Summary

The first genomic sequence for a representative of symbiotic fungi, the ectomycorrhizal basidiomycete *Laccaria bicolor*, has been published. The unravelling of this genome provides tantalizing hints about differences between this symbiotic fungus and its saprotrophic and pathogenic relatives. An expansion of several multigene families occurred in *L. bicolor*, suggesting that adaptation to symbiosis proceeded by gene duplication. Within lineage-specific genes those coding for symbiosis-regulated secreted proteins showed an up-regulated expression in ectomycorrhizas. *L. bicolor* is lacking enzymes involved in the degradation of plant cell wall components (cellulose, hemicellulose, pectins and pectates), preventing the symbiont from degrading host cells. By contrast, *L. bicolor* possesses expanded multigene families associated with hydrolysis of bacterial and microfauna polysaccharides and proteins. The genome analysis revealed the dual saprotrophic and biotrophic lifestyle of the mycorrhizal fungus that enables it to grow within both soil and living plant roots. The next stages will involve finer-scale investigation of gene networks to reveal the details of the...
I. Introduction

Just a handful of fungal species are the basis for a staggering amount of our biological knowledge of the Eumycota kingdom. From the ever-popular baker yeast (Saccharomyces cerevisiae) to the always fruitful Neurospora crassa, mycologists have cultivated a cadre of model organisms to unravel the intricate mysteries of cell communication, metabolism, genome evolution and developmental pathways. For all the laboratory tales these model species have helped to tell, there remains a wealth of evolutionary and ecological questions still to be addressed. Understanding organisms’ responses to the environment, both biotic and abiotic, in order to provide a more complete story of biological networks is one of the biggest challenges in biology today (Gewin, 2005; Ungerer et al., 2008). The field of ecological genomics seeks to understand the genetic mechanisms underlying responses of organisms to their natural environments. This is being achieved through the application of functional genomic approaches to identify and characterize genes with ecological and evolutionary relevance (Whitham et al., 2006; Ungerer et al., 2008). In addition to providing insight into ecology and evolutionary lineages, studies of nonmodel organisms are sure to reveal as-yet-unknown biological mechanisms in the interactions between species, a major driver of ecosystem evolution (Whitham et al., 2008). Thus, in the wake of the poplar genome sequencing (Tuskan et al., 2006), it was decided to investigate the mycorrhizal genomes and develop the tools to pin down the genes underlying the symbiosis interactions and ecological adaptation. The mycorrhizal community drew up a proposal for the Department of Energy Joint Genome Institute (JGI) to sequence the ectomycorrhizal Laccaria bicolor Maire (P.D. Orton) (Fig. 1) and endomycorrhizal Glomus intraradices genomes (Lammers et al., 2004; Martin et al., 2004). They obtained the go-ahead in October 2003 and the data started trickling in during early 2005. The genome of L. bicolor was publicly released by the JGI at the 5th International Conference on Mycorrhiza in Granada, Spain (Selosse & Duplessis, 2006), and the Laccaria genome consortium recently published the analysis of the high-quality draft sequence of L. bicolor (Martin et al., 2008). This will provide important new information about the genetics of the ectomycorrhizal (ECM) symbiosis.

By examining the genomic blueprint of the mycobiont and that of its host, Populus trichocarpa, and manipulating their patterns of gene expression, we can now identify the genetic control points that regulate plant-mutualist response in an effort to understand better how these interactions control plant growth and ecosystem function (Tuskan et al., 2006; Martin et al., 2008; Whitham et al., 2008). The whole genome sequence (WGS) of L. bicolor provides the first symbiont blueprint, allowing new insights into the nature of the genome of one of the largest groups of fungi. It will facilitate identification of genes (if any) that are truly innovations of symbiosis and others that have been lost during evolution, as well as the way they have been obtained during genomic evolution. Initial analyses of the 65-megabase L. bicolor genome led to a slew of insights into mycorrhizal symbiosis. Initial studies have been reported in Nature (Martin et al., 2008) and we have committed the pages of this special issue of New Phytologist to an in-depth exploration of the genome. In this review we discuss the current status of this new field. We summarize the progress that has been made in investigating the L. bicolor genome, indicate the main findings of these studies, and suggest major questions that remain to be addressed.

II. The ectomycorrhizal basidiomycete Laccaria bicolor

Ectomycorrhizal fungi form a mutualistic symbiosis with the majority of tree species in most forest ecosystems. The fungi are unique in having a simultaneous dual lifestyle, living both within the plant roots as symbionts and, at the same time, in the soil as facultative, transitory saprotrophs. Without this symbiosis, forests as we know them could probably not exist because of the essential role of the fungi for tree growth and in the cycling of essential nutrients. Thus, ECM fungi are responsible for a symbiosis of global ecological and economic importance. The trees ‘feed’ the ECM fungus with plant-derived carbohydrates and the fungus then utilizes this energy to decompose and assimilate essential nitrogen and phosphate compounds in the soil and transfer them back to the trees. Moreover, the association of different plants with the same guild of fungi mediates indirect interplant interactions, such as nutrient transfer or competition (Selosse et al., 2006). Understanding how the fungus can achieve this is essential because of the key role of forests in buffering/sequestering increased CO₂ and also for understanding how to optimize tree productivity in future biofuel production.


