Commentary

Emerging topics in stable isotope ecology: are there isotope effects in plant respiration?

The ability to distinguish between 'light' and 'heavy' forms of carbon dioxide in the atmosphere has proven to be a useful tool in the study of plant ecology and the role of terrestrial ecosystems in the global carbon cycle. Plant processes imprint unique isotopic signatures on atmospheric CO_2 such that measurements of the ratio of heavy to light carbon in CO_2 can provide a means of detecting plant physiological processes integrated over whole canopies, ecosystems, or even regions. To interpret these measurements, a mechanistic understanding of the processes that affect the proportion of $^{12}CO_2$ vs $^{13}CO_2$ in the atmosphere is required, such as the well-known model of 'discrimination' against $^{13}CO_2$ in photosynthesis caused by preferential diffusion and enzymatic reaction with $^{12}CO_2$ (Farquhar et al., 1982). In this issue, Hymus et al. (pp. 377–384) show that short-term variations in isotopes of respiratory CO_2 under natural conditions may be as large as variations in photosynthesis, and may contain additional information about plant metabolic pathways that have not been fully explored.

'Measurements of the carbon isotope composition of plant respiration may provide insight into how photosynthate is allocated into synthesis of various compounds and metabolism in different species and in response to changes in the environment'

The isotopic composition of respiratory CO_2

Photosynthetic discrimination causes plant material to be highly depleted in ^{13}C relative to the atmosphere, particularly for plants that utilize the C_3 photosynthetic pathway. When this carbon is released back to the atmosphere, it is also highly depleted in ^{13}C, providing a useful tracer of ecosystem respiration. Using a simple mixing model first described by Keeling (1958, 1961), it is possible to use atmospheric measurements to estimate the carbon isotope composition of ecosystem respiration (often abbreviated as $\delta^{13}C_r$) at night when there is no photosynthetic uptake. This approach has been utilized in a number of ecosystems around the world to gain insight into processes that affect plant gas exchange integrated over time (see review by Pataki et al., 2003). Inherent in the interpretation of these measurements has been the assumption that discrimination against heavy isotopes during respiration is negligible such that the isotopic composition of respiration reflects the isotopic composition of the respiratory substrate.

An increasing number of studies have recently challenged this assumption, which has become a topic of some debate. In particular, a common finding is that the isotopic composition of foliar respiration may be quite enriched in heavy carbon relative to all measured leaf compounds that may serve as respiratory substrates (e.g. Duranceau et al., 1999; Ghashghaie et al., 2001; Tcherkez et al., 2003; Xu et al., 2004). Most of these studies have been conducted with potted plants or controlled environments; Hymus et al. show that this effect is also observed under field conditions. By excising leaves and measuring the isotopic composition of dark respiration repeatedly over a diurnal period in two Mediterranean oak forests, they found that foliar respiration became progressively heavier over the course of the day, particularly in the upper canopy. Changes in the isotopic composition of the respiratory substrate caused by diurnal changes in photosynthetic discrimination are a possible explanation for this pattern; however, sugars, starch, lipids and cellulose extracted from excised leaves did not show much diurnal variation in isotopic composition. Despite this, cumulative leaf CO_2 uptake measured in cuvettes was well correlated with isotopic enrichment of respiratory CO_2 in the upper canopy.

Understanding plant metabolic pathways

Early measurements of the isotopic composition of respiration showed a great deal of variability relative to substrates (see reviews by O'Leary, 1981; Farquhar et al., 1982). In addition, Lin and Ehleringer (1997) showed that the isotopic composition of CO_2 evolved from isolated plant protoplasts was similar to that of their sugar substrates. This led many to conclude that isotope effects in respiration were negligible and that the isotopic composition of respiratory CO_2 was predominantly influenced by photosynthetic effects on the isotopic composition of substrates, particularly sugars. In a review, Ghashghaie
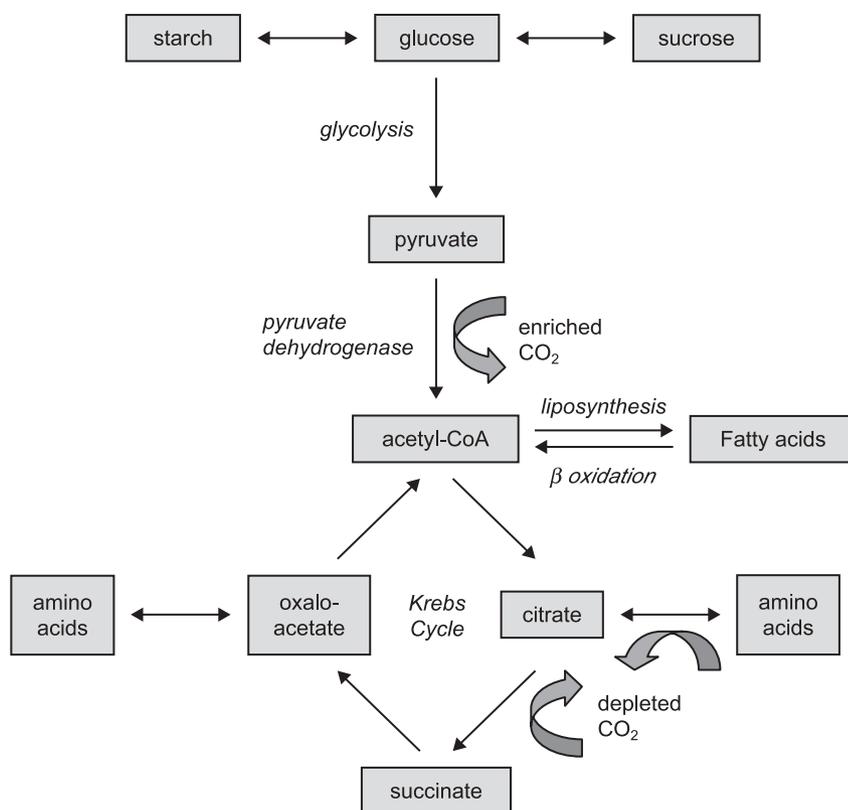
Fig. 1 Simplified schematic of respiratory and biosynthetic pathways that lead to CO_2 evolution that is enriched vs depleted in ^{13}C relative to sugars. Synthesis of fatty acids and certain amino acids will result in release of enriched respiratory CO_2, whereas full oxidation of acetyl-CoA or β oxidation of fatty acids will result in both enriched and depleted CO_2, likely with smaller net isotopes effects relative to sugars. Modified from Tcherkez *et al.* (2003).

et al. (2003) discussed several studies that measured respiratory CO_2, leaf sugars and other potential respiratory substrates directly and found poor correlations in isotopic composition. They suggested that changes in metabolic pathways from reactions that oxidize lipids to those that synthesize lipids could at least partially explain observed variations in isotopes of respiratory CO_2. This was supported by Tcherkez *et al.* (2003), who showed that isotope effects in respiration were correlated with the respiratory quotient (RQ): the ratio of carbon dioxide evolved to oxygen consumed in respiration. RQ values are known to be related to respiratory substrate, such as the oxidation of sucrose (RQ = 1) vs highly reduced compounds such as lipids (RQ < 1).

