	M E C	5 6 9 2	B	Dispatch: 15.6.12	Journal: MEC	CE: Sankara Rajan I.
	Journal Name	Manuscript No.		Author Received:	No. of pages: 12	PE: Punitha

Molecular Ecology (2012) doi: 10.1111/j.1365-294X.2012.05692.x

The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids

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Abstract

Characterizing the architecture of bipartite networks is increasingly used as a framework to study biotic interactions within their ecological context and to assess the extent to which evolutionary constraints shape them. Orchid mycorrhizal symbioses are particularly interesting as they are viewed as more beneficial for plants than for fungi, a situation expected to result in an asymmetry of biological constraints. This study addressed the architecture and phylogenetic constraint in these associations in tropical context. We identified a bipartite network including 73 orchid species and 95 taxonomic units of mycorrhizal fungi across the natural habitats of Reunion Island. Unlike some recent evidence for nestedness in mycorrhizal symbioses, we found a highly modular architecture that largely reflected an ecological barrier between epiphytic and terrestrial subnetworks. By testing for phylogenetic signal, the overall signal was stronger for both partners in the epiphytic subnetwork. Moreover, in the subnetwork of epiphytic angraecoid orchids, the signal in orchid phylogeny was stronger than the signal in fungal phylogeny. Epiphytic associations are therefore more conservative and may co-evolve more than terrestrial ones. We suggest that such tighter phylogenetic specialization may have been driven by stressful life conditions in the epiphytic niches. In addition to paralleling recent insights into mycorrhizal networks, this study furthermore provides support for epiphytism as a major factor affecting ecological assemblage and evolutionary constraint in tropical mycorrhizal symbioses.

Keywords: co-evolution, Interaction networks, modularity, nestedness, orchid mycorrhizal symbiosis, phylogenetic bipartite signal

Received 8 February 2012; revision received 10 May 2012; accepted 18 May 2012

Introduction

In ecological communities, species interact with a range of partners that can interact themselves with other species and form complex interaction networks (Thompson 2005; Proulx *et al.* 2005). The architecture of such bipartite networks has recently received much interest in ecological research, especially regarding the link between the interaction characteristics (e.g. nature, intimacy) and the pattern of network architecture (see Fontaine *et al.* 2011 for a review). It was particularly shown that networks between mutualists display higher level of nestedness and lower level of modularity (i.e. compart-

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mentalization) than networks between antagonists such as trophic links (Bascompte *et al.* 2003; Thébault & Fontaine 2010; see Fontaine *et al.* 2011 for a review), although some degree of nestedness can occasionally be found in some antagonistic networks (Kondoh *et al.* 2010). Phylogeny-based analysis further allows evaluating the inherent evolutionary or co-evolutionary processes constraining the interactions within a given network (Thompson 2005; Rezende *et al.* 2007; Cavender-Bares *et al.* 2009; Vazquez *et al.* 2009a; Mouquet *et al.* 2012). We can hypothesize that closely related species are likely to share traits and are thus likely to interact with the same partners or with closely related partners. Such inheritance may result in phylogenetic signal (Blomberg *et al.* 2003), and an asymmetry of phylogenetic signal between the partners may further reveal different evolutionary constraints for them (Ives & Godfray 2006; Vacher *et al.* 2008; Vazquez *et al.* 2009b; Jacquemyn *et al.* 2011).

Various aboveground plant–animal interactions have been surveyed in this respect (see Bascompte & Jordano 2007 for a review). However, mycorrhizal symbioses between plant roots and fungi, which involve nutrient exchanges in the plant rhizosphere (Smith & Read 2008), have only very recently been investigated (Jacquemyn *et al.* 2011; Chagnon *et al.* 2012; Montesinos-Navarro *et al.* 2012). The mycorrhizal symbiosis of Orchidaceae, which affects no <25 000 species worldwide (Dressler 2005), is particularly interesting because its mutualistic nature is controversial (Cameron *et al.* 2006, 2008; Rasmussen & Rasmussen 2009). This symbiosis is obligatory for orchids because their minute seeds are devoid of any significant reserves and require fungal colonization for germination and growth through their achlorophyllous, heterotrophic early stage (Rasmussen 1995). Conversely, the fungal symbionts that mainly belong to three fungal clades, that is, Ceratobasidiaceae, Tulasnellaceae and Sebacinales, which are called *rhizoctonias* for convenience (see Dearnaley *et al.* 2012 for a review). *Rhizoctonias* are considered as free-living saprotrophs or root endophytes of some other plant families (Selosse *et al.* 2009; Weiß *et al.* 2011; Yagame *et al.* 2012), whose major ecological niches may be independent of orchid roots. Although Cameron *et al.* (2006) could trace carbon transfer from adult orchids to their fungal symbiont in microcosm, there is no general evidence that the fungi depend on the orchids for either carbon uptake or reproduction in natural conditions.

Mycorrhizal specificity, that is, the range of fungi associated with a focus plant species over its distribution, has been extensively addressed in orchids (see Dearnaley *et al.* 2012 for a review), given its importance for conservation purposes (Cribb *et al.* 2003; Swarts & Dixon 2008). There is today much evidence that specificity varies among species, in terrestrial orchids (McCormick *et al.* 2004, 2006; Jacquemyn *et al.* 2010) and in neotropical, epiphytic orchids (Otero *et al.* 2002, 2004, 2007; Suárez *et al.* 2006, 2008). While the variation in specificity remains poorly understood, some authors argued that generalist orchids might be more prevalent in nutrient-poor or drought-stressed niches (Jacquemyn *et al.* 2010). Analysing the influence of ecological constraints on orchid mycorrhizal symbioses is therefore highly relevant, especially in the tropical context where orchid taxa are highly diversified and adapted to ecological niches that can differ considerably. Principally, epiphytic orchids have intensively diversified in tropical ecosystems (Gravendeel *et al.* 2004; Dressler 2005), where constraints of water shortage, nutrient availability, irradiation, etc., are stronger than in niches involving rooting in soil (Benzing 2008; Laube & Zotz 2003; Zotz & Hietz 2011). Testing for phylogenetic signal in orchid mycorrhizal symbioses should also enlighten the influence of evolutionary constraints on these relationships (Ives & Godfray 2006; by evolutionary constraint, we mean that the previous evolution is determining the shape of an observed pattern). It was recently shown that the mycorrhizal network of 16 *Orchis* species across Europe was highly nested and constrained by the plant phylogeny, but not by the phylogeny of their Tulasnellaceae partners (Jacquemyn *et al.* 2011). Such asymmetry in phylogenetic constraint can be explained by the more recent diversification of orchids retaining ancestors' symbionts or by a difference in evolutionary niche conservatism (Losos 2008), in particular, because the orchids may be more dependent on the symbiosis than their mycorrhizal fungi.

