Lactarius* species. For instance, *L. hengduanensis* presented a much lower TE expansion rate than the other species, a profile resembling the non*Lactarius* EcM fungi (Fig. 2b).

## *Lactarius* genomes encode expanded gene families coding for proteases

The sequenced *Lactarius* genomes displayed the highest content in protease genes among Russulales species. This is in sharp contrast with other EcM Russulales species which displayed a reduced protease gene set compared with saprotrophic species. This enrichment in proteases was mainly associated with a drastic gene expansion of the sedolisin family (S53), one of the two subtilase families (PF09286, EC3.4.14.9) (Fig. 3a; Table S3). Comparison of the sedolisin protein sequences indicated that most of the sedolisins in *Lactarius* species lacked at least one of the three canonical sedolisin regions (Fig. 3a). Several sedolisin genes were clustered (tandem duplications) in the genome. Protein orthology analysis classified all Russulales sedolisins (1951) into 46 multiple-gene families and 123 singletons. Although nearly all these families (159) contained only *Lactarius* genes, they did not evolve newly in *Lactarius*, but expanded from a more ancestral sedolisin clade (Fig. 3b). Beside sedolisins, the fungalysin family (M36) also expanded largely in *Lactarius* (13.1 copies, as compared to 1.4 copies in other EcM species, Table S3). However, fungalysin and cytophagalysin (M43B) genes were scarcely detected in *L. quietus*, the oak-associated species.

**Sedolisins are rapidly evolving in *Lactarius* species** Gene family expansion and contraction analysis within *Lactarius* species identified 229 rapidly evolving gene families (Table S4). For each of them, significant expansion/contraction was observed on multiple nodes of the phylogenomic tree (Fig. 4). Notably, seven out of the eight families with annotations were sedolisins. As it could be related to the shift/switch of host specificity, we focused on