Plant respiratory pathways provide energy for metabolism, but they also provide carbon skeletons for synthesis of plant compounds from photosynthate. Synthesis of lipids is particularly of interest in discussing isotope effects of respiration because of the known, large isotope effects, both positional and enzymatic, in the oxidation of pyruvate to acetyl-CoA. If acetyl-CoA intermediates are fully oxidized in the Krebs cycle, net isotope effects on CO_2 will not be observed, as there can be no isotopic discrimination if all of a particular substrate is converted to a product. However, if some of this substrate is synthesized into fatty acids, amino acids and other compounds, a large isotope effect may result as the products are highly depleted in heavy carbon. Conversely, to

conserve mass balance, the CO_2 evolved from the pyruvate dehydrogenase reaction must be isotopically enriched (Fig. 1). This reaction is the only known process that may explain the large enrichments of up to 7‰ reported by Hymus *et al.* and others.

Implications for understanding plant and ecosystem function

At first glance, these findings complicate interpretation of the isotopic composition of plant respiration at larger scales. The ability to estimate carbon isotope ratios in respiration with both measurements and models has been an integral component of both ecosystem and global carbon cycle science. At the ecosystem scale, the isotopic composition of respiration has been applied toward partitioning ecosystem fluxes between photosynthetic and respiratory components (e.g. Yakir & Wang, 1996), whereas at the global scale the net impact of ecosystem production on CO_2 isotopes in the global atmosphere has been utilized to distinguish ocean from terrestrial carbon sinks (e.g. Francey *et al.*, 1995). Studies at the ecosystem scale have shown that $\delta^{13}C_r$ is dynamic in response to conditions of varying temperature, humidity, soil moisture, etc. on the scale of days, weeks and months (e.g. Bowling *et al.*, 2002). Changes in photosynthetic discrimination due to the effects of environmental conditions on stomatal

conductance and photosynthetic rate have been proposed as the central mechanism underlying these variations. Now, it appears that large diurnal changes in $\delta^{13}C_r$ such as those reported in Hymus *et al.* may be expected under field conditions owing to shifts in plant metabolic and biosynthetic pathways as well as CO_2 diffusion and uptake. In particular, their study suggests that large discrepancies between the isotopic composition of sugars and respiratory CO_2 are observed late in the day when photosynthetic products have accumulated in the upper canopy and when, presumably, carbon is shunted to biosynthetic pathways.

The implications of these findings for studies of whole-plant and whole-ecosystem respiration as well as regional to global carbon cycle studies remain to be seen. Klumpp *et al.* (2005) found that while shoot respiration was enriched relative to shoot biomass, root respiration was depleted relative to root biomass, which resulted in negligible differences between $\delta^{13}C$ of biomass and respiratory CO_2 on a whole-plant basis. Further studies of this kind should be conducted under a variety of environmental conditions to determine if these results are applicable to whole plants in the field. However, even if further experiments reveal that this simplifying assumption cannot always be made, isotope effects in respiration are more than just a 'complication' in the study of plant and ecosystem carbon cycles. The work of Hymus *et al.* and others has shown that measurements of the carbon isotope composition of plant respiration may provide insight into how photosynthate is allocated into synthesis of various compounds and metabolism in different species and in response to changes in the environment. Temporal and spatial patterns of autotrophic respiration remain difficult to predict, and an improved understanding of shifts in metabolic pathways such as liposynthesis and lipid oxidation in the Krebs cycle may improve our ability to quantify growth and maintenance respiration in response to temperature and other environmental changes of interest. Although isotope fractionation in respiration has emerged as something of a contentious issue in stable isotope ecology, an alternative view is that the dynamic isotopic composition of respiratory CO_2 provides an opportunity to gain insight into the flow of carbon through plants, and will therefore continue to be an important element of plant physiological and ecological studies.

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Ectomycorrhizas and mast fruiting in trees: linked by climate-driven tree resources?

Until recently, an almost entirely overlooked aspect of tree phenology has been the likely role of ectomycorrhizas (EM) in the mast fruiting. The positive association between EM and masting appears not to be strictly universal at the genus and family level (Alexander & Högberg, 1986; Newman & Reddell, 1987), but nevertheless it is certainly striking enough to raise the important questions: does mast fruiting depend on EM symbiosis and, if so, how does the link function, and under what conditions? Mast fruiting refers to a distinct supra-annual pattern in fruiting and seedling, evidenced in most cases by large peak years separated by no or very little activity (Herrera *et al.*, 1998; Kelly & Sork, 2002). There are many well-documented examples of mast fruiting among strongly EM tree families, such as Pinaceae, Fagaceae and Betulaceae in the temperates, and likewise for certain prominent families in the tropics, such as Dipterocarpaceae in South-east Asia and Caesalpiniaceae (Leguminosae) in Central Africa and northern South America. The Rosaceae interestingly also contain many horticultural species which show alternate-bearing, a phenological pattern with the shortest possible between-fruiting interval of 1 yr. Mast fruiting can be generally viewed as an extension of this basic pattern with increasing and varying intervals between fruiting events. The EM–masting connection calls for interpetation in terms of a resource model for tree growth and reproduction, and for experimental and field observational testing. For the present, however, we have a rather small number of isolated yet telling cases that are pointing in an exciting direction. The paper by Henkel, Mayor & Woolley (pp. 543–556) in this issue makes a valuable contribution in this problem area, not only by highlighting a new example of masting in a tropical EM species but also by coupling this phenomenon to ideas about climate-driven tree resources.