This study addressed both the ecological and evolutionary constraints on the structure of orchid mycorrhizal symbioses in a tropical context. We first identified a large association matrix of mycorrhizal associations including 360 observations (210 binary links) between 73 orchid species and 95 operational taxonomic units (OTUs) of mycorrhizal fungi in Reunion Island that belongs to a biodiversity hotspot (Madagascar and the Indian Ocean islands; Myers *et al.* 2000). We investigated the levels of nestedness and modularity in this matrix and then analysed separately the epiphytic and terrestrial subnetworks because they were the most important modules of the network. We also tested whether the orchids and/or the fungi associate with closely related partners by estimating the phylogenetic signal (Ives & Godfray 2006) at two different phylogenetic resolutions: (i) the 34 epiphytic species of African angraecoid orchids occurring in Reunion Island, for which a reliable molecular phylogeny was available (Micheneau *et al.* 2008); (ii) the 25 orchid genera of the overall network.

Material and methods

Study area and sampling

Reunion Island (La Réunion; 21°09′S, 55°30′E) is a 2- to 3-million-year-old volcanic island that reaches 3070 m at the *Piton des Neiges* and 2632 m at the *Piton de la Fournaise* (Fig. 1). The climate is tropical and alternates a rainy season from December to April with a cooler, drier season. Mean annual rainfall is high in the east (1500 to >8000 mm) and lower in the west (500–1500 mm). The native vegetation includes lowland rainforests (high canopy), lowland semi-dry forests, mountain rainforests (epiphyte-rich), and subalpine heath lands (Strasberg *et al.* 2005). The native flora comprises about 150 orchid species in 30 genera including, respectively, 50% and 25% endemic of Mascarene Archipelago and Reunion Island. The richest taxon is the epiphytic group of African angraecoid orchids (tribe Angraecinae mostly). Terrestrial and epiphytic orchids occur in all habitat types, except in subalpine heath lands that are devoid of epiphytes.

We investigated the mycorrhizal symbioses in more than 50% of the indigenous species of Reunion Island, that is, 77 species belonging to 25 genera. We sampled roots of six individuals per species from three different localities whenever possible because some rare species were not found at more than one or two localities (Table S1, Supporting information). In total, 452 plant individuals were sampled from 34 forest sites between January 2007 and April 2010 (Fig. 1; Table S1, Supporting information). Each orchid species was exclusively sampled either in epiphytic position (50 spp.) or in terrestrial position (27 spp.).

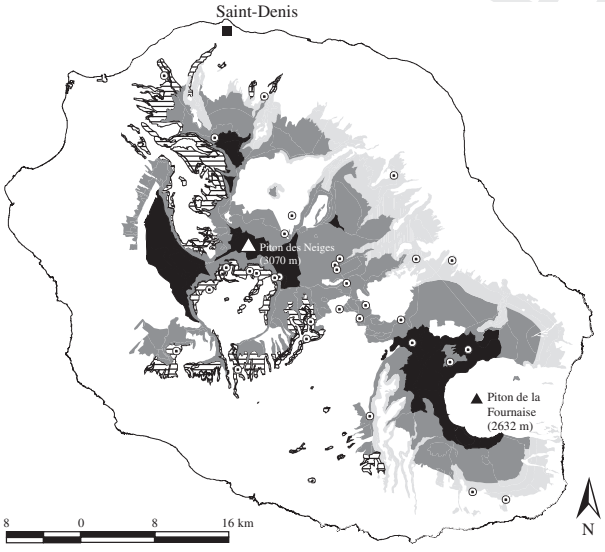

Fig. 1 Map of Reunion Island showing the forest sites with native vegetation (circles) where roots of 77 orchid species were sampled between 2007 and 2010. Hatched, lowland semi-dry forest; light grey, lowland rainforest; dark grey, mountain rainforest; black, subalpine heath land.

For each orchid individual, we collected five or more independent 2-cm-long root fragments whenever possible without dislodging the plant. Root fragments were surface-sterilized for 10s using 2% sodium hypochloride and 5% polysorbate 80, rinsed three times in sterile water and checked for the presence of typical orchid mycorrhizae, that is, intracellular hyphal pelotons (see Rasmussen 1995). A 2-mm-long root section harbouring pelotons was sampled for each root fragment, that is, five or more root sections per plant, and stored at −20 °C for molecular analyses of fungi. We randomly sampled 42 healthy-looking sections (two per 21 species) adjacent to the previous ones and quickly fixed them in 2% glutaraldehyde (10 mM Na-phosphate buffer; pH 7.2) to corroborate the molecular analyses by observation of specific features of the peloton-forming fungi in transmission electron microscopy (TEM; see Rasmussen 1995; Fig. S1, Supporting information). Ultrathin sections were obtained as in Kottke *et al.* (2009) and examined using a ZEISS TEM at 80 kV.

Molecular and phylogenetic analyses

Fungi. Total DNA was extracted from two root sections per orchid individual using a DNeasy® Plant Mini kit (Qiagen Inc., Valencia). The internal transcribed spacer (ITS) of the nuclear rDNA was tested for PCR amplification using three primer sets: the fungus-specific set, ITS 1F/4; the basidiomycetes-specific set, ITS 1F/4B; and the Tulasnellaceae-specific set, ITS 1/4-Tul, as the two previous sets do not amplify Tulasnellaceae. Whenever multiple signals were observed from a single PCR, we cloned the products as in Julou *et al.* (2005) and sequenced five clones per cloning: in total, 158 cloning reactions were successfully performed. For rhizoctonia fungi, we increased the number of molecular characters (i.e. the length of nuclear rDNA sequences) prior to phylogenetic analysis by amplifying the 5′ part of 28S nuclear rDNA using three primer sets: the fungus-specific set, ITS 1F/TW13; the Sebacinales-specific set, ITS 3S/TW13 (Selosse *et al.* 2007), or the Tulasnellaceae-specific set, ITS 5.8S-Tul/TW13 (Suárez *et al.* 2006). Sequences were edited in Geneious Pro 5.0.2, identified by Blast analysis, and deposited in GenBank (NCBI, http://www.ncbi.nlm.nih.gov, JF690991–JF691350 and JF691359–JF691537). Whenever we amplified both the ITS and the 5′ part of 28S, chromatograms were assembled to produce a unique consensus sequence (ITS-28S) that was deposited in GenBank in this way. We applied a threshold of 97% similarity between ITS sequences to

circumscribe operational taxonomic units (OTUs) among the mycorrhizal taxa, which is the usual proxy for species delimitation among basidiomycetes (Hughes *et al.* 2009; Jacquemyn *et al.* 2010). We also considered a 95% similarity threshold (Waterman *et al.* 2011) to test for the robustness of our results. A multiple sequence alignment was performed from the longest sequence of each OTU using the E-INS-i algorithm in MAFFTV6 that is recommended for sequences with multiple conserved domains and long gaps. The ITS1 and ITS2 regions that did not align convincingly were excluded, and phylogenetic analyses were performed from both the 5.8S and 28S rDNA by heuristic search using Maximum Parsimony and Maximum Likelihood criteria, followed by 1000 repetitions bootstrap analyses in PAUP 4.0b10 (Swofford 2002). Before ML analysis, we designated two models of nucleotide substitutions for the 5.8S and 28S rDNA, respectively, by calculating approximate AIC values in MRMODELTEST 2.3 (Nylander 2004).

