Breakdown and delayed cospeciation in the arbuscular mycorrhizal mutualism

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The ancient arbuscular mycorrhizal association between the vast majority of plants and the fungal phylum Glomeromycota is a dominant nutritional mutualism worldwide. In the mycorrhizal mutualism, plants exchange photosynthesized carbohydrates for mineral nutrients acquired by fungi from the soil. This widespread cooperative arrangement is broken by ‘cheater’ plant species that lack the ability to photosynthesize and thus become dependent upon three-partite linkages (cheater–fungus–photosynthetic plant). Using the first fine-level coevolutionary analysis of mycorrhizas, we show that extreme fidelity towards fungi has led cheater plants to lengthy evolutionary codiversification. Remarkably, the plants’ evolutionary history closely mirrors that of their considerably older mycorrhizal fungi. This demonstrates that one of the most diffuse mutualistic networks is vulnerable to the emergence, persistence and speciation of highly specific cheaters.

Keywords: arbuscular mycorrhizal mutualism; myco-heterotrophy; parasitism; symbiosis

1. INTRODUCTION

Cooperation between species is one of the central problems in biology. Theoretically, it is widely assumed that mutualism is prone to break down into parasitism (Trivers 1971; Axelrod & Hamilton 1981; Doebeli & Knowlton 1998; Herre *et al.* 1999; Morris *et al.* 2003; Ferrière *et al.* 2007). However, mutualisms are ubiquitous in nature and there appear to be surprisingly few examples of breakdown, that is, there are few parasitic taxa nested within mutualistic clades (Sachs & Simms 2006).

Empirically, the overwhelming majority of information about shifts from mutualism to parasitism originates from two specialized reproductive mutualisms (i.e. yucca–yucca moth and fig–fig wasp). In both of these mutualisms the interaction between the partners is obligate: the insect oviposits into the ovaries of the plant and pollinates the flower. The insect larvae then feed on a subset of the developing seeds. A shift from mutualism to parasitism by one partner would lead to an interruption in the life cycle of the other partner and eventually to the extinction of both lineages. Yet there are yucca moths and fig wasps that lay their eggs into flowers without pollinating them. These parasites evolved when their ancestors colonized a novel plant host that already had a pollinator. In these plant–insect model systems, the critical barrier to mutualism breakdown and to the evolutionary stability of the resulting parasitic species is thus thought to be a difficult requirement for three-species coexistence: a parasitic insect specialized on a mutualistic plant specialized on a mutualistic insect (Pellmyr & Leebens-Mack 1999, 2000). In fact, there are only two parasitic species emanating from the yucca–yucca moth mutualism (Althoff *et al.* 2006) and a single one from the species-rich fig–fig wasp mutualism

(Machado *et al.* 2001). The question of whether or not barriers to cheating are lowered in diffuse mutualisms, where multi-species coexistence is the rule, has received scant attention (Thompson 2005).

The most widespread diffuse mutualism is the mycorrhizal association between plants and fungi (Smith & Read 1997). In this mutualism, the plants exchange carbon for fungal nitrogen and phosphorus. Mycorrhizal plants can obtain approximately 75% of their nitrogen in exchange for approximately 15% of their carbon, and conversely, mycorrhizal fungi can obtain 100% of their C in exchange for approximately 40% of their N (Hobbie & Hobbie 2006). Sustaining this costly exchange is vital as it allows both partners to establish, grow and complete their life cycles. Mycorrhizas are diffuse symbioses because a mycorrhizal plant typically associates simultaneously with multiple fungi and a mycorrhizal fungus often associates simultaneously with multiple plants (Giovannetti *et al.* 2004; Lian *et al.* 2006).

Mycorrhizal promiscuity provides the potential for nutritional cheating when physiological continuity is established between a photosynthetic plant, its mycorrhizal fungi and a non-photosynthetic plant. The latter is referred to as a myco-heterotrophic (Leake 1994) epiparasite (Romell 1939; Björkman 1960). Myco-heterotrophy is an evolutionarily and ecologically widespread phenomenon that can provide escape from competitive exclusion in the shaded conditions of forest understorey habitats (Bidartondo *et al.* 2004); indeed, over 400 non-photosynthetic mycorrhizal plants have evolved within ten mycorrhizal plant families (Leake 1994).