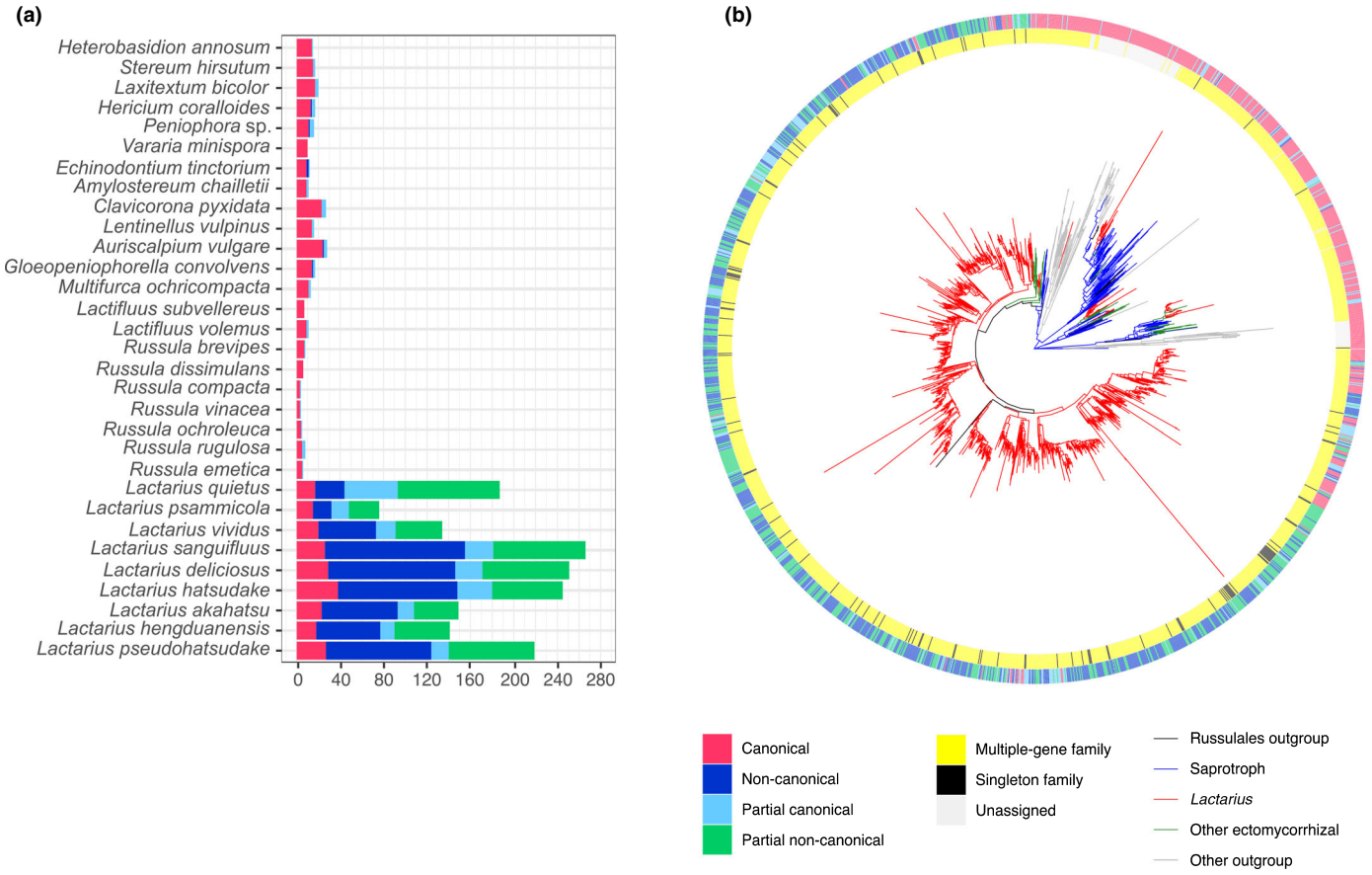


**Fig. 3** Sedolisin gene content and evolution in Russulales. (a) Sedolisin (S53) gene content in Russulales genomes. Sedolisins missing one of their three catalytic regions were labelled as partial; the sedolisin was labelled as containing a noncanonical region if at least one of the catalytic regions lacked a perfect match to the pattern described in the literature. (b) Phylogeny of sedolisins in Russulales and outgroup species.

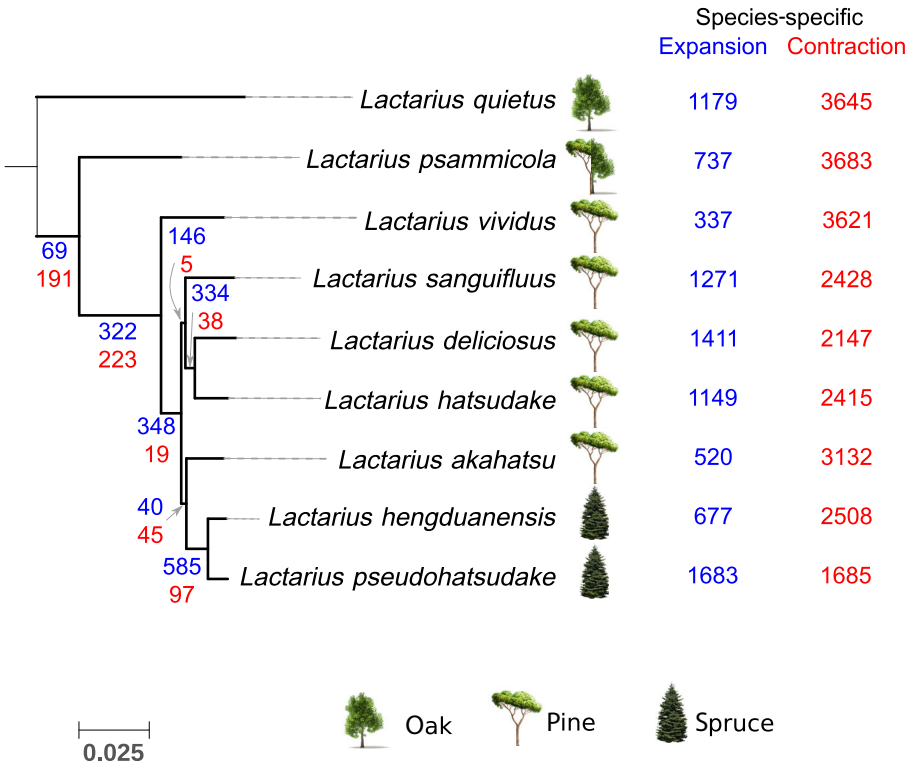


**Fig. 4** Expansion and contraction of gene families in *Lactarius* species. The number of gene families are displayed on the nodes of RAxML species tree, with expanding gene families in blue and contracting gene families in red.

three ancestral nodes: the closest ancestor of *L. quietus* and *L. psammicola*; the closest ancestor of *L. psammicola* and the species restricted to pines; and the closest ancestor of spruce-associated species. Consistently, the sedolisin families were the gene families showing major expansions or contractions.

***Lactarius* sedolisin genes are colocalized with TEs** As TEs are known to duplicate genes through transposing activity, we examined associations between TEs and sedolisin-coding genes by estimating the distance of the genes to the nearest repeat elements. Indeed, the sedolisin genes were found to be significantly closer, with a mean distance of 2.5 kb, to the repeats in *Lactarius* than in the rest of Russulales fungi (Kruskal–Wallis with Dunn's test, FDR $P$-value < 0.05; Fig. 5a). Most of the colocalized repeats within a distance of 4.5 kb, were unclassified categories (Fig. 5b).