'*The process of mast fruiting in trees has to be maintained by a tree physiological mechanism, and the search is now on to discover and understand this*'

It has been commonly supposed since Büsgen and Münch (1929), but not unequivocally demonstrated, that large trees need to build up resources to a sufficiently high level in order to trigger flowering and to enable high fruit and seed production. The most well-known case in Europe is *Fagus sylvatica* L., for which there are several sets of long-term phenological observations (e.g. Piovesan & Adams, 2001). Large trees sustain considerable nonphotosynthesizing biomass in terms of primarily stem and branch wood, roots and mycorrhizas, and this creates a large respiratory load. Further, in a masting year, dry matter allocation can be upward of half of the annual leaf production (Green & Newbery, 2002), and this presumably creates a strong additional temporary sink for tree carbohydrates and nutrients. Accumulation of resources in the intermast interval, enhanced in years of raised radiation [due to El Niño/Southern Oscillation (ENSO) years, for example], will likely increase carbohydrate concentrations in the tree, but these need to be complemented by increased nutrient concentrations. EM may therefore play a role here and in contrast to vesicular–arbuscular mycorrhized trees they may be able to store nutrients, especially phosphorus (P), for later use in fruiting. It is widely agreed that the ultimate cause of masting in trees is avoidance of predators and pathogens: strong, even irregularly spaced pulses of seed and seedling input to the population lead to satiation and increased survival (Janzen, 1974, and the recent study of Curran & Leighton, 2000). However, this process has to be maintained by a tree physiological mechanism, and this is where the search is being renewed.

Physiological control and climatic variables

Henkel *et al.* report on masting in a large tropical ectomycorrhizal tree species in the Caesalpiniaceae, *Dicymbe corymbosa* Spruce ex Benth., which locally dominates stands of lowland rain forest on the Guianan Shield in South America. It is closely related taxonomically to other genera within the tribe Amherstieae in Central Africa and shares many characteristics with them by growing on low-P, well-drained soils (Malloch *et al.* 1980; Alexander, 1989a). *D. corymbosa* allocated 3.0 tonne ha^{-1} to reproduction in the 2003 mast year (Henkel *et al.*, 2005), a very high amount: Zagt (1997) showed that *Dicymbe altsonii*, also from Guyana, invested 2.0 tonne ha^{-1} per event. *Microberlinia bisulcata* A. Chev. – another large caesalp – in Central Africa shows similar strong masting and the link to EM has been inferred too (Newbery *et al.* 1997; Newbery *et al.* 1998). There, with a shorter intermast interval compared with *D. corymbosa* (*c.* 3 vs 5 yr), investment was close to 1.0 tonne ha^{-1} (Green & Newbery, 2002). Impressive examples are to be found in the Dipterocarpaceae, for which Curran (1994) has developed extensive arguments about how masting and EM-habit may have coevolved. The seminal work by Ashton *et al.* (1988) showed how dipterocarp mass flowering (often followed by mast fruiting) was linked to

cool nights in ENSO years, and the hypothesis advanced was that low temperatures triggered floral initiation. The subset of interrelated climatic variables – low rainfall, high daytime radiation and low nighttime temperatures – may connect with resource level regulation, hormonal switches and floral initiation in woody plants.

Dominance and regeneration

The phenomenon of dominance in tropical rain forests has attracted more attention in recent years as we move away from the somewhat idealized picture of all rain forests being composed of very species-rich vegetation types. Richards (1996), in what is perhaps the cornerstone monograph for tropical ecologists, gave much thought to this aspect of local and regional dominance. Particularly clear are the forests on low-nutrient soils, especially those in Africa and South America where EM species frequently dominate (Alexander, 1989b). However, there is varying evidence as to whether regeneration at these sites is persistent or not. Connell & Lowman (1989) have argued for EM species being responsible for this dominance because the EM symbiosis aids recruitment. This appears to be the case for *Dicymbe* in the study of Henkel *et al.* (2005), but there are counter examples in Africa which accord closer to the Aubréville (1938) model of shifting mosaics, namely that regeneration is poor below adults and that new stands form away from present ones (Newbery *et al.*, 1998), and there are monodominant forests of non-EM species, e.g. *Mora* spp. in South America (Torti & Coley, 1999). Henkel *et al.* (2005) show that predation was low in their 2003 mast year and that the rate of survival of seedlings to saplings was high. So we could predict that non-EM dominant species will not have a mast fruiting strategy and, correspondingly, that predator–pathogen pressure is low. In a similar vein, we have yet to explain why the EM caesalps in East African dry forests (Högberg, 1986) have no consistent reports of mast fruiting, and there is no clear case for *Eucalyptus* (EM Myrtaceae) in Australia (Smith & Read, 1997). Could the EM–masting association be restricted to moist temperate and tropical forests? This remains to be unravelled in the coming years.

Perspectives

The link between EM and mast fruiting opens up an exciting new area of research which involves not only the well-known traits of EM in P acquisition, interplant transfer and storage (Smith & Read, 1997) but also has implications for whole-tree physiology, phenology and population dynamics. High fitness not only requires efficacious morphological and physiological adaptations but a successful life-history strategy for a tree. How climate interacts with soil conditions, especially climatic events such as dry periods (owing to intensified dry seasons in seasonal temperate and tropical climates, or

dry periods in otherwise normally aseasonal ones) is worth pursuing further. The answer will need phenological data from long-time series at several comparable sites – for example, continued observations on species like *Dicymbe*. This approach will hopefully take us closer to a better understanding of which factors determine mast fruiting, and, moreover, it will revive interest in the slumbering problem of what is the integrated control mechanism for flowering and fruiting in trees in general.

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Letters

West African legumes: the role of nodulation and nitrogen fixation

When is a nodule not a nodule?

In a recent paper, Diabate *et al.* (2005) rightly pointed out that, compared with South America, the tropical rainforest legumes of Africa have been little studied for their ability to nodulate and fix nitrogen. These authors examined 156 species in six different forests and reported on their nodulation. Overall their data are consistent with the known distribution of nodulating behaviour in the Leguminosae. Some of their new reports are on species of genera widely reported as nodulating. Others are on new genera, from sections of the Leguminosae that include nonnodulating genera. Because the criteria on which their nodule identification was based were not given, it is difficult to evaluate these data.