Laccaria bicolor is a member of the Hydnangiaceae that are placed in the order Agaricales, a large group of Basidiomycota that also includes Polyporales (e.g. *Phanerochaete chrysosporium*), Schizophyllaceae (e.g. *Schizophyllum commune*) and Psathyrellaceae (e.g. *Coprinopsis cinerea*). The Agaricales belong to the Agaricomycotina of the Agaricomycetidae (Matheny et al., 2006). By contrast, the sequenced human pathogen, *Cryptococcus neoformans*, a member of the Filobasidiales, lies within the Tremellomycetes (Agaricomycotina), whereas the plant pathogen (smut) *Ustilago maydis* belongs to the Ustilaginaceae in the Microbotryomycetes (≡ smut fungi). Comparisons between *L. bicolor* and the sequenced genomes of *C. cinerea*, *P. chrysosporium*, *S. commune*, *C. neoformans* and *U. maydis* (Table 1) therefore have the potential to illuminate features of their last common ancestor – the ancestral Basidiomycota – which lived approximately 400 to 800 Mya (Matheny et al., 2006). The *L. bicolor* genome provides what evolutionists consider an extremely useful group for understanding the evolution and life history of the Eumycota phylum.

**Laccaria** species have been a major experimental model for decades (Molina, 1982; Kropp et al., 1986; Kropp & Fortin, 1988; Massicotte et al., 1989, 2005; Ahmad et al., 1990; Gardes et al., 1990; Wong et al., 1990; Mueller & Gardes, 1991; Nguyen et al., 1992; Mueller & Ammirati, 1993; Henrion et al., 1994; Lumley et al., 1995; Frey et al., 1997; Kim et al., 1998; Selosse et al., 1998a,b; Martin et al., 1999; Baum et al., 2002; Kemppainen et al., 2005; Jany et al., 2006, Heller et al., 2008). The elucidation of its genome (Martin et al., 2008) will be of interest to scientific communities studying everything from physiology and ecology in forest ecosystems to fundamental questions in evolution and development.

### III. Sequencing and assembly of the *Laccaria bicolor* genome

In early 2005, the genomes of a dozen pathogenic fungi from the Ascomycota and Basidiomycota were sequenced (Galagan et al., 2005), but no genome of symbiotic fungi was available; therefore, the full sequence of the ECM *L. bicolor* has been eagerly awaited. In April 1988, several thousands of spores were collected from a fruiting body (Fig. 1) beneath Douglas fir seedlings inoculated with the American *L. bicolor* strain S238N (Di Battista et al., 1996) in order to survey the genetic mycorrhizal phenotypic variability (Nguyen et al., 1992).

Within this progeny, the homokaryotic strain S238N-H82 was immortalized by subculturing the vegetative haploid mycelium on agar medium for 16 yr. Then, its DNA was extracted, fragmented, cloned in plasmids and fosmids, with 3, 8 and 38 kbp inserts, and arrayed in many thousands of small wells. WGS sequencing (590 million base pairs (Mbp) of sequence) was carried out to 10 times coverage, generating a high-draft sequence. The Stanford Human Genome Sequencing Center, in collaboration with JGI, has carried out additional sequencing to close up the repetitive gaps and fix...
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misassembled regions (J. Grimwood et al., unpublished). This finished sequence has been anchored to a L. bicolor genetic map (Labbé et al., 2008). In this finished and anchored version of the assembly, the 20 largest scaffolds contain 80% of the total 60.583 Mbp of sequence. The largest assembled scaffolds with telomeric ends are 4.6 and 5.1 Mbp and likely correspond to chromosomes. The L. bicolor genome is the largest fungal genome published so far, being approx. 1.5 larger than its closest sequenced relative, C. cinerea (37.5 Mb). However, no evidence for large-scale chromosomal duplications was observed within the L. bicolor genome.

Labbé et al. (2008) have drawn up a genetic linkage map consisting of 13 linkage groups spanning 812 centiMorgans (cM) at an average distance of 2.76 cM between markers. Using RAPD, simple-sequence-repeats and single-nucleotide polymorphism markers, L. bicolor WGS assemblies were anchored on to the linkage groups. A total of 37.36 Mbp of the assembled sequences was aligned to the 13 linkage groups. Most mapped genetic markers used in alignment were colinear with the ARACHNE sequence assembly, indicating that both the genetic map and sequence assembly achieved high fidelity. The availability of a genetic linkage map integrated to the WGS will facilitate mapping of quantitative trait loci involved in ectomycorrhiza and fruiting body formation, and ecophysiological features underlying the ecological symbiosis fitness.

IV. Synteny between genomes of symbiotic and saprotrophic fungi

Assuming that symbiotic basidiomycetes derived from saprotrophic species (Hibbett et al., 2000), a limited synteny (i.e. conservation of gene order) might exist between L. bicolor and the saprotrophic Agaricales. However, no large-scale conserved synteny was identified between L. bicolor and C. cinerea or P. chrysosporium using the JAZZ sequence assembly (Martin et al., 2008; B. Cänback & A. Tunlid, unpublished), suggesting that signals of the ancestral Agaricales genome organization were erased by subsequent chromosomal breaks and translocation along the various lineages, and transposon activity. There is, however, an extensive block (330 kbp) of 119 ancestral genes that were linked to the mating type A locus in the C. cinerea/L. bicolor ancestor and which have retained that linkage in both the modern C. cinerea and L. bicolor genomes (Niculita-Hirzel et al., 2008). This may be locally explained by hitchhiking of these genes with the mating type A, for which limited recombination occurred, promoting conservatism (Charlesworth, 2006). Genomic regions of common ancestry appear to have evolved into a mosaic of syntenic blocks that may have diverged between Agaricales species owing to differential insertions of transposable elements (TE) and episodes of gene mobilization as suggested in plants (Morgante et al., 2007). This scenario is supported by the very high abundance of TE in L. bicolor genome. We have recently found significant conservation (c. 30%) of synteny between L. bicolor and C. cinerea by using the transposon-masked and linkage group-anchored L. bicolor finished genome assembly (F. Martin & B. Hilselberger, unpublished).