**Genes coding for secreted sedolisins are upregulated in host-specific symbioses** Transcript profiling using RNA-seq datasets from four compatible *Lactarius–Pinus* pairings (Tang *et al.*, 2021) revealed that nearly half of the transcripts coding for secreted sedolisins (S53) were strikingly induced during the host-specific interactions (Fig. S1). Importantly, the eight rapidly evolving sedolisin gene families were upregulated upon symbiosis. Although TEs could influence the regulation of genes nearby, we did not detect either significant proximity of these mycorrhiza-induced sedolisins to any TE category, compared with the noninduced ones (Fig. S2a), or clear association between the regulation amplitude and distance to repeats (Pearson correlation coefficient with 95% confidence; Fig. S2b).

## Secreted CAZymes

As expected from previous EcM genome analyses (Kohler *et al.*, 2015; Miyauchi *et al.*, 2020), the arsenal of enzymes involved in lignocellulose decomposition was strikingly reduced in EcM Russulales species compared with saprotrophic species (30 CAZyme families; BM test, FDR $P$-value < 0.01; Fig. 6a; Table S5). The number of secreted genes containing the chitin-binding domain CBM5 was also reduced in EcM species. However, *Lactarius* species have retained a larger polysaccharide-degrading potential than other EcM species, as they encoded more genes acting on fungal glucan (GH16, GH17, GH152), chitin (GH20, CBM5, CBM50), plant cellobiose (AA3) and cellulose (GH3, GH131) (Table S5). Besides, secreted GH25, which acts on bacterial peptidoglycan, appeared to have expanded specifically in *Lactarius* species, especially in the two spruce-specific species (Figs 6a, S3). These differences in secreted CAZymes clearly separate *Lactarius* from the other EcM fungi within Russulales (Fig. 6b).

## Effector-like SSPs

Regarding effector-like SSPs, we found 16 subgroups with known Pfam domains showing differential distribution either between saprotrophic and EcM fungi, or between *Lactarius* and other EcM species (BM test, FDR $P$-value < 0.01; Table 1). In accordance with previous results, four of them belong to CAZymes including three acting on cellulose (CBM1, GH12 and AA9) depleted in EcM and one on bacterial peptidoglycan (GH25) specifically enriched in *Lactarius* species. Two domains (PF01476: LysM and PF01522: polysaccharide deacetylase)