The term 'nodule' is widely used in geology, medicine, botany and other disciplines. With respect to legumes, its use is usually confined to swellings on roots (occasionally stems) that house unicellular bacteria with the ability to fix atmospheric nitrogen. Many woody plants, including legumes, may have swellings on roots that superficially appear to be nodules, but are not. This has led to false reports of nodulation in the literature. In a detailed survey of leguminous trees in a tropical rain forest in Guyana, Perreijn (2002) divided species into three categories: those with nitrogen fixing nodules, those with nodule-like structures on roots and those without either. In a careful examination of the nodule-like swellings on the roots of the caesalpinioid species *Eperua falcata* Aubl. and *Mora excelsa* Benth., no nitrogenase activity was found, nor could nitrogen-fixing bacteria be isolated from them. Photographs of these structures are shown in Sprent (2001). Small, nodule-like structures were also seen on *Chamecrista adiantifolia* (Benth.) Irwin & Barneby, by Perreijn (2002), a species that is known to nodulate (Corby, 1988). The caesalpinioid genus *Chamaescrista* has at least 50 known nodulating species (Sprent, 2001), and for a number of these there is both structural (Naisbitt *et al.*, 1992) and functional (Sprent *et al.*, 1996) evidence that they fix nitrogen. It is thus possible that the structures recorded by Perreijn (2002) on *C. adiantifolia* were young or ineffective nodules, as she suggested. No structural studies were performed on them. As has been pointed out on a number of occasions (e.g. de Faria *et al.*, 1989; Sprent, 2001), it is very difficult to be certain from field specimens that a swelling on a root is a nodule. It can be equally easy to decide on external appearance that some structures that are nodules are not, because their morphology and, in some cases, their internal structure are unlike those of more familiar agricultural species (Sprent, 2001).

In order to confirm that a species nodulates, one or more of a number of lines of additional evidence are needed. Field observations should ensure that the nodulated roots were attached to the putative host plant; position of nodules on the root systems and their external shape should be recorded

(e.g. de Faria *et al.*, 1984; Corby, 1988). In many cases, nodule morphology, position on roots (whether or not associated with lateral roots) and nodule structure are taxonomic characters (Corby, 1988; Sprent, 2000). In addition, seedlings should be grown in soil from their native habitat and observation of development of swellings on roots should be made, and this is planned by Diabate *et al.* (2005) for their material (A. Galiana, INRA, Montpellier, pers. comm.). Following this, or on field specimens, evidence of nitrogenase activity (usually by the acetylene reduction assay) and/or simple sectioning to show internal colouration of nodules (usually pink when active, due to haemoglobin, but not always, as some nematode galls are also pink) and location of infected tissue (central in legumes) are simple checks that can be followed up with more detailed microscopy, including immunolocalization of nitrogenase and other key enzymes (Chen *et al.*, 2003).

Not all laboratories in countries with the most interesting legume trees have the facilities to carry out a full suite of tests for true nodulation and nitrogen fixation, but a subset of morphology and simple light microscopy, coupled with a knowledge of host plant taxonomy, can help to support visual observations until more detailed studies on fixed material and isolation of bacteria can be carried out. Fortunately, isolation can be achieved quite simply from nodules dried over a suitable desiccant and light microscopy on material immersed in old-fashioned fixatives such as 95% ethanol.

In the same way that observation of swellings on roots is not sufficient to establish nodulation, isolation of particular bacteria from nodules is not sufficient to prove that these bacteria can induce nodules. Isolated bacteria should be re-inoculated on to their putative hosts and shown to produce nodules, as Diabate *et al.* (2005) have done for species of *Albizia*, *Erythrophleum* and *Millettia*. This basic bacteriological technique, which has been taught at undergraduate level for nearly a century, has been used by some (e.g. Sy *et al.*, 2001) but not all (e.g. Moulin *et al.*, 2001) recent publications reporting new genera of bacteria that may nodulate legumes. Modern methods, such as tagging bacteria with green fluorescent protein, make observations of nodulation processes much easier (Chen *et al.*, 2003).

Can legume taxonomy help?

The family Leguminosae (Fabaceae) is generally subdivided into three subfamilies, Caesalpinioideae, Mimosoideae and Papilionoideae, and the extent of nodulation varies widely within these, from uncommon, through common to very common, within subfamilies, considerable correspondance with tribal affinities. Thus it is particularly important to be careful when reporting nodulation in groups where it is uncommon. Diabate *et al.* (2005) took this into consideration when discussing some of their observations, pointing out that the monotypic caesalpinioid genus *Chidlowia* (reported to nodulate) is in the tribe Caesalpinieae, where seven of the eight confirmed nodulating caesalpinioid legumes are placed. However, tribe Caesalpinieae has about 50 genera, for some of which there are no reports on nodulation status, others for which there are unconfirmed reports and yet others with several reports of nonnodulation. The tribe also includes *Eperua* and *Mora*, discussed above, as well as the only confirmed African caesalpinioid nodulating genus, *Erythrophleum*. It will be particularly interesting to see whether the field observations on *Chidlowia* are confirmed and whether any nodules produced have the characteristic primitive structure associated with nodules from all tree species in the Caesalpinioideae that have been studied (Sprent, 2001).

Within the subfamily Mimosoideae, nodulation is much more common, but not universal. Genera considered to be basal are trees and are usually included in tribes Parkieae and Mimoseae. However, Parkieae, which contains only two genera (*Parkia* and *Pentaclethra*) is now considered to be polyphyletic (Luckow *et al.*, 2003), with *Parkia* (nonnodulating) more closely related to members of tribes Ingeae and Mimosae than to *Pentaclethra* (nodulating in Africa, but not South America) (Sprent, 2001). Relations within tribe Mimoseae have also been revised by Luckow *et al.* (2003), and this is of interest with respect to nodulation generally and to the reports of Diabate *et al.* (2005), who give new positive reports of nodules on species of *Aubrevillea*, *Calpocalyx*, *Tetrapleura* and *Xylia*. There are no published molecular data on *Aubrevillea*, which was tentatively put in a group of its own within the Mimoseae by Luckow *et al.* (2000). This genus of two species is endemic to the Guineo–Congolean forest, so it will be interesting to see further information on its nodulation status. Genera *Calpocalyx*, *Tetrapleura* and *Xylia* all have negative reports on nodulation in the literature. On molecular and other criteria, they appear to be fairly closely related to each other and also to the genus *Adenanthera*, which is generally considered not to nodulate (Sprent, 2001; Luckow *et al.*, 2003). Again, confirmatory data are needed to verify nodulation in these genera.