V. What’s there and what’s not

The short intron length in coding sequences and the frequent occurrence of TE-associated sequences close to genes complicated the process of automated gene modelling in L. bicolor. Thus, repetitive elements in the WGS were identified by a series of dedicated programs (Quesneville et al., 2005). The corresponding TE database was used in a multistage genome masking strategy to minimize the negative effects of TE and other repeated sequences before gene finding. Using a combination of gene-prediction programs (e.g. FGENESH, EUGENE, GENEWISE, TWINSCAN) and iterative manual inspection of predicted gene models relative to a training set, a total of 20 614 protein-encoding genes have initially been identified. These predictions used ab initio algorithmic predictions as well as the coding sequences from known fungal genomes, and L. bicolor cDNA sequences and their predicted translations. Although multiple models with overlapping sequences were generated for each locus, a single model was chosen for the gene catalogue set. Model selection was based on maximizing protein sequence relationship and expressed sequence tags (EST) support for splice sites, coding sequences and model completeness (i.e. inclusion of 5′ methionine, 3′ stop codon, and UTRs). After a first automatic filtering, the catalogue was refined by the annotators, including through generation of ad hoc gene models. On August 2008, the gene repertoire contains 19 102 protein-coding gene models, 279 tRNA, 18 splicesosomal RNA and 50 copies of the ribosomal RNA genes. Over 1000 gene models having sequences with a similarity to TE fragments have been demoted as TE relics and pseudogenes. Even the fairly deep sequencing (39 000 Sanger ESTs and 186 000 ‘454’ pyrosequencing ESTs) of randomly selected cDNA clones from various tissues (free-living mycelium, ectomycorrhizas, fruiting body) (Peter et al., 2003) failed to validate the structure of c. 45% of genes that are likely expressed only at low levels or under developmental (e.g. spores) and specific nutritional conditions (e.g. nutrient starvation). The resulting predictions are distributed through web portals at the JGI Laccaria genome browser (http://www.jgi.doe.gov/laccaria) and the INRA Laccaria DB (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome). Of these predicted gene models, c. 76% showed significant similarity to sequences in protein databases, particularly those from other Basidiomycetes. While functions of many genes may be inferred from sequence homology to genes in other organisms, a majority of predicted genes still have no known function and genes unique to L. bicolor were nearly 24% of the total predicted gene set. This is a surprisingly high percentage that probably reflects both the symbiotic lifestyle and the
VI. Expansion of multigene families

The relative rates of gene duplication, gene loss, horizontal transfer events and transposable element proliferation have an impact on the size differences between fungal genomes (Tunlid & Talbot, 2002). Two evolutionary trends that lead to larger genomes among fungal pathogens are the consistent expansion of certain gene families, as well as pathogens' apparent affinity for gene acquisition through horizontal transfer (Powell et al., 2008). Predicted protein-coding genes were compared with the gene sets of the sequenced basidiomycetes C. neoformans (Loftus et al., 2005), U. maydis (Kämper et al., 2006), Malassezia globosa (Xu et al., 2007), P. chrysosporium (Martinez et al., 2004), C. cinerea (Stajich et al., 2006) and Melampsora larici-populina (Melampsora Genome Consortium) to derive a common set of gene families attributable to the basidiomycete ancestors (Table 2). This dataset has been used to identify conserved and novel, lineage-specific gene families (Martin & Tunlid, in press; Martin et al., 2008; B. Hilselberger & F. Martin, unpublished) and infer evolutionary insights into the conservation and diversity of cellular functions in the symbiont. The L. bicolor genome encodes both more and larger gene families than any other basidiomycetes sequenced so far. Amongst families shared with other basidiomycetes, 1064 families expanded, 3694 showed no change and 112 families experienced a contraction (Martin & Tunlid, in press). Innovation in protein-coding genes and expansion in gene families is largely the result of diversification and rapid turnover in gene families involved in environmental/symbiosis interactions (Martin et al., 2008).

VII. A niche for transposons

At 60 Mbp, the finished genome of L. bicolor is bigger than that of previously published fungal genomes. The size may be partly explained by the large number of repeated sequences, including mobile DNA sequences, known as TEs that constitute more than 22% of the genome. One unexpected finding is that L. bicolor harbours unprecedented transposon diversity – more types than any other fungi studied to date (Martin et al., 2008; J. Labbé & H. Quesneville, unpublished). The most abundant TEs are class 1 elements (e.g. Copia, Gypsy), which are collectively represented by 962 complete sequences and c. 17 000 remnant degraded copies. Class 2 elements, including MITE, Pogo, Ant1/Tc1 and helitrons, account for a total of 5738 sequences, including 355 complete sequences. This abundance of MITE elements has not yet been reported in other fungal genomes. In contrast to C. cinerea (Stajich et al., 2006), TE clusters are not restricted to subtelomeric or centromeric chromosomal regions, but they are distributed throughout the genome where they form ‘islands’ of diverse types of TE. It appears that these TE-rich regions contain a low density of expressed genes (A. Kohler & Y-C. Lin, unpublished). Genomic regions of common ancestry appear to have evolved into a mosaic of syntenic blocks that may have diverged between Agaricales species owing to differential insertions of TEs and episodes of gene mobilization as suggested in plants (Morgante et al., 2007). This scenario is supported by the very high abundance of TE in the L. bicolor genome. The paucity of TE in C. cinerea indicates that episodes of recent (within the past 50 million yr) and lineage-specific amplification of TEs (or, alternatively, purge of transposons) occur in fungal genomes.