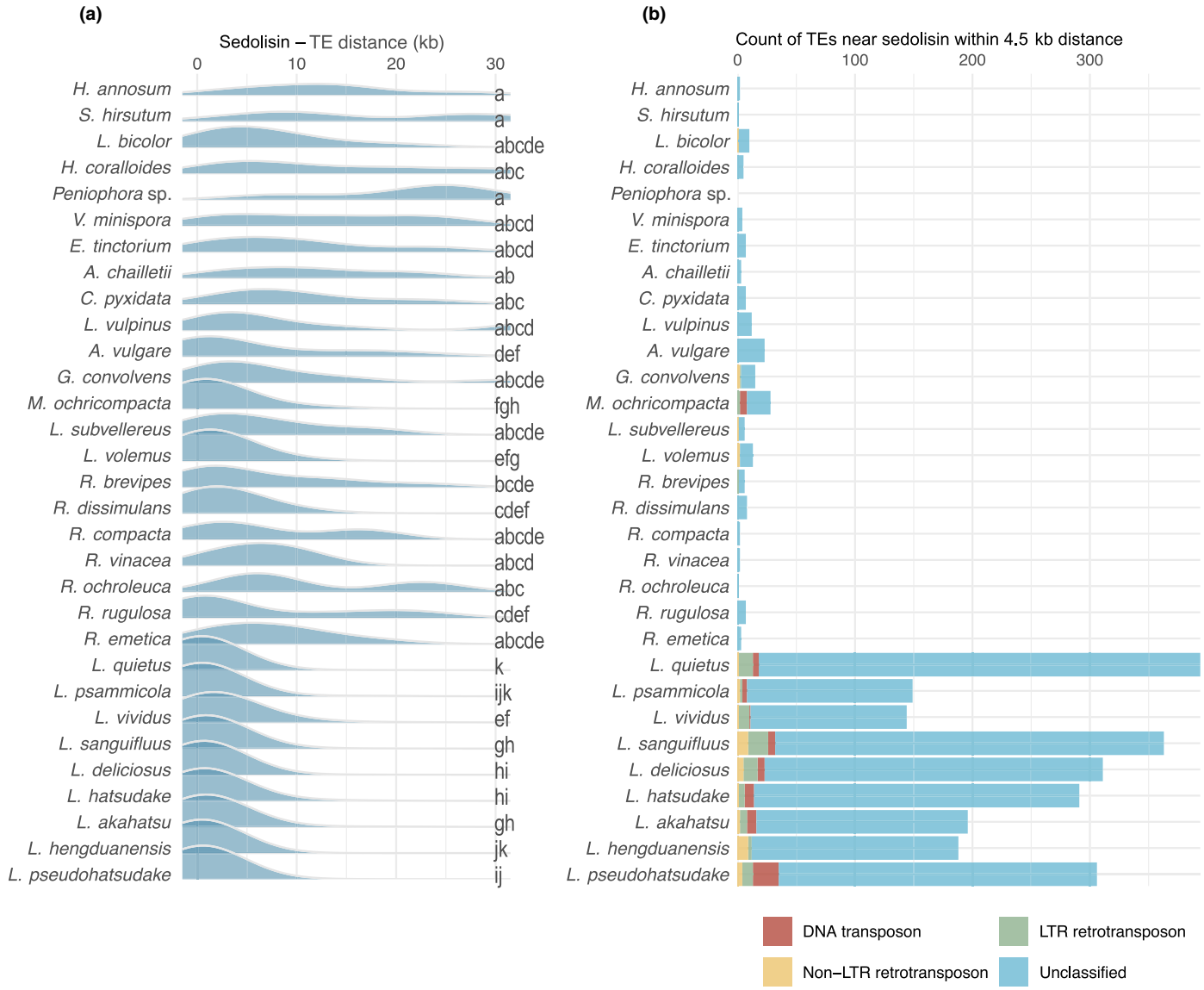


**Fig. 5** Colocalization of sedolisin genes with transposable elements (TEs) in *Lactarius*. (a) Sedolisin gene–TE distances in Russulales species. Gene–TE distances were plotted for each species and comparisons were performed among all species. Significant differences (Kruskal–Wallis with Dunn's test, *P*-value < 0.05) are indicated by the letters on the right side of each species. (b) Number of each TE category found within a distance of 4.5 kb to sedolisin genes. LTR, long terminal repeat.

involving chitin binding and modification were also found to be significantly enriched in *Lactarius*. Another domain (PF00314: Thaumatin), possibly acting on the beta-1,3-glucans in fungal cell walls (Sakamoto *et al.*, 2006), was enriched in *Lactarius* species as well. Consistent with the overrepresentation of protease genes, we detected three protease-associated domains (PF09286, PF13582 and PF13688) that were enriched in *Lactarius* SSPs. However, it should be noticed that there was a clear difference for some domains among these *Lactarius* species. For instance, *L. vividus* and *L. hatsudake* contained no pro-kumamolisin activation domain (PF09286), and the oak-specific *L. quietus* harboured the lowest number of SSPs containing LysM domain. When the distance between SSP genes and TEs was investigated, they appeared significantly closer to TEs than did other genes. However, those TEs were mainly unclassified.

## Lectins

Given the potential role of lectins in determining host specificity in several plant–fungus interactions, including EcM symbiosis (Guillot *et al.*, 1991; Giollant *et al.*, 1993; Varrot *et al.*, 2013), we surveyed the lectin gene distribution in Russulales. We found six lectin families with differential gene contents between saprotrophic and EcM fungi, or between *Lactarius* and other EcM species within Russulales (BM test, Bonferroni *P*-value < 0.01; Fig. S4). Among these, the PVL-like family was only detected within *Lactarius* species and restricted to species associated with pine and spruce hosts. The H-type lectin genes, rarely found in non*Lactarius* genera, mainly expanded in *Lactarius* species, with six copies in *L. sanguifluus* to 18 copies in *L. psammicola*.