Within the subfamily Papilionoideae, nodulation is recorded by Diabate *et al.* (2005) for *Amphimas*, a genus found not to be nodulated in Cameroon (D. Nwaga, University of Yaounde 1, pers. comm.; see Sprent, 2001). This genus is currently placed in the polyphyletic tribe Sophoreae, which contains both nodulated and nonnodulated genera (Sprent, 2001). However, *Amphimas* is considered to be basal within this tribe and the subfamily (Pennington *et al.*, 2001). Recent molecular studies on base papilionoid genera are grouping together several genera from various tribes, including Sophoreae, which appear unable to nodulate (Pennington *et al.*, 2001; Sprent, 2001). Molecular data are not yet available for *Amphimas* and there are no reports on nodulation for a number of other genera in this basal group. More information on these is needed to assess any relationship between nodulation criteria and molecular and other taxonomic characters. The utility of nodulation characters in legume

taxonomy has been discussed by Sprent (2000), and in a review of the dalbergioid group of papilionoid legumes, nodulation characters were among those most strongly related to molecular evidence (Lavin *et al.*, 2001).

The significance of nodulation in African rainforest legumes: a longitudinal perspective

As Diabate *et al.* (2005) point out, a large proportion of trees in humid forests of West Africa are legumes and of these many are important timber species. Sprent (2001) grouped genera listed from Guineo–Congolean forests by Lock (1989) with some updates on taxonomy from later work and from the International Legume Database & Information Service (ILDIS) database (http://www.ildis.org/LegumeWeb/). These data are summarized and extended in Table 1. Clearly there is a lot of work to be done to check the nodulation status of genera in these forests. Even then, as Diabate *et al.* (2005) and others have reported, not all species known to nodulate will do so in a particular area, owing to factors such as soil type and presence of suitable endophytic bacteria.

Taking a longitudinal perspective of tropical rain forests, those in Asia appear to have a relatively low proportion of legumes, but both nodulated and nonnodulated species are found there (Sprent, 2001). African forests have a high proportion of legumes, but many of them are caesalpinioid, base mimosoid and papilionoid and lack the ability to nodulate (Table 1). In South America, legumes are also very common, but there appears to be a higher proportion of nodulating species from all subfamilies (Sprent, 1999,

Table 1 Woody legumes (trees and lianas) of the Guineo–Congolean forests and their putative nodulation status

Subfamily, tribe	nodulated	nonnodulated	unknown
CAESALPINIOIDEAE			
Caesalpinieae	1	1	4
Cassieae	0	2	2
Cercideae	0	1	1
Detarieae	0	23	19
MIMOSOIDEAE			
Acacieae	1	0	0
Ingeae	2	0	0
Mimoseae	2	5	4
Parkieae	0	1	0
PAPILIONOIDEAE			
Aeschynomeneae	1	0	0
Dalbergieae	3	0	0
Millettieae	10	0	3
Phaseoleae	6	0	1
Sophoreae	1	3	5
Swartzieae	1	2	0

These data are summarized and modified from Sprent (2001). The numbers of genera and their tribal affinities are currently under review.

2001). The occurrence and significance of mycorrhizas in African rainforests has been widely studied, especially with respect to phosphorus dynamics (e.g. Newbery *et al.*, 1997). However, their nitrogen dynamics has been largely ignored. Does the small proportion of nodulated trees play an important role? Recent work from the Cameroon, as reported briefly by Sprent (2001, 2002), suggests that it may. In the Bayang-Mbo forest, large numbers of nodules were found in the litter layer and nodulated roots were often seen growing up trunks of trees of nonnodulating legumes and other species (Fig. 1a,b). These were traced for many metres (up to at least 30 m in some cases) until they could be connected to their parent trees or lianas (Fig. 1c). Surpisingly, mature trees were found to have copiously nodulated roots (Fig. 1c). It is generally considered that large trees recycle much of their nitrogen and therefore have little need for nodulation. This is one reason why it is recommended (e.g. de Faria *et al.*, 1989) that it is best to look for nodulation in seedlings rather than in mature trees. Nodules found in this Cameroon forest were of a variety of types, but fortunately these included classical desmodioid and aeschynomenoid forms (Corby, 1988; Sprent, 2001), known to be associated with particular genera. Desmodioid nodules are more or less spherical, with prominent lenticels and pronounced internal pink colouration. Nodules of this type were traced to a liana (probably a species of *Mucuna*), with a stem that went vertically up to the canopy (Fig. 1d). Aeschynomenoid nodules are oblate and formed in the axils of lateral roots; these were traced back to a large tree of *Pterocarpus*, a genus known to have this type of nodule (Corby, 1988; Sprent, 2001). In the same area of forest, most of the legume trees were non-nodulated caesalpinioid species, often from tribe Detarieae (Table 1). In other parts of the forest, nodulated species of papilionoid genera *Baphia*, *Millettia* and *Erythrina* were found, the former two also reported from Guinea by Diabate *et al.* (2005).