Evolutionary trajectories have yet to be analysed in the cheaters of arbuscular mycorrhizas. In the relatively more specialized ectomycorrhizas, the ability to obtain carbon from neighbouring photosynthetic plants has led cheater plants to loose evolutionary codiversification (Bidartondo

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et al. 2004). Here we test whether evolutionary codiversification is tighter when plants cheat the more diffuse, much older and more pervasive arbuscular mycorrhizal mutualism. To accomplish this, we identified the mycorrhizal fungi in myco-heterotrophic plant species of Burmanniaceae and Triuridaceae from three regions in Cameroon. For five closely related *Afrothismia* species (Burmanniaceae), we compared a multi-gene plant phylogeny with a multi-gene phylogeny of their associated fungi. We used penalized likelihood (PL; Sanderson 2002) and Thorne & Kishino's (2002) method to estimate the divergence times in both *Afrothismia* and its arbuscular mycorrhizal symbionts. Finally, we propose a model for the evolution of non-photosynthetic mycorrhizal plants which explains the delayed cospeciation pattern observed in *Afrothismia*.

2. MATERIAL AND METHODS

(a) Taxon sampling

The Burmanniaceae family has evolved non-photosynthetic lineages from within photosynthetic lineages at least five times (Merckx *et al.* 2006). Within the Burmanniaceae, the non-photosynthetic tribe Thismieae (approx. 40 species) represents one of those events and includes the genus *Afrothismia*. Populations of five *Afrothismia* species (electronic supplementary material, figure 1) and three co-occurring myco-heterotrophic plant species were sampled from three different regions in Southwest Cameroon: Mount Kupe, Diongo (western foothills of Mount Cameroon) and Korup National Park. These three regions are approximately 100 km apart from each other. *Afrothismia hydra*, *Afrothismia winkleri* and *Afrothismia foertheriana* plants were collected from two distinct locations approximately 1–2 km apart within each region. Sampled species, voucher information and sampling localities are listed in the electronic supplementary material, table 1. We extracted genomic DNA from 3 to 12 individual approximately 2 mm long sections of roots or whole enlarged bases of roots from each plant using methods described elsewhere (Gardes & Bruns 1993) with a purification step using GeneClean (QBiogene). We then amplified (Pico-Maxx, Stratagene or JumpStart, Sigma), cloned (TOPO TA, Invitrogen) and sequenced (BigDye v. 3 on ABI3730 Genetic Analyzer, Applied Biosystems) the fungal 18S ribosomal RNA gene from NS1/ITS4 amplicons (White *et al.* 1990) for all plants, part of the fungal translation elongation factor 1 α gene (*EF1 α*) from EF1-526f/EF-1567r amplicons (S. Rehner 2001, unpublished data) for *Afrothismia*, and part of a homologue of the fungal *TOR2* gene from GmTOR-1071f/GmTOR-1638r amplicons (Stuckenberg & Rosendahl 2005) for *Afrothismia*. With the exception of *TOR2*, we used nearly universal eukaryotic primers; nonetheless, the only non-*Glomus* organism we detected was an unidentified stramenopile (*EF1 α* from *A. foertheriana*). Stramenopiles do not have any mycorrhizal lineages and this is probably a pathogen. For *Glomus*, we examined variation in rDNA by DNA sequencing at least four clones from each plant and in non-rDNA two to eight clones from each plant. From plants, we amplified 18S rDNA with primers NS1, NS2, NS3, NS4, NS5 and NS8 (White *et al.* 1990), ITS with primers ITS1 and ITS4 (White *et al.* 1990), and *atpA* with primers ATPA-F1 and ATPA-B1 (Eyre-Walker & Gaut 1997). All PCR products were purified and sequenced on an ABI310 Genetic Analyzer (Applied Biosystems).

(b) Datasets

To test for mycorrhizal specificity and for subsequent molecular dating analyses, a large 18S rDNA (109 taxa; 1721 bp) was compiled with all our fungal symbiont DNA sequences obtained from the roots of *Afrothismia*, *Burmannia*, *Kupezia*, *Sciaphila* and *Tacca* as well as representative Glomeromycota 18S rDNA accessions from GenBank. Accessions of Ascomycota, Basidiomycota and Zygomycota were included for out-groups (table 4 in the electronic supplementary material).