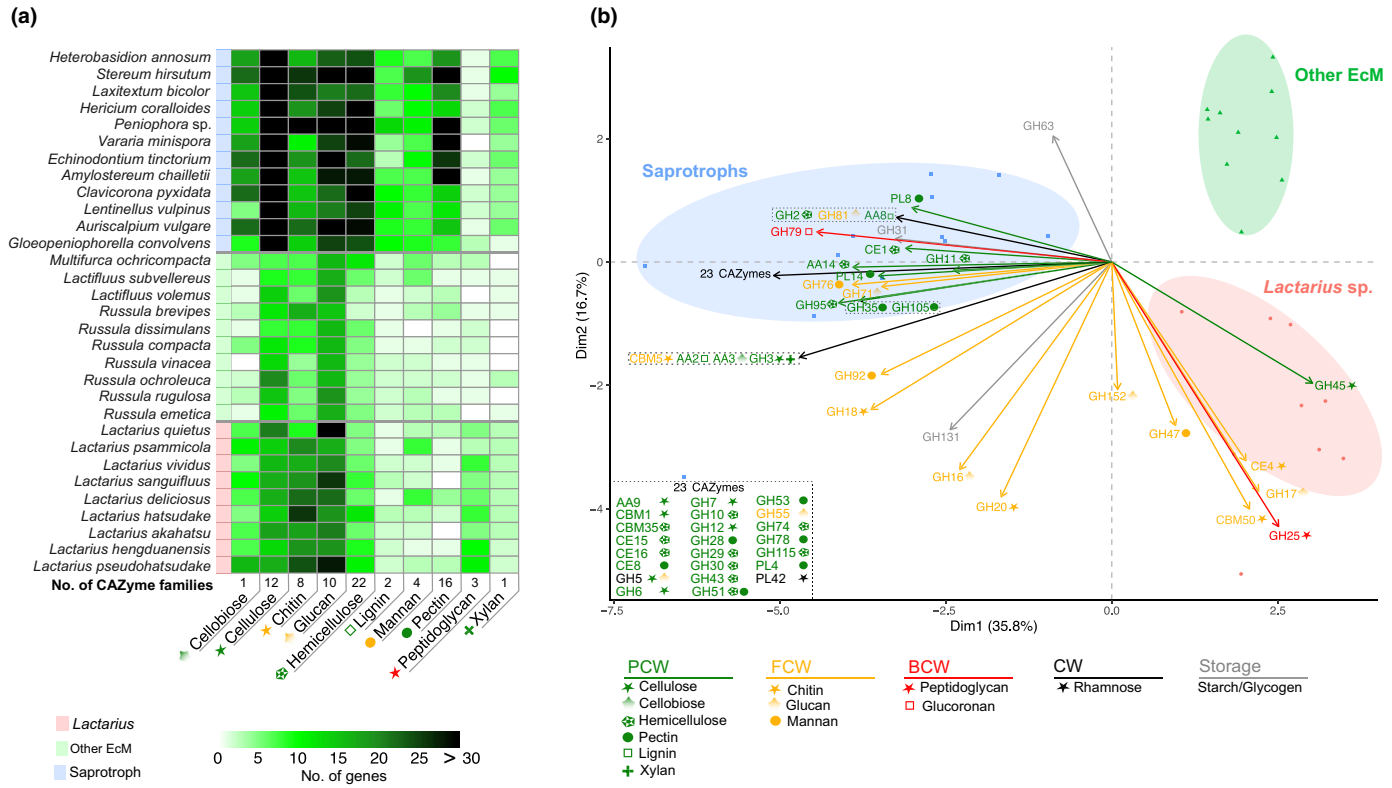


**Fig. 6** Differential distribution of secreted carbohydrate-active enzyme (CAZyme) genes among Russulales fungi. The number of genes coding for secreted CAZymes was compared among Russulales fungi (*Lactarius*, other ectomycorrhiza and saprotroph). (a) Secreted CAZyme categories, grouped by their potential substrates, showing differential distributions either between saprotrophic and ectomycorrhizal (EcM) fungi, or between *Lactarius* and other EcM species, are shown (Brunner Munzel test, false discovery rate (FDR) *P*-value < 0.01, detailed in Supporting Information Table S5). (b) Principal component analysis of secreted CAZyme genes showing differential distributions among the groups of interest. For display purpose, gene families with a Spearman correlation > 0.8 were binned together. BCW, bacterial cell wall; FCW, fungal cell wall; PCW, plant cell wall.

## G-protein coupled receptors

Given their high upregulation during EcM colonization in *Laccaria bicolor*, *Tuber melanosporum* and *Suillus* species (Voiblet *et al.*, 2001; Martin *et al.*, 2010; Plett *et al.*, 2012; Liao *et al.*, 2016), GPCRs were considered as candidates related to host specificity or associated with EcM colonization more generally. In the present Russulales genome dataset, no specific expansion was detected in EcM species, with a mean of 14 ± 2 copies, and the host-specific genus *Lactarius* contained the lowest number of GPCRs (BM test, *P*-value < 0.01; Table S6). During EcM development involving *L. akahatsu*, *L. sanguifluus*, *L. deliciosus* or *L. vividus* with a compatible host, only one GPCR gene was significantly upregulated in *L. akahatsu* and another one downregulated in *L. deliciosus* (Tang *et al.*, 2021).