Apart from the few reports of nodulation that need to be confirmed, Diabate *et al.* (2005) generally found lack of nodulation in large numbers of woody members of caesalpinioid tribes Amherstieae, Cassieae, Cercideae and Detarieae. These observations, together with those from Cameroon, cited above, raise the question as to whether a few copiously nodulated species can fix enough nitrogen for the needs of the whole forest, supplementing that which is recycled. If this is the case, it raises another possibility, that mycorrhizas may have a function in transferring nitrogen between species. This possibility is supported by recent work of He *et al.* (2003) showing that there can be two-way transfer of nitrogen between nodulated and nonnodulated woody species. It has important practical implications. If nodulated trees are selectively logged, which may be the case (for example, for *Pterocarpus*, which is prized for its reddish-coloured wood), then the nutrient dynamics of the whole forest could be at risk.
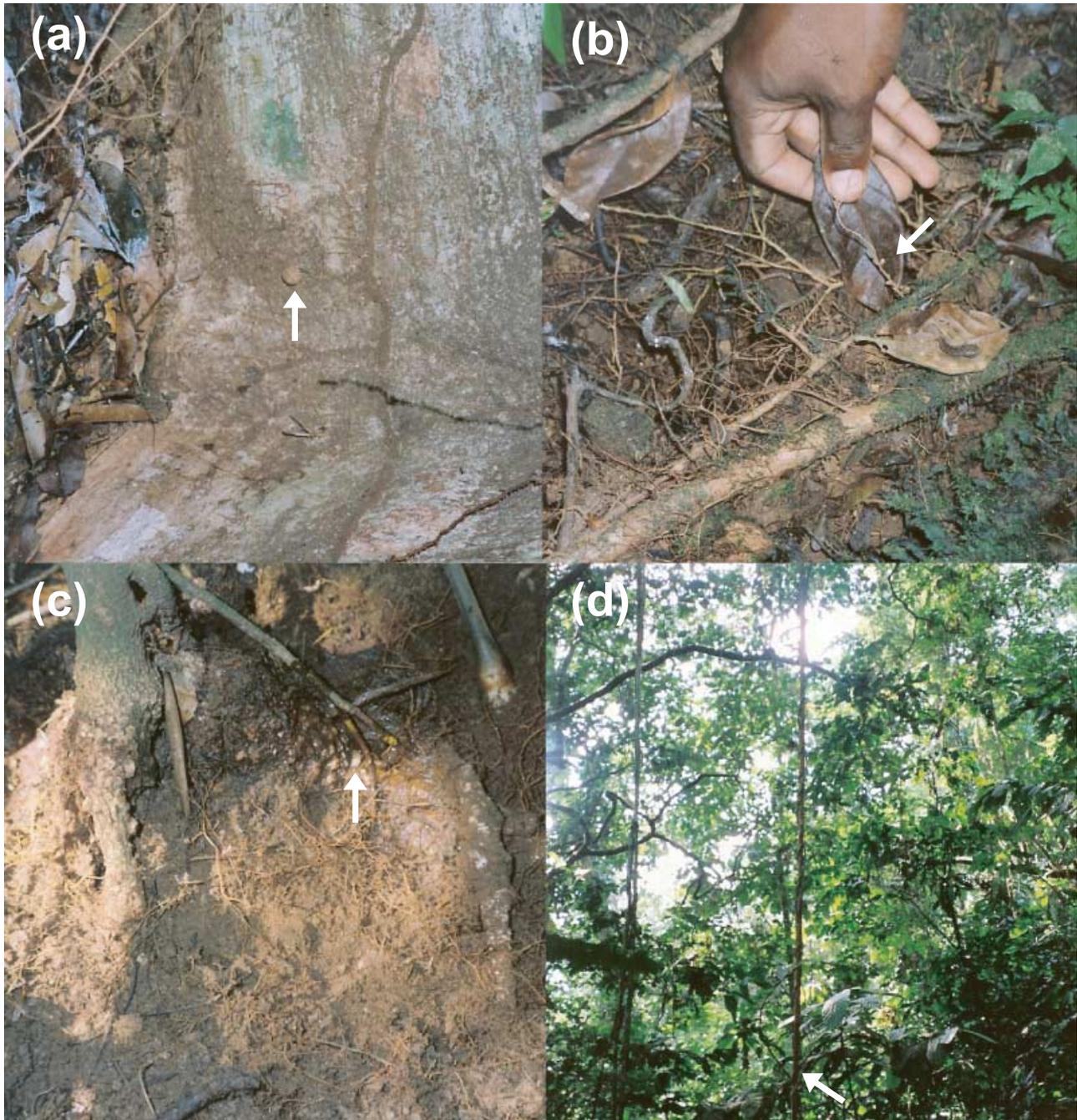
Fig. 1 Nodulated legumes in the Bayang-Mbo Forest, Cameroon. (a) A nodulated root climbing up the trunk of a nonnodulating caesalpinioid legume – the spherical nodule has a lenticel (seen as a stripe) typical of the desmodioid type of nodule found in papilionoid tribes Phaseoleae and Desmodieae (now usually grouped as one tribe). (b) A continuous root system, with desmodioid nodules attached, within the litter layer was traced approximately 25 m back to the base of a liana where many more nodules were attached (c). The stem of this liana went vertically up to the forest canopy (d).

Clearly, as Diabate *et al.* (2005) stress, we need to know much more about the nodulation status of West African legumes if forests are to be properly understood and managed. This will require much careful work on the part of many people, particularly those who live in the countries concerned.

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The evolution of mycorrhiza-like associations in liverworts: an update

Although liverworts do not have roots, many of them are associated with mycorrhiza-forming fungi (see compilation in Nebel *et al.*, 2004). The cellular structures of the associations and the fungi involved provide good arguments for the hypothesis that these are mycorrhiza-like associations (Read *et al.*, 2000; Kottke *et al.*, 2003), although physiological experiments revealing nutrient exchange are still missing. Because of convenience, the associations may even be termed 'mycorrhizas' (Brundrett, 2004), but to avoid unfruitful discussion about terms we prefer to speak of 'mycorrhiza-like associations'. A recent commentary on the symbiotic fungal associations of liverworts entitled 'Are liverworts imitating mycorrhizas?' (Selosse, 2005) asked whether liverworts established the symbiosis by obtaining the fungi through host shifts from mycorrhizas of higher plants, which would automatically include a rather recent evolution. The overview presented by us (Nebel *et al.*, 2004), however, indicated strong congruency between the evolution of liverworts and their specific symbiotic fungi, tempting us to hypothesize that 'the symbiotic fungal associations of liverworts are the possible ancestors of mycorrhizae' (Nebel *et al.*, 2004). The term 'ancestors' was not meant in a strict phylogenetic sense, as there is no heritage and thus no phylogeny of the symbiosis; instead, the symbiosis has to be individually established each time. The term should mean that liverworts developed the

symbiosis with fungi before the true mycorrhiza, the root–fungal symbiosis, evolved, and that the fungi originally switched from the gametophytes of liverworts to the roots of the sporophytes of tracheophytes and not vice versa. The extant situation might, however, include both transfer directions.

Molecular phylogeny of liverworts and fungal symbiosis

The most recent molecular phylogenies of liverworts (Davis, 2004; He-Nygren *et al.*, 2004) appear to substantiate our hypothesis. We therefore present an overview (Fig. 1) updated on the basis of the phylogenetic tree of liverworts given by Davis (2004), which was obtained from a 12-gene backbone data matrix and in which most nodes appeared to be highly supported (Davis, 2004). The revision of several main clades therein was also supported by results from other authors (Forrest & Crandall-Stotler, 2004; He-Nygren *et al.*, 2004). The overview (Fig. 1) reveals much more clearly than before (Nebel *et al.*, 2004) that: (1) all the basal groups of liverworts are associated with Glomeromycota; (2) the more derived

liverworts clades lost the mycorrhiza-like association with Glomeromycota at one common event probably connected to change of the habitat from terrestrial to epiphytic; and (3) the symbiotic state was re-gained at least twice and independently by species growing terrestrial on rotten wood or humus, but this time with Basidiomycota and/or Ascomycota.