Orchids. We obtained a phylogenetic tree of the 25 orchid genera by retrieving *matK* sequences from the GenBank database; *Vanilla* was added as external group. For the angraecoid orchids, we further included information at species level, on the basis of four plastid loci (*matK*, *trnL* intron, *trnL-F* intergenic spacer and *rps16* intron) as published in Micheneau *et al.* (2008), and we restricted our analysis to the 34 species occurring in Reunion Island; *Polystachya mauritiana* was used as outgroup.

Network architecture analysis

The matrix of the species network, S, included $L = 210$ binary links relating 95 *rhizoctonia* OTUs (on rows) and the 73 orchid species (on columns; Fig. 3) that showed associations with *rhizoctonia* taxa. We assessed its levels of nestedness and modularity to get insights into the overall architecture. Nestedness is the trend of specialist species to associate with partners that form well-defined subsets of the partners with which generalist species associate (Bascompte *et al.* 2003), and modularity conversely represents the degree of clustering in the network, which yields distinct communities (Guimerà *et al.* 2004; Guimerà & Amaral 2005a,b; Newman 2006; Barber 2007).

Nestedness. We considered the NODF measure of nestedness (Almeida-Neto *et al.* 2008), which is among the most appropriate metric to analyse bipartite networks (Ulrich *et al.* 2009). We tested the significance of NODF values against a swap null model (Gotelli & Entsminger 2001), where the probability of drawing an interaction is proportional to the specificity of the species. A nested structure can even occur in the null model when species establishing many relationships more easily grasp rare species ('passive sampling', see Ulrich *et al.* 2009). Deviations from the swap null model then convey the signature of further biological and ecological constraints on the structure. Though conservative, this null model showed the best statistical performances (Ulrich & Gotelli 2007). We used both the *trial swap* (sequential) and *quasi swap* (not sequential) algorithms of the function *oecosimu* in *vegan* R package and performed 999 simulations to get the null distribution of NODF values. We applied the same procedure to analyse the level of nestedness in the two subnetworks including, respectively, terrestrial and epiphytic orchid species and their mycorrhizal fungi.

Modularity. For a given set of r modules within the network, the modularity was measured as follows:

$$M = \sum_{s=1}^{r} \left[\frac{l_s}{L} - \left(\frac{d_s}{2L} \right)^2 \right],$$

where l_S was the number of links between nodes in module s, and d_S the sum of the degrees of the nodes in module s (Guimerà & Amaral 2005a,b; Newman 2006; Barber 2007). We obtained the set of modules displaying the largest modularity by applying the simulated annealing algorithm of Guimerà & Amaral (2005a,b) as implemented in the program *Netcarto*. The modularity of the network was compared with that of 999 randomized networks to assess its significance (Guimerà *et al.* 2004). Furthermore, the modularity could be measured for any other arbitrary set of modules. We thereby assessed the modularity related to the epiphytic and terrestrial position of the partners (two modules), M_{pos}, and the modularity related to the preferred forest type (four modules), M_{hab}. The significance of M_{pos} and M_{hab} was assessed by randomizing manifold the composition of the modules, so as to compare the observed modularity to the corresponding null situation.

Phylogenetic constraint analysis

Methods to quantify phylogenetic constraint (or phylogenetic signal; Blomberg *et al.* 2003) rely on averaging the phylogenetic relatedness of the partners interacting with a particular species (Ives & Godfray 2006), and null models are used to test for the significance of this signal against randomized relationships. In the context of a bipartite network, the signal can be addressed for one or the other partner, and a global signal of the network can also be assessed (Ives & Godfray 2006). We tested whether closely related orchids were more likely

to associate with related mycorrhizal fungi, and whether closely related fungi were more likely to associate with related orchid hosts.

Model. We applied the phylogenetic bipartite linear model of Ives & Godfray (2006). Using an estimated general least square (EGLS) analysis, the structure of the association matrix is decomposed into a phylogenetically corrected mean association strength and a vector of residuals depending on the phylogenies (Ives & Godfray 2006; : E3). The reference evolution model used to calculate the phylogenetic structure is the Ornstein–Uhlenbeck (OU) process that can incorporate stabilizing selection (Blomberg *et al.* 2003). The significance of the phylogenetic structure is discussed by comparing the mean square error (MSE) on the basis of this model of evolution (MSE_d) with the MSE derived under the assumption of no phylogenetic signal (i.e. a star phylogeny, MSE_s) and with the MSE assuming maximum phylogenetic signal (i.e. Brownian motion evolution, MSE_b). The smaller the MSE, the better the model (Ives & Godfray 2006; : E6), and hence, the comparison informs on the overall strength of the phylogenetic signal. The bipartite model further yielded two independent parameters of phylogenetic structure based on plant and fungal phylogenies, respectively, d_p and d_f, which allowed detecting any asymmetry in phylogenetic constraint. Bipartite linear models were performed using the *pblm* function in the *picante* R package (Kembel *et al.* 2010).

Application. We applied the model to a submatrix (110 links) of the matrix *S* (210 links) relating 54 mycorrhizal OTUs (on rows) and 34 angraecoid epiphytic species (on columns), for which a reliable molecular phylogeny was available (Micheneau *et al.* 2008). We also applied the model to a version of matrix *S* including the 95 mycorrhizal OTUs (on rows) and the 25 orchid genera (on columns; 159 binary links), because a validated phylogenetic tree was lacking for the non-angraecoid species. Finally, we applied the model to the subnetworks including, respectively, the terrestrial and epiphytic orchid genera and their mycorrhizal fungi.

Results

Mycorrhizal fungi

The 452 orchid individuals all displayed typical orchid mycorrhizae within their root cortical cells, although fungal coils were more easily found in terrestrial orchids than in epiphytic ones. We obtained one or more fungal ITS sequences in each of the 77 orchid species for a total of 547 diverging sequences (Fig. 2): 360