## Secondary metabolism pathways

Based on the possible relevance of secondary metabolites (SMs) in determining host specificity in *Suillus* species (Lofgren *et al.*, 2021), we compared the repertoire of SM-related genes among Russulales species. Compared with other EcM fungi, *Lactarius* species harboured a higher number of terpene synthase (TPS) genes (BM test, bonferroni *P*-value = 0.018; Fig. S5). However, the TPS gene content varied among *Lactarius* species (from nine copies in *L. quietus* to 20 copies *in L. pseudohatsudake*). Besides, TPS genes were also enriched in the basal EcM fungus *Multifurca ochricompacta* (21 copies) and some of the most related saprotrophic species such as *Clavicorona pyxidate* and *A. vulgare* (17 and 12 copies, respectively). Among these genes, three were identified as upregulated during mycorrhiza formation: one in *L. sanguifluus* and two in *L. deliciosus* (Tang *et al.*, 2021).

## Biosynthesis of latex rubber

Latex production is a well-known feature of milk-cap fungi including species in *Lactarius*, *Lactifluus* and *Multifurca* genera. Considering its ecological importance, such as the resistance to fungivorous predation (Taskirawati & Tuno, 2016), genes potentially involved in fungal latex rubber biosynthesis were surveyed. Genes of the cytosolic mevalonate (MVA) pathway, rubber initiation and elongation, were found in all Russulales genomes (Fig. S6; Table S7). No genes coding for the plastidial methylerythritol phosphate (MEP) pathway were found in these fungi. We observed a slight enrichment of latex biosynthesis genes in *Lactarius* spp. compared with other Russulales species (BM test, Bonferroni *P*-value = 0.034).

**Table 1** Small secreted protein (SSP) domains differing in abundance among Russulales species.

| | PFAM | Domain description | Saprotroph | EcM | Adjusted *P*-value |
|---|---|---|---|---|---|
| EcM vs saprotrophs | PF00445 | Ribonuclease T2 family | 0.17 | 1.21 | 6.82E−03 |
| | PF00734 | CBM1 | 2.67 | 0 | 3.28E−05 |
| | PF01670 | GH12 | 2.42 | 0 | 0.00E+00 |
| | PF03443 | AA9 | 5.58 | 0.16 | 3.21E−20 |
| | PF10342 | Kre9/KNH-like N-terminal Ig-like domain | 9.50 | 5.84 | 3.38E−04 |
| | PF11937 | DUF3455 | 1.33 | 4.21 | 6.32E−03 |
| | | | Other EcM | *Lactarius* | |
| *Lactarius* vs other EcM | PF00314 | Thaumatin family | 0.90 | 5.33 | 2.79E−03 |
| | PF01183 | GH25 | 0.50 | 5.33 | 6.09E−05 |
| | PF01476 | LysM domain | 2.30 | 11.56 | 3.07E−05 |
| | PF01522 | Polysaccharide deacetylase | 0 | 1.33 | 3.49E−03 |
| | PF02265 | S1/P1 nuclease | 0.30 | 1.89 | 5.90E−04 |
| | PF08590 | DUF1771 | 0.20 | 1.33 | 7.45E−03 |
| | PF09286 | Pro-kumamolisin, activation domain | 0 | 2.67 | 3.27E−04 |
| | PF09419 | Mitochondrial PGP phosphatase | 0 | 0.78 | 6.15E−04 |
| | PF13582 | Metallo-peptidase family M12B Reprolysin-like | 0.20 | 2.89 | 3.00E−04 |
| | PF13688 | Metallo-peptidase family M12 | 0.10 | 1.78 | 2.42E−04 |

Pfam domains contained in SSPs showing differential distributions either between saprotrophic and ectomycorrhizal (EcM) fungi, or between *Lactarius* and other EcM species are listed (Brunner Munzel test, false discovery rate *P*-value < 0.01); the mean number of each Pfam domain contained in each group is shown.

## Discussion

The shift from the saprophytic to symbiotic lifestyle of ancestral Russulales species took place at *c.* 70 Ma, during the third wave of plant root diversification (Strullu-Derrien *et al.*, 2018). It has been suggested that this event was linked to a global climate change, as well as an increase in potential habitats and soil complexity, which presumably resulted in a competitive advantage for more specialized root types. In association with this root diversification, multiple saprophytic fungi in various fungal lineages shifted to an EcM lifestyle (Looney *et al.*, 2018). As a result of convergent evolution, EcM lineages in most fungal orders share similar genomic features, including a larger genome size resulting from TE proliferation, a restricted set of PCWDEs and a specific suite of effector-like SSPs (Kohler *et al.*, 2015; Miyauchi *et al.*, 2020; Lebreton *et al.*, 2021b). These convergent sets of genetic traits are the hallmarks of the EcM lifestyle and they are shared by the symbiotic Russulales. However, we found a series of idiosyncrasies that distinguish *Lactarius* species from other EcM lineages as discussed in the following.