These findings pose several interesting questions. Why is the L. bicolor genome effectively a niche for transposons? Did they enter the genome after divergence from C. cinerea ancestor – or were they lost from the later species? Has their presence resulted in extensive genomic rearrangements? In particular, did LTR-mediated retroposition contributed to the expansion of large L. bicolor gene families? Do other mycorrhizal/
biontrophic genomes harbour a similar number of transposons? This last question will soon be answered with the imminent availability of the Black Truffle of Perigord (*Tuber melanosporum*) and the poplar leaf rust (*Melampsora larici-populina*) genome sequences which appear to have even larger sets of TEs (*Melampsora* and *Tuber* Genome Consortia). Based on their high sequence similarities, several *L. bicolor* TEs have moved in very recent evolutionary times or are still moving (Martin *et al.*, 2008), suggesting that they might contribute to extant intraspecific sequence variation.

**VIII. The trading post for symbionts**

Analysis of multigene families has shown an expansion of sugar transporters, amino acid permeases and secondary metabolite genes amongst the filamentous plant pathogenic fungi (Powell *et al.*, 2008; Soanes *et al.*, 2008). The ability to extract nutrients from a host is a defining feature of biontrophic pathogens and symbionts, and sugar and amino acid transporters are necessary for this key process. The symbiotic interface, comprising the plant and fungal cell wall materials, has long been thought to be the zone by which mycobionts derive nutrients from their hosts (Smith, 1993; Martin, 2007). Owing to the difficulty in studying these membrane transporters, only 20 proteins involved in nutrient transport (N, C, K, P) have been characterized in ECM fungi over the last decade (for a review, see Chalot *et al.*, 2006; Nehls *et al.*, 2007; Lucic *et al.*, 2008). This should rapidly change in the near future as a comprehensive annotation of membrane transporters has been carried out (Fajardo López *et al.*, 2008; Lucic *et al.*, 2008; Martin *et al.*, 2008). As expected from the complex exchange of ions and metabolites between the mycobiont and its hosts, there is an especially large number (c. 500) of predicted membrane-bound transporter proteins in the *L. bicolor* genome (Martin *et al.*, 2008). They are likely important for acclimation of the mycelium to the fluctuating, often nutrient-poor, conditions of soil environments (Marschner, 1995). The 127 ATP-dependent transporter and 36 ion channel families are larger than in other sequenced basidiomycetes, and several families underwent a lineage-specific expansion (Lucic *et al.*, 2008; Martin *et al.*, 2008).

Sugars play a crucial role in the interaction and the mycobiont activities rely on a constant import of carbohydrates delivered via the symbiotic interface by the host plant. It is thought that sucrose is the main transport form for carbon partitioning between the partners (Nehls *et al.*, 2001). Upon release from the rhizodermal and cortical cells to the symbiotic apoplast, sucrose is cleaved by the plant extracellular invertase and the monosaccharides glucose and fructose taken up by the fungus (Nehls *et al.*, 2007). The lack of invertase in *L. bicolor* (Deveau *et al.*, 2008) implies that the decision as to whether sucrose is inverted by the host plant and monosaccharides are delivered to the mycobiont greatly influences further developmentnal and metabolic activities in the fungal partner. The secreted acidic invertase activity of the host root therefore has the ability to control the whole symbiosis outcome, although nonenzymatic inversion of sucrose likely takes place in the acidic symbiotic apoplast. Although uptake studies show that sugars and amino acids are transferred from the host plant into the mantle and extramatrical hyphae of symbiotic fungi (Nehls *et al.*, 2007), and strongly support the idea that the plasmamembrane of Hartig net hyphae play a major role in nutrient assimilation for the mycobiont, the intraradicular locations of this structure and the complexities of the fungal–host interface of ECM (Chalot *et al.*, 2006) have made it difficult to determine what membrane transporters and translocation processes are involved. This distribution of sugars at the subcellular level in fungal and root cells, but also for long-distance transport in the hyphal webs, requires several essential transport mechanisms across fungal and plant membranes. Now Uwe Nehls’s group has characterized the full complement of sugar porters in *L. bicolor* (Fajardo López *et al.*, 2008).

Carbohydrate starvation and ectomycorrhiza development resulted in a strongly enhanced expression of several genes. From gene expression patterns and import kinetics, two functions have been advocated by the authors: reduction of carbon leakage under conditions of carbohydrate starvation and formation of a strong hexasome uptake capacity in ectomycorrhizas. Interestingly, the *L. bicolor* genome encodes a trehalose phosphorlyase, an enzyme able to efficiently convert imported glucose to trehalose (Deveau *et al.*, 2008). All glycolytic and carbohydrate storage pathways have been identified and seem to be functional, as they are all transcribed in *L. bicolor* (Deveau *et al.*, 2008). The evolution towards mycorrhizal symbiosis did not lead to the loss or to the expansion of gene families involved in the primary carbon metabolism as is often observed in obligatory symbiosis (Moran, 2007).