A three-gene dataset was constructed for all fungi that were associated with *Afrothismia*, including *Glomus intraradices*, with *Glomus mosseae* as out-group. After excluding all ambiguously aligned positions, we obtained a total matrix of 2748 bp (18S rDNA: 1691 bp, *EF1 α* : 464 bp, *TOR2*: 593 bp; see table 2 in the electronic supplementary material for GenBank accessions). In parallel, a three-gene dataset of *Afrothismia* was compiled with *Thismia panamensis* as out-group. Once combined, we obtained a dataset of 3113 bp (18S rDNA: 1566 bp, ITS: 501 bp, *atpA*: 1046 bp; see table 3 in the electronic supplementary material for GenBank accessions).

To allow inclusion of multiple calibration points for molecular dating analyses, the *Afrothismia* 18S rDNA and *atpA* data were combined with DNA sequences of all monocot orders and basal angiosperm groups with *Amborella trichopoda* as an out-group (table 5 in the electronic supplementary material). This resulted in a matrix of 2888 bp (18S rDNA: 1666 bp and *atpA*: 1222 bp). Individual alignments of all datasets were manually created with MACCLADE v. 4.06 (Maddison & Maddison 2001).

(c) Phylogenetic reconstruction

Both maximum parsimony (MP) and Bayesian (BI) analyses were performed with each of the above-mentioned datasets. MP analyses with PAUP* v. 4.0b10 (Swofford 2002) involved heuristic searches with 1000 replicates, tree bisection reconnection branch swapping and holding 100 trees in memory with the multrees option in effect. Clade support was assessed via non-parametric bootstrapping using 1000 replicates and the same search strategies. BI analyses were performed with the GTR+I+G model of DNA evolution, identified as the best-fitting model for each gene fragment under the Akaike information criterion implemented in MODELTEST v. 3.06 (Posada & Crandall 1998). BI analyses were performed with MRBAYES v. 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Multi-gene datasets were analysed under a mixed model approach partitioned over the different gene fragments. Each BI analysis consisted of two parallel Markov Chain Monte Carlo (MCMC) runs of four chains performed with a length of five million generations, a burn-in corresponding to the first 1.25 million (25%) and a sample frequency of one tree for every 1000 generations. In each analysis, the potential scale reduction factors for all model parameters (approx. 1.0) and the split frequency standard deviations (less than 0.01) confirmed convergence of runs. Convergence of the chains was also checked using TRACER v. 1.4 (Rambaut & Drummond 2007). For all analyses the effective sampling size of each parameter was found to exceed 100, suggesting acceptable mixing and sufficient sampling. During phylogenetic analyses of the 18S rDNA+*atpA* monocot dataset, the relationships between the orders were constrained to a seven-gene monocot topology (Chase 2004). All other datasets were analysed without constraints.

Figure 1. *Glomus* group A and B clades from the Bayesian phylogenetic analysis on 18S rDNA sequences of Glomeromycota (see figure 2 in the electronic supplementary material for the phylogeny with all taxa). Bayesian posterior probabilities of main clades are shown above branches, and non-parametric bootstrap percentages are placed below branches. The inset map shows the sites where collections were made. A *Glomus* group A symbiont 18S rDNA sequence from the roots of a *Tacca* species, the nearest extant photosynthetic relative of *Afrothsmia*, is found in a clade with 18S rDNA sequences from *Sciaphila ledermannii* fungal symbionts.