The new molecular concept of the liverworts phylogeny relieves us from several problems we noticed in the older version (Nebel *et al.*, 2004). These problems were the position of *Blasia* as a taxon without a fungal symbiont, the occurrence of different symbiotic fungal associations (Glomeromycota and Basidiomycota) in the former Order Metzgeriales, and the question of dating the loss of the symbiotic state in the leafy liverworts as basal or derived. In the recent liverworts phylogeny, *Blasia* was transferred to the Complex thalloid clade (Davis, 2004; Forrest & Crandall-Stotler, 2004; He-Nygren *et al.*, 2004). In the phylogenetic tree of Davis (2004), *Blasia*, *Sphaerocarpos* and *Riccia*, which have no fungal symbionts, appear each as sister groups to the Glomeromycota-associated *Marchantia*, *Dumortiera* and *Monoclea*, indicating a common symbiotic ancestor and the multiple, independent
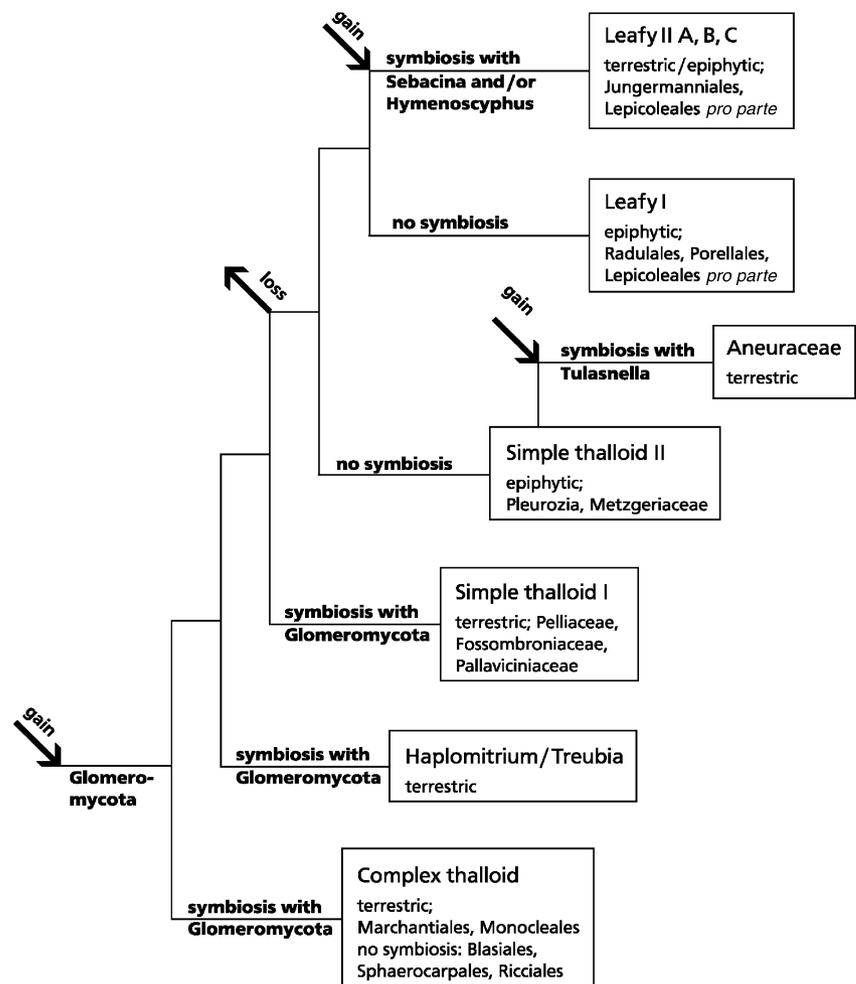

Fig. 1 Occurrence of mycorrhiza-like associations in the main clades of liverworts. The phylogenetic tree of liverworts was based on the maximum likelyhood tree obtained from a 12-gene backbone data matrix by Davis (2004). The figure has been redrawn, simplified and slightly modified. (Treubia is included in a tentative position.) For further explanations, see text.

loss of the symbiosis. It would be difficult to accept a non-symbiotic *Blasia* or *Sphaerocarpos* as the most ancestral member of the liverworts because this would imply a multiple establishment of the Glomeromycota associations at the basis of the liverworts. All the basal groups which are sister to the Complex thalloids now, the Haplomitriales and the Treubiales as well as the Simple thalloid I (Pelliaceae, Fossombroniaceae, Pallaviciniaceae), are associated with Glomeromycota. Thus, the hypothesized ancestral situation can, most likely, be indicated by 'gain of symbiosis with Glomeromycota' for a common ancestor of the monophyletic liverworts (Fig. 1).

The new molecular liverworts phylogeny separates the former Simple thalloids (Orders Fossombroniales, Metzgeriales) into two independent clades, the Simple thalloid I and the Simple thalloid II (Davis, 2004; see also He-Nygren *et al.*, 2004). The Simple thalloid I contain only Glomeromycota-associated species (*Pellia*, *Fossombronia*, *Petalophyllum*, *Pallavicinia*, *Jensenia*, *Symphyogyna*) and few with no fungal symbiosis (*Hymenophyton*, *Podomitrium*; M. Nebel, unpublished results). The Simple thalloid II comprise the nonsymbiotic Metzgeriaceae, the nonsymbiotic genus *Pleurozia* and additionally the Aneuraceae, which form a symbiosis with *Tulasnella* species (Basidiomycota) (Bidartondo *et al.*, 2003; Kottke *et al.*, 2003). The more detailed molecular phylogenetic tree of Davis (2004), in which *Metzgeria* and *Apometzgeria* appear basal to *Aneura*, suggests that the Simple thalloid II lost the mycorrhizal state first, and later established a new symbiosis with Basidiomycota. It is tempting to speculate that the gain of *Tulasnella*, a member of the Basidiomycota with some saprophytic capabilities, supported the development of *Aneura* and *Riccardia* on rotten wood, and consequently supported the evolution of the myco-heterotrophic state of *Cryptothallus*. In the latter case, *Tulasnella* forms additionally ectomycorrhizas with *Betula*, *Cryptothallus* thus appearing as an epiparasite on *Betula*, gaining carbon via the fungus (Bidartondo *et al.*, 2003).