Lucic *et al.* (2008) identified 128 genes encoding putative transport protein for N-containing compounds in *L. bicolor*, most of them (92%) having a detectable transcript in agar-grown mycelium, ectomycorrhizas or fruiting body. These authors pointed out the expansion of several multigene families, including the ammonium transporter (AMT) family. The dramatic induction of *LbAMT2.2* expression in ECM (Martin *et al.*, 2008) combined with the expansion of the AMT family suggests higher ammonium uptake capacities in symbiotic hyphae and therefore a key role of ammonium in the ECM symbiosis.

**IX. Saprotrrophic capabilities**

Mycorrhizal hyphae networks constitute the functional interface between litter decomposition (i.e. the release of carbon and nitrogen compounds from organic substrates) and production of biomass for both above- and below-ground communities. The web of ECM hyphae likely uses nitrogen and carbon compounds released by bacterial and fungal decomposers, such as white rots (Lindahl *et al.*, 2007). Plant cell walls
Most fungi colonizing forest litter and living plants secrete enzymes that break down cellulose, hemicellulose and possibly xylans chains within a matrix of pectins (Schwarze, 2007). Surveys of cellulolytic fungi, such as Laccaria bicolor, have revealed that they provide this fungus with a range of strategies to maintain an efficient functioning under a vast array of environmental conditions – from the high number of genes encoding various glycoside hydrolases, polysaccharide lyases, chitinases (Martin et al., 2008; Seidl, 2008; Danchin et al., unpublished) and secreted proteinases (Lilly et al., 2008) that are predicted in the genome may indicate a capacity to use alternative nutrient sources, such as insects, bacteria and decomposing organic matter. Indeed, evidence exists that ECM fungi use organic matter from soil (Lindahl et al., 2007) or even soil microfauna for L. bicolor (Klironomos & Hart, 2001) and that they transfer the released nitrogen and phosphorus to the plant. Interestingly, L. bicolor belongs to a group of fungi that form fruiting body on soils where decomposition of animal wastes has occurred, which are thus rich in organic N (Sagara, 1995).

The wide range of hydrolytic activities encoded by the L. bicolor genome may represent an ecological adaptation to soil local environmental heterogeneity and is thought to provide this fungus with a range of strategies to maintain an efficient functioning under a vast array of environmental conditions – from the in planta highly protected and nutrient-rich niche to the soil litter crowded by microbial competitors. The genome exploration led to the discovery that L. bicolor lacks the enzymes involved in degradation of the carbohydrate polymers of PCW, but maintains the ability to degrade non-PCW polysaccharides.

Laccaria bicolor also has a wide repertoire of reducing and thiol-dependent antioxidant systems involved in ROS production and detoxication (Morel et al., 2008). They are likely activated for coping with the stress (pH, ROS, nitrative species) that the mycelium encounters during its progression in the soil litter, the rhizosphere and the root apoplastic space. Interestingly, mapping H$_2$O$_2$ at the subcellular level (Gafur et al., 2004) showed that mycorrhizal and non-mycorrhizal Paxillus involutus isolates differed in their H$_2$O$_2$ production.

These observations point towards the dual life of mycorrhizal fungi, such as L. bicolor, and their ability to grow in soil, fending off pathogens and using decaying organic matter (except PCW polysaccharides) while serving as a custodian of living plant roots. L. bicolor is like Janus, the mythological caretaker of gates, doors and hallways; beginnings and endings. Janus, with his two faces looking in opposite directions, is ever aware of opportunity.

X. Laccaria secretome

Another intriguing similarity with other biotrophic fungi, such as U. maydis and leaf rusts (Catanazari et al., 2006), is the impressive arsenal of small secreted proteins (SSPs). An in silico pipeline, including the SignalP, TargetP, WolfPSort
By contrast to some other plant–microbe interactions, including AMF symbiosis (Kämpf et al., 2006), the genes for these SSPs are extensively clustered and the proteins are implicated in pathogenesis (Kämper et al., 2006). SSPs are often not clustered in *L. bicolor*. The whole-genome expression microarrays (Martin et al., 2008) showed that a dozen of genes encoding SSPs are dramatically induced during symbiosis or are ECM-specific. Tissular localization of the most highly expressed and induced SSP, the 7 kDa mycorrhiza-induced SSP (MISSP7), using indirect immunofluorescence microscopy showed that this protein is localized to hyphae of the finger-like, labyrinthine Hartig net (Martin et al., 2008). Whether some of these SSPs are similar to those found in other fungi during hyphal fusion (anastomosis) and homing (Glass et al., 2004), and aggregation of hyphae leading to the formation of sexual organs (Moore, 1998), remains to be investigated.