(d) Diversification time estimates

For both Glomeromycota and monocot datasets, a χ^2 likelihood ratio test rejected the hypothesis of evolution according to a strict molecular clock (for Glomeromycota $\chi^2_{109} = 276.22$; $p = 3.12 \times 10^{-16}$; for monocots $\chi^2_{135} = 2032.48$; $p = 5.15 \times 10^{-287}$). Therefore, we used two different relaxed molecular clock methods: PL as implemented in r8s (Sanderson 2002, 2003) and Thorne & Kishino's (TK) method (Thorne & Kishino 2002) with multidivtime (<http://statgen.ncsu.edu/thorne/multidivtime.html>). With the program ESTBRANCHES DNA, we calculated substitution branch lengths for each gene fragment using the F84+G model with parameters estimated using PAML (Yang 1997) and the 50% majority-rule topology from the Bayesian analysis (Rutschmann 2005). For each analysis, a single MCMC chain was run for 1.1 million generations with a burn-in of 0.1 million generations and a sampling frequency of one per 100 generations. To check for convergence of the MCMC chain, we ran analyses at least twice

from different starting points. For the PL analyses, we used the 50% majority rule BI tree with branch lengths as input for r8s. Analyses were run with a Truncated-Newton optimization algorithm and optimal rate-smoothing parameter determined by the statistical cross-validation method in r8s ($1.6e^7$ for Glomeromycota, 10.0 for monocots). We calculated credibility intervals and standard deviations for the PL age estimations of the main nodes by repeating the PL analysis on 1000 trees randomly sampled from the trees sampled during the BI analysis (Roelants *et al.* 2007). Details about priors and calibration points are given in the electronic supplementary material.

3. RESULTS

(a) Mycorrhizal specificity

Bayesian and parsimony phylogenetic analysis of Glomeromycota 18S rDNA produced highly congruent estimates of phylogenetic relationships (figure 1; figure 2 in the

Figure 2. Comparison of multi-gene phylogenies for *Afrothismia* plants (right) and *Glomus* fungi (left) shows evidence of delayed cospeciation by the plants. *Afrothismia* species are extreme specialists that have tracked ancient fungal lineages over considerable evolutionary time. Thus, the plants' evolutionary history closely mirrors that of mycorrhizal fungi. Each photograph shows a flower of the plant species named above. Bayesian posterior probabilities (more than 95%) are shown above branches and non-parametric bootstrap percentages (more than 85%) are shown below branches. Branch lengths represent the number of substitutions per site. Divergence time estimates are indicated in boxes.

electronic supplementary material). The topology is well resolved and shows high congruence with previous 18s rDNA-based hypotheses for Glomeromycota (Schwarzott *et al.* 2001). All fungal symbionts are placed within a maximally supported *Glomus* group A lineage and there is no fungal lineage overlap among the different myco-heterotrophic plant species. The mycorrhizal fungi obtained from the roots of *Sciaphila ledermannii*, *Kupezia martinetugei*, *Burmannia hexaptera*, *Afrothismia gesnerioides*, *A. hydra* and *Afrothismia korupensis* are all placed in strongly supported monophyletic groups. The fungal symbionts of *A. winkleri* and *A. foertheriana* are part of paraphyletic groups due to the inclusion of the *A. hydra* and *A. korupensis* symbiont clades, respectively.

(b) Multi-gene *Afrothismia* and fungal symbiont phylogenies

The three-gene phylogenies of *Afrothismia* and its fungal symbionts are both well resolved and strongly supported (figure 2). No incongruence was observed between parsimony and Bayesian analyses. The three-gene *Glomus* phylogeny shows improved resolution over the single 18s rDNA tree with fungal symbionts of each *Afrothismia* species in strongly supported clades. There is one topological difference between the plant and the fungus phylogenies: *A. foertheriana* is sister to *A. hydra* and *A. winkleri* while its fungal symbionts are sister to those of *A. korupensis*.

(c) Diversification time estimates

The chronograms obtained from the 18s rDNA of the fungi are shown in the electronic supplementary material, figure 3. Using the Heckman *et al.* (2001) interval, the stem node of the Glomeromycota is estimated at 1399 ± 6 (PL) and 1304 ± 44 Mya (TK). The crown node of this

clade is estimated at 1307 ± 30 (PL) and 1223 ± 63 Mya (TK), much older than the oldest known Glomeromycota fossils (Redecker *et al.* 2000). Under the same priors, the split between Ascomycota and Basidiomycota is estimated at 1208 ± 53 Mya (PL) and 1135 ± 92 Mya (TK). This is congruent with prior estimates (Heckman *et al.* 2001; Padovan *et al.* 2005) when using the calibration date of 1576 Mya for the plant–animal–fungus split for this node. In contrast, when applying a 590–600 Mya interval constraint for calibration point I, we inferred an estimate of 600 ± 38 (PL) and 595 ± 32 Mya (TK) for the origin of the Glomeromycota. Table 6 in the electronic supplementary material lists the divergence time estimates obtained for the *Afrothismia* symbionts with both calibration strategies.