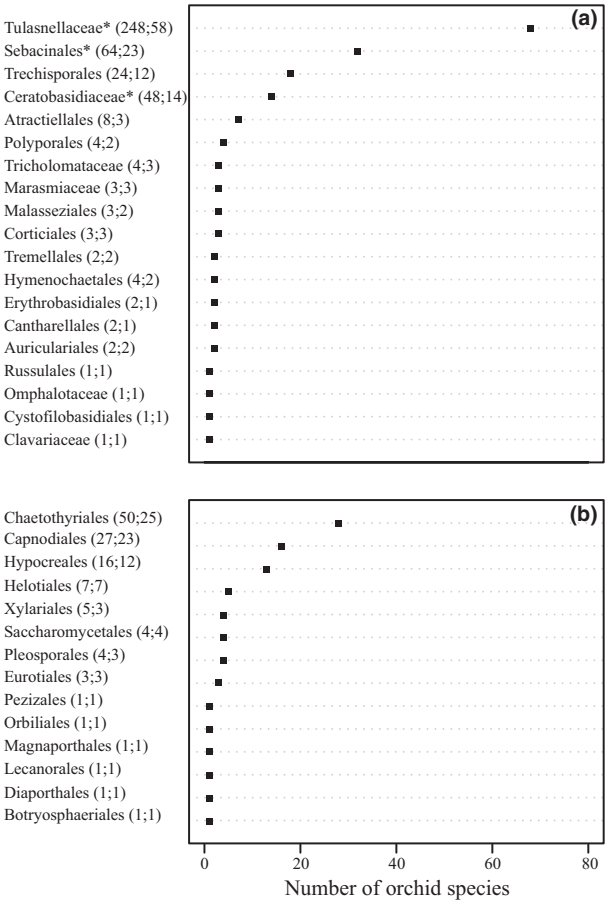

Fig. 2 Fungal taxa against number of orchid species harbouring these taxa: (a) basidiomycetes, (b) ascomycetes. Numbers of ITS sequences and operational taxonomic units (OTUs) are shown in brackets in the *Y*-axis column. *Taxa seen in transmission electron microscopy to form typical orchid mycorrhizae and then considered as mycorrhizal fungi in this study.

sequences of *rhizoctonias* were identified in 73 (95%) orchid species and in all orchid genera, that is, 248 sequences of Tulasnellaceae, 64 sequences of Sebacinales belonging to the clade B *sensu* Selosse *et al.* (2009) and Weiß *et al.* (2011), and 48 sequences of Ceratobasidiaceae (Fig. 2a). We circumscribed 95 *rhizoctonia* OTUs at the 97% sequence similarity threshold, including 58 OTUs of Tulasnellaceae, 23 OTUs of Sebacinales, and 14 OTUs of Ceratobasidiaceae (Fig. 2a), and 85 of them were found only in either epiphytic or terrestrial orchid taxa, while only 10 were found in both. We thereby ascribed them to three classes: 'terrestrial' (31 OTUs), 'epiphytic' (54 OTUs), or 'ubiquist' (10 OTUs). We also ascribed them to the main forest type in which they were found, namely the lowland rainforest (59 OTUs), mountain rainforest (17 OTUs), semi-dry forest (15 OTUs), and subalpine heath land (four OTUs). A few sequences of non-*rhizoctonia* basidiomycetes were

also identified, mainly Trechisporales and Atractiellales (Fig. 2a), plus a vast diversity of ascomycetes sequences related to putative parasitic, endophytic, or saprotrophic taxa (mainly Chaetotyriales, Capnodiales and Hypocreales; Fig. 2b). To test the robustness of our molecular sampling, we reapplied the same procedure to ten independent root sections from 15 randomly selected orchid individuals that had been already investigated: 12 individuals showed the same *rhizoctonia* OTUs, and three revealed a new *rhizoctonia* OTU but from the same *rhizoctonia* clade. Ascomycete fungi were rarely identified in more than one root section per individual, further excluding that they were the main mycorrhizal taxa of these orchids. Moreover, whenever pelotons were seen in the 26 root sections examined in TEM, they displayed fungal hyphae with septal pores and cell walls proper to the same *rhizoctonia* clades that those identified using molecular sampling (Fig. S1, Supporting information), except for two root sections showing Tulasnellaceae that never amplified by PCRs. Other basidiomycetes and ascomycetes were not seen in TEM. Therefore, although we could not totally exclude that some other fungal taxa formed mycorrhizae with the Reunion orchids, we restricted this network analysis to the three *rhizoctonia* taxa.

Architecture of orchid–rhizoctonia tropical networks

The nestedness in the overall association matrix *S* (95 fungal OTUs × 73 orchid species; 210 binary links) was not significant (NODF = 4.512, $P > 0.2$; same results for the *quasiswap* and *tswap* null models). Conversely, the modularity of the matrix *S* was highly significant ($M = 0.715$, $P < 0.01$; Fig. 3). The clusters displaying the largest modularity mostly included either epiphytic or terrestrial orchids or fungi, and the modularity associated with the epiphytic-terrestrial position, $M_{epi} = 0.312$, was indeed significant (randomization of the epiphytic-terrestrial labels, $P < 0.01$). We found distinct guilds of *rhizoctonia* OTUs associated with epiphytic and terrestrial orchids, because only 10 of the 95 OTUs were shared between the epiphytic and terrestrial subnetworks. We thus analysed separately the nestedness of the epiphytic and terrestrial subnetworks. We found the network of epiphytic orchids to be significantly nested (NODF = 5.61, $P < 0.01$), while the network of terrestrial orchids was not (NODF = 10.37, $P > 0.05$). Moreover, the modularity associated with the preferred habitat type was not different from random (randomization of the habitat labels, $P = 0.99$) in the overall network ($M_{hab} = -0.08$), as well as in the two subnetworks ($M_{hab} = -0.14$ for epiphytic and −0.13 for terrestrial subnetworks). Therefore, despite the diversity of forest types, the epiphytic or terrestrial position was the main ecological factor structuring the overall association matrix. When we alternatively delineated the OTUs at the 95% similarity threshold, the resulting matrix *S* (86 *rhizoctonia* OTUs × 73 orchid species; 207 binary links) revealed exactly the same architectural properties (Nestedness: NODF = 5.448, $P > 0.2$; Modularity: $M = 0.675$, $P < 0.01$), so did the subnetworks (data not shown).

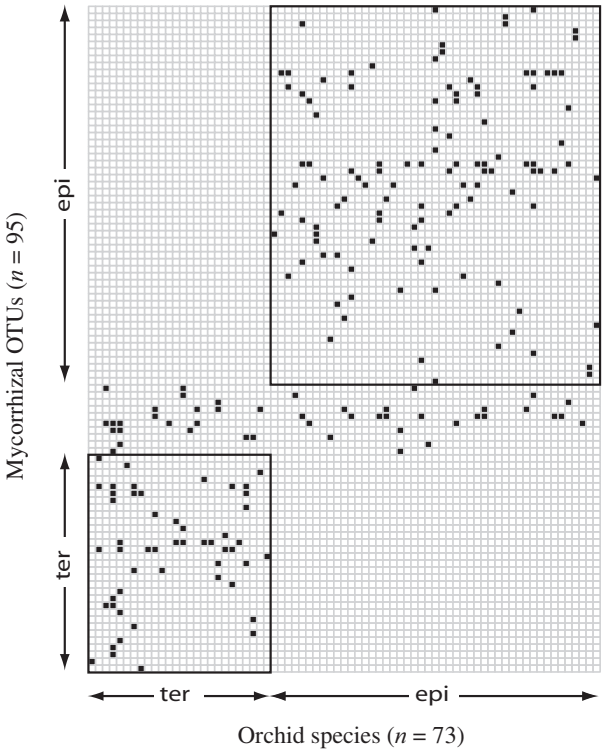

Fig. 3 Modularity of the association matrix *S* (210 binary links) relating 73 orchid species (on columns) and 95 *rhizoctonia* OTUs (on rows). The clusters displaying the largest modularity of *S* mostly include either epiphytic or terrestrial orchid–fungus interactions.