Although symbiosis development induces dramatic alterations of the mycobiont transcriptome, no ectomycorrhiza-specific genes have been identified so far (Martin et al., 2007). This apparent lack of ectomycorrhiza-specific genes has recently been challenged in experiments using a *L. bicolor* genome-wide expression oligoarray (Martin et al., 2008). This array contains probes for the whole set of predicted gene models. In ectomycorrhizal root tips of Douglas fir or poplar plantlets, the most highly up-regulated *L. bicolor* transcripts displayed fold values (ectomycorrhizas vs free-living mycelium) exceeding 1000; but only six amongst 520 symbiosis-regulated transcripts were not detected either in the free-living vegetative mycelium or in the fruiting body. Many of these ectomycorrhiza-specific transcripts were not detected because their size was below the cut-off value for EST sequencing (e.g. MISSP7) and they were thus missing from the cDNA spotted arrays (Duplessis et al., 2005; Le Quéré et al., 2005). The apparent paucity of ectomycorrhiza-specific genes and the moderate induction of most symbiosis-regulated genes is striking, however, and suggests that ontogenetic and metabolic programmes that lead to the development of symbiosis are mainly driven by the differential expression/activity of pre-existing transcription factors and/or transduction pathways, rather than by the expression of symbiosis-specific gene arrays. Several of these symbiosis-regulated genes belong to lineage-specific or expanding multigene families (Martin et al., 2008).

**XI. Signalling pathways**

By contrast to some other plant–microbe interactions, including AMF symbiosis (Akiyama et al., 2005; Besserer et al., 2006), the signalling molecules and the molecular basis of signal perception and transduction in ectomycorrhiza are poorly known (Salzer et al., 1997; Martin, 2008). Identifying the processes that regulate the information flow between the ECM mycobiont and its host root is however an active research area.

Host plants release into the rhizosphere critical metabolites that are able to trigger basidiospore germination, growth of hyphae towards the root and the early developmental steps of mycorrhiza formation (Martin et al., 2001). Development of the symbiotic tissues requires temporally and spatially controlled activity of genes and proteins participating in the morphogenetic process and cell differentiation. The proliferation of short roots and Hartig net fungal tissues, and the need to adapt to a rapidly changing environment (changes in pH, enhanced fluxes of nutrients, presence of ROS) likely involve a cascade of gene networks, including signalling pathways. A comparison of the *L. bicolor* signalling pathway genes with other basidiomycetes confirmed the adaptation of its enzyme repertoire to symbiosis (Martin et al., 2008; S. Duplessis et al., unpublished). Up to 32 α-GTPases were identified, whereas *C. cinerea* and *P. chrysosporium* only contain 16 and 11 members of this GTPase family, respectively. These new types of G-alpha proteins, supported by ESTs and expression profiling, may be candidates for the complex, fine-tuned molecular crosstalks that must occur between the mycobiont and its host plant during mycorrhiza establishment. Interestingly, four tetraspanin families have been identified in *L. bicolor* (Lambou et al., 2008). Tetraspanins are small membrane proteins that act as organizers of membrane-signalling complexes.

**XII. Sex genes in *Laccaria bicolor***

In Eumycota, dichotomy between males and females is rare, but instead outcrossing is promoted by mating-type loci with a high number of alleles (Fraser & Heitman, 2004). In *L. bicolor*, single meiospores germinate to produce haploid, homokaryotic mycelia. *L. bicolor* has a bifactorial (tetrapolar) mating system (Kropp et al., 1986; Kropp & Fortin, 1988; Niculita-Hirzel et al., 2008; Fig. 2). Two complex mating-type factors control sexual compatibility in the homokaryons and regulate the maintenance of the dikaryotic state. Fusion of sexually compatible haploids (i.e. with different alleles of mating-type loci) results in the formation of the diploid (dikaryotic) mycelium. The loci are multiallelic, with more than 45 A and 24 B mating-type alleles (Raffle et al., 1995), but the molecular mechanisms of mating control are unknown. Having multiple alleles to determine mating promotes outcrossing and genetic recombinations in natural populations, but does allow some homogamy to occur, since each meiotic spore is compatible to 25% of the other meiotic spores. The hyphae of *L. bicolor* dikaryons develop clamp connections at each septum, while the hyphae of homokaryons do not. Dikaryon is the predominant vegetative structure in *L. bicolor*, as in most other Agaricomycetes (Gardes et al., 1990). The filamentous dikaryotic mycelium forms ectomycorrhiza and, under appropriate conditions, produces the fruiting bodies (Fig. 1) within which karyogamy and meiosis occur during the formation of the spores. The homokaryotic and dikaryotic mycelia are capable of indefinite growth in vitro, allowing for their maintenance and duplication.
for research purposes or field inoculation; unfortunately, the fruiting bodies have not hitherto be obtained in the nonsymbiotic state. The ECM symbiosis (Fig. 1) is generally induced by dikaryotic mycelia interacting with short roots of the host tree (Kropp & Fortin, 1988).

Niculita-Hirzel et al. (2008) identified the genes that govern the establishment of cell-type identity and orchestrate mating. Using genome comparison, molecular phylogeny, allele-specific PCR and gene expression, they demonstrated that the mating specificity in *L. bicolor* is encoded by the two mating-type loci known in other Agaricomycotina (Brown & Casselton, 2001): the *A* mating-type locus encodes homeodomain transcription factor genes, while the *B* mating-type locus encodes pheromone peptides and pheromone GPCR receptors. *A* and *B* loci are located in two genetically independent genomic regions that have a very different evolutionary history. The *A* locus lies in a region where the synteny is well conserved across the Agaricales, suggesting a strong selection pressure and a low recombination rate. On the other hand, the *B* locus lies in a region where no synteny was observed with gene duplication and translocation, and transposon insertions. This initial dataset on mating-type genomic structure and expression will help in elucidating the entangled molecular pathways leading to mating and to symbiosis development.