### Divergent evolution of the symbiotic lifestyle within Russulaceae

Despite descending from a single lineage in Russulaceae, *Lactarius* and *Russula* species display divergent genomic traits that may impact the development and functioning of their EcM associations. The large expansion of sedolisin proteases is unique to *Lactarius* species and was not reported in other EcM fungal lineages sequenced so far (Kohler *et al.*, 2015; Peter *et al.*, 2016; Murat *et al.*, 2018; Miyauchi *et al.*, 2020; Lofgren *et al.*, 2021). Their colocalization with TEs suggests that this expansion was probably caused by a recent TE proliferation that occurred in the last 10 Myr. These proteases may play a role in releasing organic N nutrients (i.e. amino acids or oligopeptides) from soil organic matter (SOM) via protein cleavage. However, their extensive induction during EcM symbioses suggests that these sedolisins are more likely to be involved in the interaction with host plants (Tang *et al.*, 2021). Secreted proteases in several plant pathogens could dampen the host defence reactions via cleaving immunity-related proteins, such as chitinases, secreted by the host roots (Naumann *et al.*, 2011; Jashni *et al.*, 2015; Sanz-Martín *et al.*, 2016; Ökmen *et al.*, 2018). This protease-based strategy is supported by our finding that several other proteases, such as fungalysins, were also strongly induced during the symbiosis (Tang *et al.*, 2021).

Within the Russulaceae family, *Russula* species are known for their broad range of hosts, that is, most of them are known as host generalists. Their species diversification has been linked to frequent host switching between angiosperms and Pinaceae with subsequent host expansion (Looney *et al.*, 2016). By contrast, many *Lactarius* species, such as the ones in the section of *Deliciosi*, have long been considered as host specialists (Nuytinck *et al.*, 2007; Verbeken & Nuytinck, 2013; Wang *et al.*, 2019). This divergent host selection provides a unique opportunity to explore the molecular determinants involved in host specificity. In pathogenic fungi, a restricted host range is often accompanied by gene losses (Spanu *et al.*, 2010; Baroncelli *et al.*, 2016). However, a recent study comparing the gene repertoires of host-specific species in *Suillus* (Boletales) with other less host-specific fungal symbionts reported no significant gene loss, but suggested that secondary metabolites synthesized by terpene- and nonribosomal polyketide synthases (NRPS) may play a role in determining host specificity (Lofgren *et al.*, 2021). Interestingly, we also found a slight enrichment of terpene synthase (TPS) genes, but not of NRPS genes in *Lactarius* species. Strikingly, a dramatic expansion of sedolisin proteases was observed in *Lactarius*. Moreover, our analysis of the expansion and contraction of gene families indicated that several sedolisin gene families were rapidly evolving in multiple phylogenetic nodes where host switches probably occurred. This evidence, together with their unique regulation in various EcM symbioses (Tang *et al.*, 2021), supports an important role for sedolisins in the host specificity of *Lactarius* EcM associations. In addition, other protein categories showing a significant enrichment in *Lactarius* species or a unique symbiotic regulation, such as the LysM domain-containing SSPs and lectins, may also be involved in the

interaction with specific host species, owing to their biochemical role in ligand-binding mechanisms (Guillot *et al.*, 1991; Giollant *et al.*, 1993; Kombrink & Thomma, 2013; Labbé *et al.*, 2019; Bozsoki *et al.*, 2020).

### Heterogeneity among *Lactarius* genomes

We found a substantial heterogeneity in genome size and gene composition among the sequenced *Lactarius* species, even though they belong to a single section. A nearly two-fold variation in genome size and gene content was observed among *Lactarius* species, which is in contrast to the homogeneity of *Russula* genomes (Fig. 1a,b). As comparable BUSCOcompleteness was reported for all these genomes, this variation is not related to differential quality scores in genome assemblies or gene annotations among *Lactarius* species. The absence of whole-genome duplication indicates that this variation mainly results from duplication of specific gene families. Indeed, the protein orthology analysis revealed that the oak-specific species *L. quietus* and the other four Pinaceae-specific species (*L. sanguifluus*, *L. deliciosus*, *L. hatsudake* and *L. pseudohatsudake*) present higher rates of duplications of dispensable genes than the others, *L. quietus* itself having a higher content of species-specific genes than the rest. The smaller genomes of *L. psammicola*, *L. vividus* and *L. akahatsu* can also be explained by large gene reductions (Fig. 1). There is also a large difference in the genome size and gene content among the species associated with a single genus of hosts (i.e. *Pinus* or *Picea*). Interestingly, a large variation within a single EcM genus has also been observed in *Suillus* and *Amanita*, the latter in which both EcM and nonEcM species have evolved (Hess *et al.*, 2018; Lofgren *et al.*, 2021). However, unlike the large amplification of species-specific gene families in *Amanita*, *Lactarius* presents more duplication of dispensable families shared by at least two species, whereas its species-specific families have a very limited amplification. This difference highlights the diversity of genome evolution in different EcM fungal lineages. The high heterogeneity found in both specialistic lineages (i.e. *Suillus* and *Lactarius*) may, on the other hand, suggest an important role for host specialization in shaping EcM fungal genomes, as frequently observed in plant pathogens (Vries *et al.*, 2020).