The resulting monocot topology based on 18s rDNA and *atpA* data is shown in the electronic supplementary material, figure 4. Our analysis shows a maximally supported placement for *Afrothismia* as sister clade to *Tacca–Thismia–Haplothismia*. The paraphyly of Burmanniaceae tribe Thismieae is unexpected, yet a previous study has indicated that in Burmanniaceae tribe Burmanniaceae many lineages lost their chlorophyll independently leading to convergent morphological evolution (Merckx *et al.* 2006). In general, many myco-heterotrophic plants, despite the very diverse range of families and genera, show remarkable convergence (Leake 1994). The stem and crown node age of the Dioscoreales are estimated at 120 ± 6 Mya (TK) and 139 ± 15 Mya (PL), and 113 ± 8 Mya (TK) and 135 ± 14 Mya (PL), respectively (figure 5 in the electronic supplementary material). These estimates bracket previous estimates for the stem (124 Mya) and the crown node (123 Mya) of Dioscoreales obtained with *rbcL* DNA sequence data (Jansen & Bremer 2004). Our analyses recover an estimate of 91 ± 11 Mya (TK) and

Figure 3. A model for the evolution of non-photosynthetic mycorrhizal plants. (a) A community of generalist mycorrhizal mutualists. Black squares represent mycorrhizal plants in two distant lineages ((a,b),c) and (w,x). Circles represent mycorrhizal fungi in two distant lineages ((1,2),3) and ((9,8),7). Double-ended arrows show mutualistic links. For example, 1 and 2 are sister taxa and their closest relative is 3. All plants must be linked to fungi and all fungi must be linked to plants. Double-ended arrows show mutualistic mycorrhizal links (i.e. plants provide carbon and fungi provide mineral nutrients). (b) A mycorrhizal community where a plant species x (grey) has lost the ability to photosynthesize, so it cannot provide carbon to fungi, breaking down the

120 $\pm$ 11 Mya (PL) for the origin of *Afrothismia* (table 6 in the electronic supplementary material). Diversification of the genus, which should be treated as a minimum age since our sampling is incomplete, started between 50 $\pm$ 13 Mya (TK) and 78 $\pm$ 9 Mya (PL).

(d) Comparison of fungi and plant divergence time estimates

The *Afrothismia* chronogram is comparable with its *Glomus* host chronogram by three nodes (table 6 in the electronic supplementary material). No matter which calibration strategy or relaxed clock method we applied to obtain the divergence estimates, *Glomus* nodes are significantly older than corresponding nodes in *Afrothismia* (figure 6 in the electronic supplementary material).

4. DISCUSSION

All mycorrhizal fungi in the roots of the sampled myco-heterotrophic plants belong to *Glomus* group A, a relatively species-rich group of arbuscular mycorrhizal fungi. This supports previous observations in myco-heterotrophic Burmanniaceae, Triuridaceae, Corsiaceae and Gentianaceae (Bidartondo *et al.* 2002; Franke *et al.* 2006). It is noteworthy that we did not observe any fungal overlap among the different plant species, not even between closely related *Afrothismia* species. Specificity towards narrow groups of arbuscular mycorrhizal fungi has been detected in one species of Corsiaceae in South America (Bidartondo *et al.* 2002) and our data show that African species of Burmanniaceae and Triuridaceae are also associated with extremely narrow subsets of arbuscular mycorrhizal fungi. Parasites are generally more specialized than mutualists (Thompson 2005) and accordingly breakdown of the arbuscular mycorrhizal mutualism has led to extreme specialization towards fungi by myco-heterotrophic plants.