The leafy liverworts comprise two sister clades in the new molecular phylogenetic tree, the Leafy I and the Leafy II, representing an early dichotomy according to Davis (2004). According to our previous data collection (Nebel *et al.*, 2004), the Leafy I contain only nonsymbiotic species. Interestingly, molecular phylogeny places this group of epiphytic species basal to the Leafy II (Davis, 2004; He-Nygren *et al.*, 2004), whereas classical systematics considered the species-rich epiphytics to be a 'crown group' (Schuster, 1984; Crandall-Stotler & Stotler, 2000). Thus, the molecular results would support a hypothesized loss of the symbiotic state as one main event by a common ancestor of the Simple thalloid II and the Leafy liverworts (Fig. 1). This event might have been linked to the change of the habitat from terrestrial to epiphytic.

The Leafy II contain many species that are symbiotically associated with Ascomycota and/or Basidiomycota, and all these species grow terrestrial or semiterrestrial on rotten wood

or humus. Where the fungi were identified, they came out as members of the *Hymenoscyphus ericae* aggr. (Ascomycota) or as *Sebacina* species (Basidiomycota) (see literature in Nebel *et al.*, 2004). Species with mycorrhiza-like associations occur in all the three subclades (A, B, C) of the phylogenetic tree presented by Davis (2004). The re-gain of the symbiosis, thus, was most likely by a common ancestor. It is not possible so far to indicate definitely the node of the gain, as the symbiotic state of *Ptilidium* is unclear. According to our re-investigation, *Ptilidium* is not associated with mycorrhizal fungi, and neither is *Schistochila*; no data are available for *Temnoma*. These three genera form the basal branches of the Leafy II according to Davis (2004); however, their position is critically discussed (Davis, 2004). We tentatively position the re-gain of the mycorrhiza-like state at the main node of the Leafy II (Fig. 1).

So far, only about 5% of the species in the Leafy II have been investigated for symbiotically associated fungi. It is therefore too early to obtain a definite picture of the distribution of *Sebacina* and *Hymenoscyphus*. However, it is remarkable that both are equally present in Leafy II C, the most basal clade, but the Ascomycota predominate in Leafy II B and A, the more derived clades (Cephaloziineae, Lepidoziineae). The Leafy II A include also several species for which a symbiosis with fungi was not found (*Herbertus*, *Lepicolea*, epiphytic *Plagiochila* species, *Trichocolea*). These observations indicate a secondary loss of the symbiotic state in the 'crown group'.

Systematic position of symbiotically associated fungi

When we consider the systematic position of the associated fungi, the coincidence in evolution is striking. The Glomeromycota unambiguously comprise the most basal group of symbiotic fungi of land plants (Schüßler *et al.*, 2001), and they form the only group that is associated with the basal groups of liverworts. The Basidiomycota associated with the more derived liverworts belong to the Orders Sebacinales and Tulasnellales. The Sebacinales, according to recent molecular phylogeny, is the most basal group of the Hymenomycetes in Basidiomycota (Weiß & Oberwinkler, 2001; Weiß *et al.*, 2004). The Tulasnellales were placed within the Cantharelloid clade (Hibbett & Thorn, 2001), the most basal clade in the Homobasidiomycetes of Basidiomycota (Larsson *et al.*, 2004). *Sebacina* and *Tulasnella* species are also grouped with other basal Basidiomycota as 'Heterobasidiomycetes', all of them containing a doliporus with imperforate parenthesomes (Weiß *et al.*, 2004). No other, more derived symbiotic Basidiomycota have been detected in liverworts so far, although these could be easily recognised at the ultrastructural level because of their perforate parenthesomes.

The restriction of liverworts to basal groups of symbiotic fungi can, in our opinion, be best explained by an 'old

heritage'. There is good molecular support for the liverworts as the most ancestral land plants (Qiu *et al.*, 1998; Groth-Malonek *et al.*, 2004). Fossil spores of Glomeromycota and of liverworts were recently dated to the Ordovician (Redecker *et al.*, 2000; Wellmann *et al.*, 2003). Although there are up to now no fossils of mycorrhiza-like associations before the Lower Devonian, the more ancestral liverworts probably already etablished the symbiosis with Glomeromycota in the Ordovician. The gain of the symbiotic state with *Sebacina*, *Tulasnella* and *Hymenoscyphus ericae* occurred later in geological times, indicated from the more derived liverworts clades and the derived positions in the phylogenetic tree of fungi (Schüßler *et al.*, 2001). Because there are no fossils and no molecular clock dating these fungi, it is highly speculative, but we might suggest that this event should be dated to the time before the Pangaea continent was split in parts. This would help to explain the worldwide occurrence of *Sebacina*, *Tulasnella* and the *Hymenoscyphus ericae* aggr. (Leotiales) as symbionts of liverworts (Allen *et al.*, 2003; Kottke *et al.*, 2003; Nebel *et al.*, 2004, and literature therein).

Fungal host switch

A host switch from higher plant mycorrhizas to the liverworts cannot be excluded *a priori.* However, it could apply for the late gain of *Sebacina* and *Tulasnella* symbionts (Fig. 1), as suggested by Selosse (2005). *Marchantia foliacea*, according to molecular sequences, shared a *Glomus* species with *Podocarpus* sp. in the surroundings (Russel & Bulman, 2004). Turnau *et al.* (1999) observed hyphal connections between liverworts and plant roots. A share of the symbiont between *Cryptothallus* and *Pinus/Betula* was demonstrated in field material and in pure cultures (Bidartondo *et al.*, 2003). The symbiotic state has to be established individually each time during the life cycle of a plant. Thus, the independent phylogeny of plants and fungi will allow new combinations. We should probably not expect that the liverworts are only associated to the oldest lineages of Glomeromycota and the most basal *Sebacina* or *Tulasnella* species. Our argumentation for an originally old establishment of the mycorrhiza-like associations in liverworts does not exclude a possible recent and ongoing exchange of the symbiotic fungi. However, the majority of the more than 5000 ectomycorrhiza-forming fungi is composed of Homobasidiomycetes (Basidiomycota) or Pezzizales (Ascomycota). It is rather difficult to believe that just *Sebacina* and *Tulasnella* would have recently switched from higher plants to liverworts. Even in the neotropical mountain rain forest, liverworts are associated with *Tulasnella* and *Sebacina* (Nebel *et al.*, 2004). Ectomycorrhizal trees are extremely rare there, and these few Nyctaginaceae do not form ectomycorrhizas with *Tulasnella* and *Sebacina* but with *Thelephora*, *Russula* and *Lactarius* (Haug *et al.*, 2005).

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