Phylogenetic bipartite signal of orchid–rhizoctonia tropical networks

We first analysed the phylogenetic signal of the angraecoid–*rhizoctonia* subnetwork. It proved weak for fungi ($d_f = 0.01$), its confidence interval including zero (95% confidence interval, [0–0.10]), while it was significantly stronger for orchids ($d_p = 0.27$; [0.19–0.61]; Fig. 4a). For instance, the subclade *Aerangidinae* regrouping the species *Cryptopus elatus*, *Oeonia rosea*, *Beclardia macrostachya*, and *Beclardia* sp. TP84 was mainly linked to Ceratobasidiaceae, and a clade encompassing *Jumellea* and *Aeranthes* spp. was linked to a same Sebacinales OTU (Fig. 4a). The strength of the overall phylogenetic signal ($MSE_d = 0.24$) was similar to that of a star phylogeny ($MSE_s = 0.24$) and lower than that of the maximal

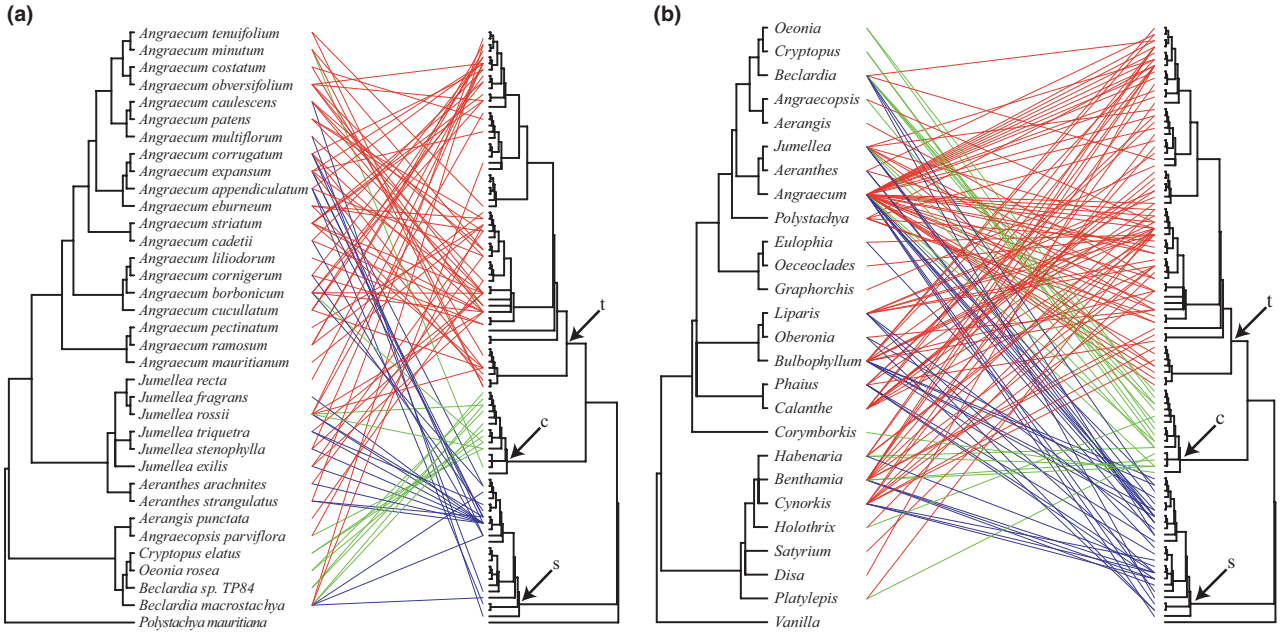

Fig. 4 Orchid against fungal phylogenies showing mycorrhizal links between 95 *rhizoctonia* OTUs and 34 epiphytic angraecoid species (a, 110 binary links) or 25 orchid genera (b, 210 binary links). Mycorrhizal links with Tulasnellaceae (t), Ceratobasidiaceae (c) and Sebacinales (s) clades are shown in red, green, or blue, respectively.

inertia (MSE_b = 0.38). Therefore, phylogenetic relationships among angraecoid species imposed some structure on the association matrix, but the phylogenetic relationships among fungi did not, so that the overall phylogenetic signal of the association matrix still remained weak.

We further analysed the phylogenetic signal of the overall association matrix at broader phylogenetic resolution, that is, the 25 orchid genera. In this case, the phylogenetic signal was small and not significantly different from zero for orchids (d_p = 0.12; [0–0.23]) and fungi (d_f = 0.07; [0–0.16]; Fig. 4b). Furthermore, the model fitted using actual phylogenetic data (MSE_d = 0.54) was smaller but did not strongly depart from a star phylogeny (no phylogenetic structure, MSE_s = 0.57) and was far from the maximal inertia (MSE_b = 1.06). At this resolution, there was no clear phylogenetic structure in the overall network. We finally analysed the phylogenetic signal of the epiphytic and terrestrial subnetworks separately. The terrestrial subnetwork did not reveal any phylogenetic structure for both partners (d_p = 0.03, [0–0.16]; d_f = 0.03, [0–0.13]), whereas the signal was higher and significant for both partners in the epiphytic subnetwork (d_p = 0.18, with confidence interval [0.12–0.25]; d_f = 0.24, [0.06–0.32]). Furthermore, for the terrestrial subnetwork, MSE_d was smaller but close to that of a star phylogeny (MSE_d = 0.83, MSE_s = 0.85, and MSE_b = 2.05), whereas it was slightly higher for epiphytes (MSE_d = 1.25, MSE_s = 1.12, and MSE_b = 1.44 for epiphytes). Therefore, a stronger phylogenetic signal was found for associations in the epiphytic subnetwork.

Discussion

We addressed the ecological and evolutionary constraints on orchid mycorrhizal symbioses, in the tropical context where the highest heterogeneity in orchid habitat is expected, using the communities of indigenous orchids of Reunion Islands as a model. We built the largest association matrix of orchid symbioses available to date, which included 360 observations corresponding to 210 binary links between 73 orchid species and 95 *rhizoctonia* OTUs across the gradient of habitats in the study area. This study reached an original view of the main ecological and evolutionary constraints on tropical orchid–*rhizoctonia* associations, especially providing the first evidence for the role of epiphytism in the structure of orchid mycorrhizal symbioses.

Mycorrhizal diversity and specificity of tropical orchids

The diversity of fungal symbionts and, more importantly, the low mycorrhizal specificity were both noticeable in the tropical orchids of Reunion Island. The three *rhizoctonia* clades, and mostly the Tulasnellaceae, were dominant fungal symbionts. Tulasnellaceae symbionts had often been identified in terrestrial orchids world-

wide (McCormick *et al.* 2004; Jacquemyn *et al.* 2010; Waterman *et al.* 2011; Yuan *et al.* 2010), as well as in some Neotropical epiphytic orchids (Suárez *et al.* 2006). We suggest that it may be the major fungal lineage involved in orchid mycorrhizal symbioses, although associations with other Basidio- or Ascomycetes may be more restrictively found (Dearnaley *et al.* 2012). This further emphasizes the need to investigate the ecological diversity of Tulasnellaceae – for example, based on phylogenetic reconstruction using available sequences of orchid symbionts, associates of other plant families and environmental records – which is poorly understood in comparison with other *rhizoctonias* (Weiß *et al.* 2011; Yagame *et al.* 2012).