**Fig. 2** The life cycle of *Laccaria bicolor*.

**XIII. Impact on the understanding of symbiotic and ecological evolution**

*Laccaria* belongs to the Hydnangiaceae (Agaricales), a family recently shown to occupy a basal position within a clade of the Agaricoids (in Agaricales) that also encompass the genus *Coprinopsis* (Garnica et al., 2007) and several ECM clades. The most likely ancestral state for this clade is saprophytism and, in addition to Hydnangiaceae, at least five other independent emergences of ECM association occurred in it (e.g. in Cortinariaceae, Inocybaceae, *Descolea* spp.; Matheny et al., 2006). This clade illustrates the frequent shift from saprophytism to ECM life – a shift pervasive in evolution of Agaricomycetes (Hibbett et al., 2000). This makes comparison with the genome of *C. cinerea* very relevant since many differences are thus likely the result of ecological divergence.

Horizontal gene transfers allowed acquisition of virulence genes by some plant pathogenic fungi (Friesen et al., 2006; Richards et al., 2006). Although this mechanism seems open for soil fungi that co-exist with diverse other soil microbiota, there is little evidence that it strongly acted during evolution to symbiosis in *L. bicolor* (A. Deveau, B. Cänbäck, A. Tunlid & F. Martin, unpublished). Congruently, analyses of Ascomycetes genomes also show that horizontal gene transfers remain rare (Richards et al., 2006), with duplication as the main driver of gene diversification and specialization (Wapinski et al., 2007).

Mutualistic symbiosis is unstable, since each partner can gain more in developing a purely exploitive (‘cheating’) interaction. Indeed, the potential similarity with pathogenesis is emphasized by effector-like SSPs found in *L. bicolor* (see earlier), suggesting that similar traits act in pathogenesis and symbiosis. Cheaters are often avoided, and thus counter-selected in the longer term, by refusal of the host to collaborate or sanctions imposed by the host (Kiers & van der Heijden, 2006). Little is known about the possible sanctions in ECM, but the
discovery that \textit{L. bicolor} lacks invertase genes opens an interesting perspective from the plant side. A long coevolution with plants whose cell wall acidic invertases give access to sucrose may have driven the fungus to dependency on this C source. Now, deprivation of hexoses to cheating ECM variants would be a stringent counter-selection. The carbon flow may be an important tool for sanction in plant symbioses: hydrolysis of nectar sucrose has already been shown to allow partner choice in ant–plant mutualisms (Heil et al., 2005).

\textbf{XIV. Tools and questions for population genetics and ecological genomics}

The availability of a genome also offers powerful tools, such as microsatellites (Labbé et al., 2008), for research into population dynamics and biology in ECM fungi that will boost population analyses. Interestingly, \textit{L. bicolor} and congeneric species are already models for population genetics of ECM fungi (Selosse et al., 1998a,b; Gherbi et al., 1999; Fiore-Donno & Martin, 2001; Jany et al., 2006; Roy et al., 2008). Comparison of WGS sequence with ESTs available from the parental (diploid) strain, \textit{L. bicolor} S238N, allowed estimates of allelic variation and has provided hundreds of single-nucleotide polymorphisms (SNPs) (J. Labbé et al., unpublished), which will be invaluable for population and evolutionary genetic analyses. The high heterozygosity of the parental strain corroborates the trend to outcrossing reported from natural \textit{Laccaria} spp. populations (Roy et al., 2008).

As an example of a relevant biological question, there is controversy relating to the exact outcrossing level in \textit{Laccaria} spp., an interesting issue both theoretically and in view of using \textit{L. bicolor} as an inoculant to improve tree growth (Selosse et al., 1998a,b). In previous analyses of populations of the related \textit{L. amethystina}, there was some discrepancy as to whether heterozygotes were in excess (Gherbi et al., 1999) or in small deficit (Fiore-Donno & Martin, 2001; Roy et al., 2008), so that a preference for mating with unrelated or related strains is unclear. This may have resulted from the limited number of markers used in these studies, and their biases (Roy et al., 2008). Similarly, the role of sexual reproduction in \textit{Laccaria} spp. is thus far solely inferred from high population diversity (Gherbi et al., 1999), with no data on linkage disequilibrium, the only relevant feature to test for sex in populations. Here again, having more markers will allow such studies.

The cosmopolitan genus \textit{Laccaria}, with up to 100 species worldwide (G. Mueller, pers. comm.), is an interesting model for phylogeography studies using multiple-gene analysis, comparative genome hybridization or high-throughput sequencing. Moreover, the existence of transcontinental species is now strongly questioned by the increase of international scientific exchanges. Do they exist (i.e. does gene flow maintain biological species at large scale) or do they represent collections of populations undergoing progressive allopatric diversity (Geml et al., 2006)? Local mycologists with a limited knowledge of species from other continents may well overlook the latter situation. Here, the genus \textit{Laccaria} and tools derived from the genome of S238N-H82 offer exciting possibilities. For example, there is some evidence that \textit{L. amethystina} from Europe and Asia belong to two distant species (Roy et al., 2008). On the other hand, and unexpectedly, \textit{L. bicolor} seems to be a possible transatlantic species, with evidence that alleles are shared between European and American strains (Selosse et al., 1998b; G. Mueller, pers. comm.). Beyond these simple questions lies the issue of gene flow between populations over large distances in fungal populations.