### Concluding remarks

To better understand the evolution of EcM symbiotic lifestyle and host specificity, we sequenced several milk-cap fungal species and performed genomic comparisons with their ancestral saprotrophs and symbiotic sister genera (*Russula*, *Lactifluus* and *Multifurca*) within Russulaceae. *Lactarius* species have significantly larger genomes than the other clades, as a result of TE proliferation. They also convergently lost PCWDEs, but retained a number of CAZymes acting on microbial cell wall components, especially the bacterial peptidoglycan. Most remarkably, *Lactarius* harbours a drastically expanded sedolisin protease family, a feature absent from any other EcM fungal lineages sequenced so far, including its sister genera within the same family. The expansion and rapid evolution of sedolisin genes, together with their extensive symbiotic upregulation, suggest that milk-cap fungi use a protease-based toolkit to dialogue with their specific host species, a strategy adopted by some plant pathogens yet not reported in plant symbionts. Ongoing functional analysis of symbiosis-induced sedolisins will provide the needed information on the substrate of these proteases and their role in EcM development. Besides, other gene products with known high ligand-binding specificity may also play a role in the host specialization. Meanwhile, this long-term host specialization/adaptation may have, in turn, reshaped fungal genomes, causing large interspecific differences in their size and gene repertoire. Taken together, this study casts a new light on the evolution of EcM lifestyle and highlights an important role for secreted proteases in host-specific *Lactarius* symbioses. The uniqueness of *Lactarius* revealed here thus warrants diverse lineages to be investigated in the future for a full view of EcM evolution.

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## Author contributions

FMM conceived and coordinates the Mycorrhizal Genomics Initiative. NT, FY, AG-L and FMM designed the present project. A Lebreton, NT and FMM wrote the manuscript with input from FY and YD. RW and DM isolated and identified the fungi. NT produced the materials for sequencing. IVG coordinated genome sequencing and annotation at JGI. AK, KL, WA, KB, AC, A Lipzen and VN performed transcriptome sequencing, assembly and gene annotation at JGI. ED and BH performed CAZyme annotations. A Lebreton, NT and SM performed comparative genome analyses. A Lebreton and NT contributed equally to this work.

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## Data availability

Genome assemblies and gene annotations used in this study are available via the JGI fungal genome portal MycoCosm (see the Russulales page: https://mycocosm.jgi.doe.gov/Russulales/Russulales.info.html) and NCBI Genome database under the BioProject of PRJNA500114 to PRJNA500118, PRJNA500120 and PRJNA500123 (accession nos. JAKELG000000000, JAKELH000000000, JAKELI000000000, JAKELK000000000, JAKELL000000000, JAKEYE000000000 and JAKEYF000000000). RNA-seq read data are available at the NCBI Sequence Read Archive (SRA) under the BioProject of PRJNA706172. All other data supporting the findings of this study are included within the article and its additional files.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Sedolisin gene number regulated in four *Lactarius* ectomycorrhizal symbioses.

**Fig. S2** Noncorrelation of gene–transposable element distance to the regulation of sedolisin genes.

**Fig. S3** Evolution of peptidoglycan-degrading GH25 genes in Russulales.

**Fig. S4** Lectin gene abundance in Russulales.

**Fig. S5** Secondary metabolism-related genes in Russulales.

**Fig. S6** Possible latex rubber biosynthetic pathway in Russulales.

**Methods S1** *Lactarius* genome and transcriptome sequencing, assembly and annotation.

**Table S1** Details on Russulales and outgroup species used in this study.

**Table S2** General information of Russulales and outgroup genomes.

**Table S3** Proteases differing in abundance between *Lactarius* and other Russulales ectomycorrhizal species.

**Table S4** Rapidly evolving gene families (OGs) on *Lactarius* clade.

**Table S5** Distribution of secreted CAZymes in Russulales fungi.

**Table S6** G-protein coupled receptor gene abundance in Russulales.

**Table S7** Latex rubber biosynthesis-related genes in Russulales.

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