Mycorrhizal specificity has been a central issue in the research on orchid mycorrhizal ecology, given that specific fungi may be required for promoting seed germination and plant growth for conservation purposes (Cribb *et al.* 2003; Dearnaley *et al.* 2012). Orchid species were shown to range from specialists to generalists (McCormick *et al.* 2004; Otero *et al.* 2004, 2007; Martos *et al.* 2009; Jacquemyn *et al.* 2010), although some mycorrhizal preference may better characterize this symbiosis. Most species here showed more than one mycorrhizal associate, which could co-occur within a single individual or even within a single root section. And, although the majority of individuals hosted several Tulasnellaceae, multiple associations with more than one *rhizoctonia* clade were possible. It is also noteworthy that these multiple associations seen by molecular sampling were confirmed by TEM evidence. We did not detect any repulsion among the *rhizoctonia* clades during colonization of orchid roots: the co-occurrence of *rhizoctonia* taxa in roots did not depart from random expectation (data not shown). Therefore, these results suggest that low specificity may be more prevalent in Reunion orchids, whatever their terrestrial or epiphytic ecology. Moreover, the trend of low specificity in this network is consistent with some recent observations, like in the mycorrhizal network of temperate *Orchis* spp. (Jacquemyn *et al.* 2010, 2011). The identification method may be an important issue; indeed, the recent studies that used thorough molecular sampling (see Suárez *et al.* 2006, 2008; and Kottke *et al.* 2009; for neotropical orchids; Jacquemyn *et al.* 2010, 2011 for temperate orchids; this study), but not *in vitro* isolation or partial molecular sampling, concluded on low specificity.

Epiphytic and terrestrial compartments of orchid–fungus interactions

In the only network approach on orchid mycorrhizal symbioses available to date, Jacquemyn *et al.* (2010, 2011) showed that the mycorrhizal associations of 16 *Orchis* spp. were highly nested, and no module could be detected. More recently, Montesinos-Navarro *et al.* (2012) and Chagnon *et al.* (2012) reported a similar pattern of nestedness in the widespread arbuscular mycorrhizae (Smith & Read 2008), and some level of modularity could be detected by Chagnon *et al.* (2012) because of a few cases of reciprocal specialization between some plant and fungal species. The nested architecture of mycorrhizal networks indicates that specialist plant species associate with mycorrhizal fungi that form well-defined subsets of the mycorrhizal fungi with which generalist plant species associate (Bascompte *et al.* 2003; Bascompte & Jordano 2007). In other words, more specialist plant species associate with more generalist mycorrhizal fungi, and such an architecture well characterizing positive interactions may contribute to the persistence of specialists and to the overall stability of the network (Bascompte *et al.* 2006; Thébault & Fontaine 2010; Fontaine *et al.* 2011).

Contrastingly, the mycorrhizal network of Reunion orchids challenges this view by displaying a much higher level of modularity. This alternative pattern had usually been observed in antagonistic interactions, such as trophic interactions (Thébault & Fontaine 2010; see Fontaine *et al.* 2011 for a review), where it emerges because of high reciprocal specialization between the partners contrary to mutualisms and which might be driven by the co evolutionary arm race between defences and counter defences (Thompson 2005). However, the high modularity of our network rather relates to the contrast between coexistent epiphytic and terrestrial guilds, so that it reflects the strong ecological isolation of orchid and fungal partners between these two contrasting niches although in the same forest habitats (Newman 2006). Furthermore, although forest habitats widely vary along the altitudinal gradient in Reunion Island, they fail to explain the modularity of the overall network. Therefore, the epiphytic-terrestrial contrast appears to be the key ecological constraint on these tropical orchid mycorrhizal symbioses.

Analysing separately the epiphytic and terrestrial subnetworks further highlighted differences in the network organization. Interestingly enough, the epiphytic subnetwork was significantly nested, whereas the terrestrial subnetwork was not. The matrix of terrestrial associations may be still not large enough to detect significant nestedness. For the interpretation, this means that assemblages in soil are more random than on the tree bark on the present data set. As a hypothesis, epiphytic niches may have favoured cooperation between orchids and fungi, that is, positive interactions, perhaps more than in soil niches. Epiphytes have indeed adapted to grow in extreme abiotic conditions—such as

water storage, long drought period, high irradiation, low nutrient availability (Benzing 2008; Laube & Zotz 2003; Zotz & Hietz 2011)—where mycorrhizal symbioses that allow water and nutrient sharing (Smith & Read 2008) may have been a major selective pressure towards facilitation between orchids and fungi (see Tirado & Pugnaire 2005 for other plant interactions). Furthermore, epiphytic orchids may have a greater photosynthetic activity as a result of less shaded habitats than forest floor orchids, and as such may supply their fungal partners with more carbon in return for nutrients. Together with the likelihood of stronger nutrient limitation for epiphytic than soil-rooted orchids, it may increase the frequency of truly mutualistic mycorrhizal interactions. Conversely, the moderate stress in ground niches, especially in tropical rainforests, may have favoured emergence of antagonisms, such as competition (Grime 1977). We suggest that the environmental stress characterizing the epiphytic niches may explain both the finding of different guilds of mycorrhizal fungi and the higher nestedness in the epiphytic subnetwork. Comparing the cooperativity between plants and mycorrhizal fungi in terrestrial *versus* epiphytic models shall deserve further research in orchids and other plant families (Kessler *et al.* 2010).

Evolutionary constraints on mycorrhizal symbioses of tropical orchids

In our analysis of phylogenetic signal at narrow resolution, that is, from the phylogeny of 34 angraecoid orchid species, we detected an asymmetry of evolutionary constraint between orchids and fungi. More precisely, the phylogenetic signal was significantly stronger for orchids than for fungi. Such asymmetry in phylogenetic constraint indicates that closely related orchids tend to associate with the same or with closely related fungi, whereas fungi are much less constrained on their host choice. The strength of phylogenetic signal in the orchid phylogeny is consistent with the results of Vazquez *et al.* (2009b) on plant-pollinator networks, but it is not as strong as the signal of the mycorrhizal symbioses in the phylogeny of *Orchis* spp. (Jacquemyn *et al.* 2011). Such difference can be explained by the choice we made to include the three *rhizoctonia* clades in our phylogeny-based analysis, whereas Jacquemyn *et al.* (2011) restricted their analysis to the more frequent clade of mycorrhizal fungi, the Tulasnellaceae precisely. Both these network analyses corroborate the presence of some degree of phylogenetic conservatism of fungal partners, which had been previously seen by mapping them onto phylogenies of various orchid genera (Shefferson *et al.* 2007, 2010; Roche *et al.* 2010; Waterman *et al.* 2011). The fact that orchids are evolutionary constrained on their choice of fungal partners suggests the presence of some heritable traits in these plants, which may control range of suitable partners. But, it may also suggest a trend for closely related orchids to be ecologically similar, that is, to retain to some extent ancestors' ecological niche (Losos 2008); hence, they may meet the same guilds of mycorrhizal fungi.