A surprising result emerges when we focus on the specialization towards narrow fungal lineages in *Afrothismia*, where mycorrhizal overlap is lacking even between very closely related species growing in the same regions: the phylogenetic relationships between the *Afrothismia* species closely mirror those of its fungal associates (figure 2). However, the divergence time estimates obtained by us for fungal nodes are significantly older than for their corresponding nodes in the plant phylogeny, thus reflecting our current knowledge about the macroevolutionary diversification of fungi and plants. Fossil evidence shows arbuscular mycorrhizas to be at least as old as the earliest land plants (Remy *et al.* 1994; Redecker *et al.* 2000) and it

mycorrhizal mutualism. Non-photosynthetic plants depend on fungi that link them to photosynthetic plants. One-ended dashed arrows show mycorrhizal links where fungi provide carbon to plants. Non-photosynthetic plants have a reduced mycorrhizal range and only associate with related fungi 1, 2 and 3. (c) Speciation of the non-photosynthetic plant lineage x into x', and x' leads to further specialization on fungal lineages; x' depends on fungus 3 and x'' depends on fungi 1 and 2. The fungi and the photosynthetic plants remain generalist mycorrhizal mutualists. (d) Speciation of plant lineage x'' into y and z. Plant y specializes on fungal host 2 and plant z specializes on closely related fungal host 1. This form of phylogenetic tracking by non-photosynthetic mycorrhizal plants towards pre-existing fungal lineages produces an evolutionary pattern of delayed cospeciation.

has been suggested that colonization of land by plants was dependent upon symbiosis with arbuscular mycorrhizal fungi. The evolutionary barrier to cheating has indeed been lowered exceptionally in the arbuscular mycorrhizal mutualism. Not only have arbuscular mycorrhizal cheaters evolved independently multiple times and diversified as cheaters, but they have also achieved exceptional evolutionary stability (e.g. outliving cheaters in plant-insect mutualisms by 25–48 Mya; Pellmyr & Leebens-Mack 2000; Rønsted *et al.* 2005).

Our observations indicate a cospeciation pattern between *Afrothismia* and its mycorrhizal fungi, but not in a synchronous way as in some host-parasite interactions where speciation of the host triggers speciation in the parasite. Our analyses show that when the photosynthetic ancestor of *Afrothismia* began specializing on particular fungal lineages, initiating or following the shift from mutualism to parasitism, most extant *Glomus* lineages had already diverged. However, once adapted to a group of related arbuscular mycorrhizal fungi, subsequent specialization and a lack of host-switching forced the plants to track the *Glomus* phylogeny, resulting in a delayed cospeciation process (*sensu* Hafner *et al.* 2003; figure 3). Phylogenetic congruence between *Afrothismia* and *Glomus* is not perfect; this incongruence can be explained by host-switching over short phylogenetic distance in the lineage leading to *A. foertheriana*, or by 'rough' phylogenetic tracking where the ancestor of the *A. foertheriana*, *A. hydra*, *A. winkleri* clade was temporarily associated with a paraphyletic *Glomus* lineage.

While this study shows that the arbuscular mycorrhizal mutualism has given rise to coevolving extreme specialist cheaters, the selective forces behind the specialization process remain speculative. The pattern observed cannot be explained by an evolutionary arms race because then we would expect a synchronous, or stepwise, cospeciation process. Unlike ectomycorrhizal cheater plants, which show both narrow host shifting and host jumps to distant fungal lineages (Jackson 2004), the phylogenetic congruence we detected shows a lack of host jumping to distant fungal lineages that is probably due to the effects of interspecific interactions in which fungal detection (e.g. seed germination cues) and/or fungal metabolic usage by plants (e.g. carbon transporters) are species-specific.

5. CONCLUSIONS

The interaction between plants and arbuscular mycorrhizal fungi is probably the most important symbiotic association of terrestrial ecosystems. Its promiscuous character has created opportunities for diverse plant lineages to successfully exploit these mycorrhizal symbioses. The shift from a mutualistic plant to a non-photosynthetic cheater is accompanied by specialization towards narrow groups of arbuscular mycorrhizal fungi. This study shows that extreme specialization and a lack of host-switching in closely related species of *Afrothismia* have led to a pattern of conservative evolutionary tracking hitherto unknown in mycorrhizal symbioses and horizontally transmitted symbioses in general.

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