Many other questions will also begin to be answerable if new genome sequences from ECM fungi continue to emerge. One such is the following: what is speciation? The large polymorphism of several ECM taxa, such as the Agaricomycetes, invites a direct test of the idea that most genes diverge continuously within and among species, whereas sharper changes in a minor fraction of genes account for the adaptive differences (e.g. symbiosis vs saprotrophism) that separate diverging species.

When considering the interaction of mycorrhizal fungi with their environment, we would like to identify the genes and genetic pathways that underlie important ecological responses and interactions, determine the extent to which those genes and pathways exhibit functional variation in nature, and characterize the ecological and evolutionary consequences of that variation (Ungerer et al., 2008). Another goal of ecological genomic research is to understand how mycorrhizal genomes interact at higher levels of organization; for example, is there a Gemeinheit genome and, if so, can we understand how it functions (Whitham et al., 2006, 2008)? One approach is to investigate the role(s) of candidate genes whose sequence identity suggests they might be important for an ecologically relevant process or phenotype (e.g. N and P transporters, secreted hydrolytic enzymes). Transcriptional profiling using microarrays can identify genes whose expression changes in response to environmental perturbations and thus become candidate genes for being involved in the response. This is one of the primary methods currently being used in ecological genomics research to identify important genes.

\textbf{XV. More genomes of ectomycorrhizal fungi to come}

The availability of a WGS for a representative mycorrhizal, particularly an ECM species, provides much grist to the comparative genomics mill and is certainly a milestone (Cullen, 2008). However, one potential danger lies in confusing the genome of a representative organism (such as \textit{L. bicolor}) with that of symbiotic ancestors. The structures of ancestral genomes can be inferred by compiling lists of shared genes, but this cannot be extrapolated to absences, both because genome sequences are sometimes incomplete and because...
secondary losses seem to have occurred in all organisms. The lack of genes coding for carbohydrate-active enzymes acting on PCW polysaccharides in *L. bicolor*, for example, has been taken to indicate that this feature allowed the ECM ancestor to occupy a carbon-rich niche in the host roots. This finding emphasizes the importance of having sequence data for more than one representative of each phylum of ECM fungi, particularly in a diverse phylum such as Agaricomycetes. For comparative purposes, the WGS of a representative ECM Boletale is required, as is that of a large phylum of symbiotic basidiomycetes, given how successfully boletales have exploited this evolutionary strategy. The model organisms nominated by the ECM symbiosis community are *Patellus involutus* and *Rhizopogon salebrosus*. Similarly, the availability of the genome of the ECM ascomycete *T. melanoporum* (Pezizales), sequenced by the French Génoscope and the *Tuber* genome consortium, will provide new insights into mycorrhizal symbiosis.

The arrival of massively parallel sequencing technologies, such as 454 and SOLID sequencing and polony multiplex analysis of gene expression (PMAGE), heralds a digital transformation of genome sequencing, gene expression analyses and other genetic measurements. These high-throughput sequencing technologies are currently used for producing > 200 000 ESTs for the model mycorrhizal species *L. bicolor* and *T. melanoporum*, but also highly ecologically relevant species, such as *L. amethystina*, *Cenococcum geophilum*, *Lactarius quietus*, *Scleroderma citrinum* and *Thelephora terrestris* within the framework of the European EVOLTREE programme (F. Martin, unpublished). These advanced sequencing technologies will enable the genome sequencing of additional species taxonomically related to species with WGS genome, such as other *Laccaria* species. However, both better coverage and higher representation across a broad range of phyla are needed before it is possible to reconstruct the gene set of the various ECM ancestors.

**XVI. Conclusions**

Over the last few years, there has been a considerable investment in the generation of genomics tools for *L. bicolor*. In addition to the genome sequence, there is an abundance of cDNA libraries and corresponding ESTs available. Several microarray platforms, including NimbleGen whole-genome expression oligoarrays, are now available to the mycorrhizal community and may be combined with immunocytolocalization and laser capture microdissection of symbiotic tissues to greatly refine and expand analyses of ECM development and metabolism. With the aid of the meiotic, genetic linkage map using SSR and SNP markers (Labbé *et al*., 2008), genes and QTLs can now be mapped. The amalgation of these data into interactive web databases, such as the INRA LaccariaDB, will facilitate the analysis of genes involved in mycorrhiza development and functioning. The acceptance of *L. bicolor* as a standard model organism for symbiosis genetics will, however, depend strongly on the availability of additional genetic, genomic and molecular biological resources. Tagged-mutant lines and routine protocols for RNA-based gene inactivation (Kemppainen *et al*., 2008) will place *Laccaria* research on a firm genetic footing.

**Acknowledgements**

We would like to thank Igor Grigoriev (JGI), Jane Grimwood (SHGC) and their teams for their outstanding efforts in sequencing and annotating the *L. bicolor* genome. FM would like to acknowledge the members of the *Laccaria* Genome Consortium for the days and nights they dedicated to deciphering the *L. bicolor* code. Investigations carried out in FM’s laboratory were supported by grants from the INRA (project ‘Genome Sequencing of Poplar and Associated Microorganisms’), the European Commission Network of Excellence EVOLTREE, the CEA-Génoscope Genomics Institute (project ‘ForeST’) and the Région Lorraine. M-AS is funded by the CNRS and the French Agence Nationale de la Recherche. The genome sequencing and analysis of *L. bicolor* was funded by grants from the US. Department of Energy. Critical reading of the manuscript by our fellow *New Phytologist* editors, Holly Slater, Andrea Polle and Natalia Requena, was greatly appreciated.

**References**


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