In our analysis of phylogenetic signal at broader resolution, that is, from the phylogeny of 25 orchid genera, a noteworthy result was the significant signal in the fungal phylogeny in the epiphytic subnetwork only, which would indicate that the fungi retained ancient associations with orchids but became more labile with the descendants on shorter evolutionary resolutions (see previous analysis showing the absence of signal in the phylogeny of fungal partners of angraecoid species). Phylogenetic resolution is an important issue for detecting the phylogenetic signal and discussing the evolutionary constraint on interaction networks. The relative values of phylogenetic signal, as measured by d_f and d_p, are indeed dependent on the rules used to delineate the fungal OTUs on one hand and on the taxonomic resolution retained for both partners on the other hand. Although phylogenetic niche conservatism has often been thought to be a common evolutionary trend and then assumed in several studies (see Losos 2008 for a review), it may be related to evolutionarily heterogeneous processes, with varying spatial and temporal scales (Mouquet *et al.* 2012). Specifically, the angraecoid orchid species have recently diversified in the young Reunion Island, where speciation has mainly occurred towards shifts of habitats along the altitudinal gradient (Micheneau *et al.* 2008). Fungi may have retained more ancient association traits with epiphytic orchids, before this radiation, which would explain the absence of phylogenetic signal with the angraecoid species but the stronger phylogenetic constraint with epiphytic genera.

Finally, we again found a discrepancy between the epiphytic and terrestrial subnetworks when considering the respective phylogenetic structure, which suggested that differential evolutionary processes might have driven the mycorrhizal symbioses in epiphytic and terrestrial niches. The stronger signal was found for epiphytic orchid genera: we hypothesize that they could have been more evolutionary constrained on their partner choice than terrestrial genera have, as a result of extreme environmental constraints imposed by life in epiphytic niches. It is likely that *rhizoctonia* species, on their side, secondarily evolved ability to colonize the epiphytic niche: these fungal species, more recently than the terrestrial ones, would have engaged a closer relationship with orchids to achieve this niche shift towards harder life conditions. Conversely, the more favourable terrestrial niches could have favoured more

labile associations for both orchids and fungi. From this and other observations, we feel that a comparative analysis of the mutualism level in terrestrial *versus* epiphytic orchids' mycorrhizae deserves further studies.

Conclusions and perspectives

This article first provided support for the imprint of some ecological constraints on the orchid mycorrhizal symbioses, in particular, the role of the epiphytic/terrestrial contrast in tropical communities. We therefore call for further researches that shall focus on the variation in mycorrhizal diversity, specificity, and functioning between epiphytic and terrestrial plants (Alexander & Selosse 2009), in orchids and in other taxonomic groups, such as the bromeliads, bryophytes, ferns (Kessler *et al*. 2010), to better understand the organization of mycorrhizal networks in rainforests and to understand the possible divergence in their characteristics. This article also corroborates some previous results revealing the imprint of evolutionary constraints on the orchid mycorrhizal symbioses, especially the constraint imposed by the plant phylogeny, which may result from a higher dependence for plants upon the symbiosis hence a higher specialization. Further researches shall also focus on the link between dependence, specialization, and phylogenetic constraint in interaction networks.

Acknowledgements

The authors warmly thank R. Guimerà and C. Micheneau who kindly provided the program *Netcarto* and a consensus tree of the African angraecoid orchids, respectively; M.P. Dubois for assistance during the molecular analyses. We thank three anonymous referees and Dirk Redecker for useful comments on this manuscript. We also thank the *Parc National de La Réunion* for authorizing access to protected areas of Reunion Island. F. Martos was funded by the *Région Réunion*, and M.A. Selosse by the *Centre National de la Recherche Scientifique* and the *Société Française d'Orchidophilie*.

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This study was part of the Ph.D. thesis of F.M. on the mycorrhizal ecology of tropical orchids. It was designed by M.-A.S. and T.P. in the framework of their research on plant mycorrhizal symbioses and ecology of tropical orchids, respectively. F.M. carried out the molecular analyses, with the help of C.G., and analysed the data. I.K. performed the TEM observations. F.M. supervised the network analyses. F.M., F.M., and M.-A.S. wrote this article.

Data accessibility

ITS or ITS-28S DNA sequences: GenBank accessions JF690991–JF691350 and JF691359–JF691537. Accessions JF691304–JF691308 were isolated from four orchid individuals (fm174-fm177) of the Asian species *Cymbidium aloifolium*, which were found to naturalize in some Reunion forests: they were not considered in our analysis on mycorrhizal networks of indigenous orchids.

Phylogenetic data: TreeBASE Study Accession no. S12721.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Orchid species and individuals sampled from 34 forest sites across Reunion Island, and their root-associated fungi.

Fig. S1 Ultrastructural features seen in transmission electron microscopy allowing identification of the main mycorrhizal fungi, that is Tulasnellaceae, Sebacinales and Ceratobasidiaceae.

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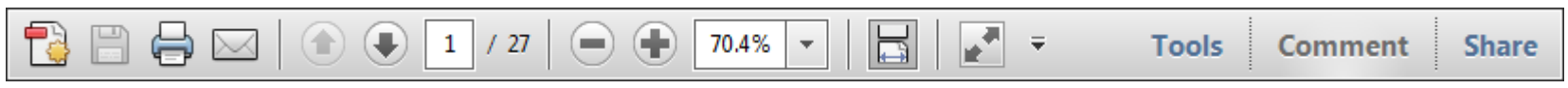

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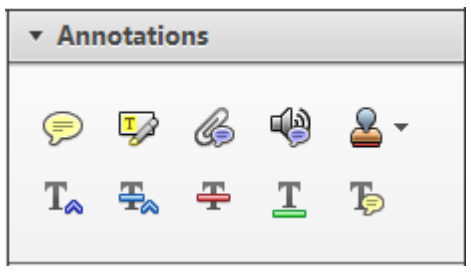

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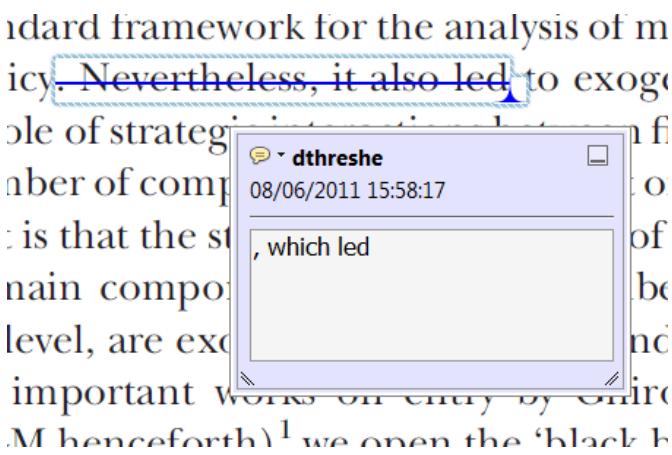
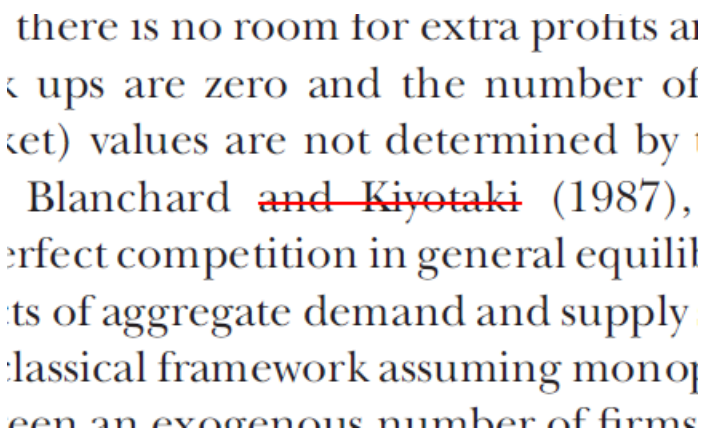


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

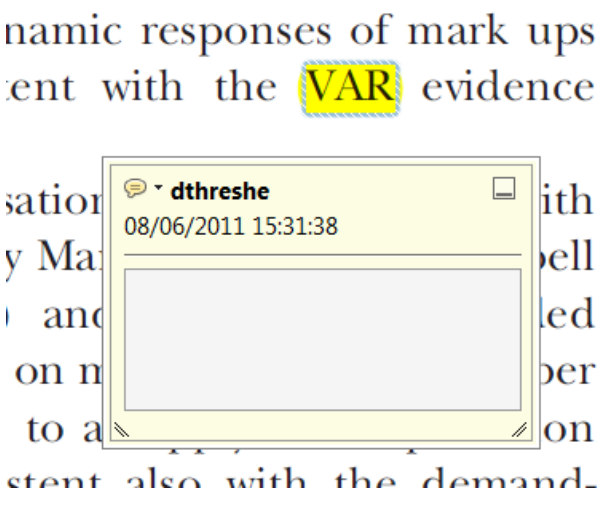


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

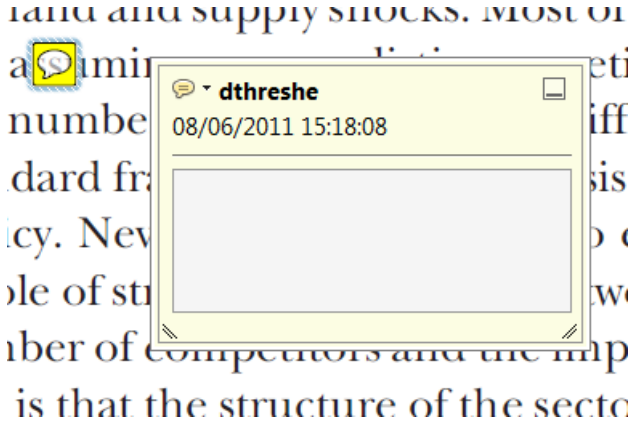


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



WILEY-BLACKWELL

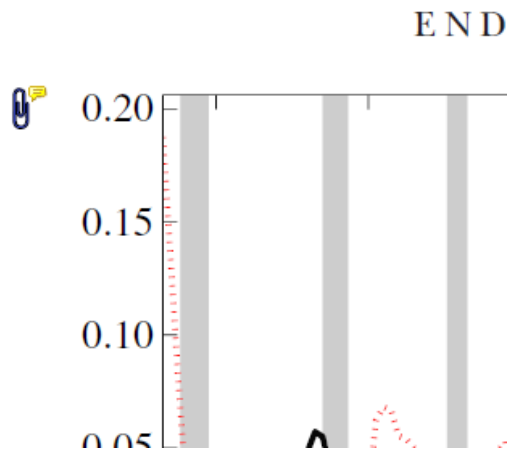
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

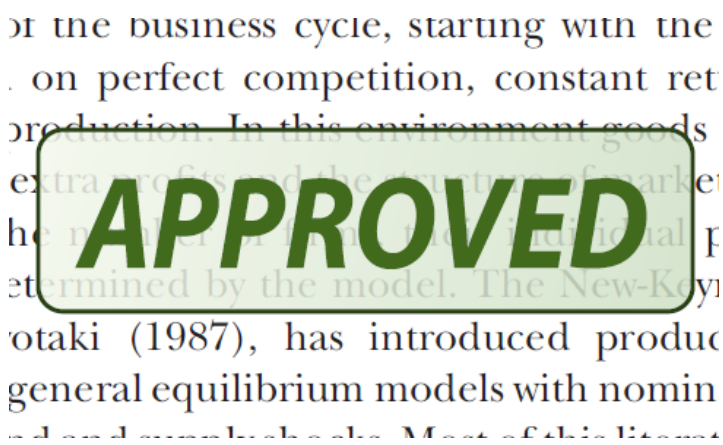


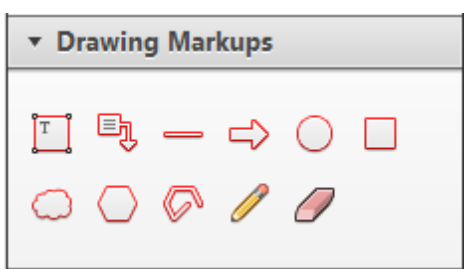
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

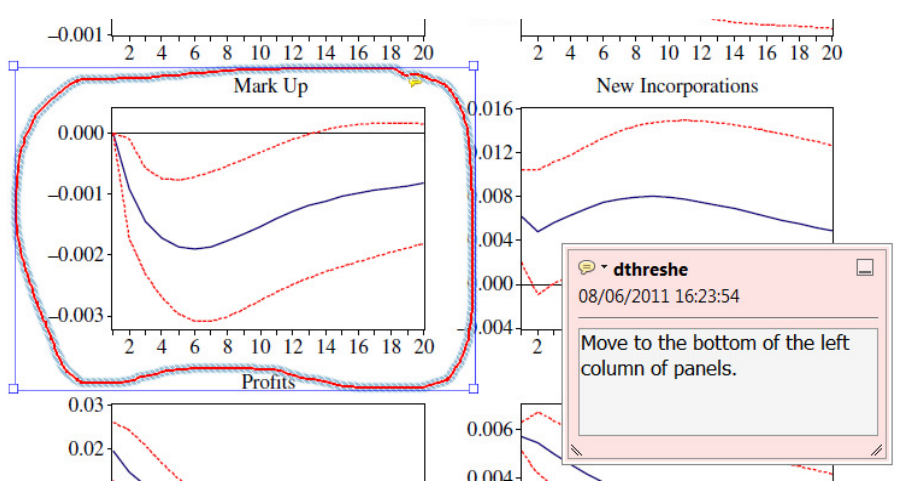
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: