doi:10.1111/j.1558-5646.2012.01611.x

# EVOLUTIONARY STABILITY IN A 400-MILLION-YEAR-OLD HERITABLE FACULTATIVE MUTUALISM

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Received September 27, 2011
Accepted February 10, 2012

**Many eukaryotes interact with heritable endobacteria to satisfy diverse metabolic needs. Some of these interactions are facultative symbioses, in which one partner is not essential to the other. Facultative symbioses are expected to be transitional stages along an evolutionary trajectory toward obligate relationships. We tested this evolutionary theory prediction in *Ca.* Glomeribacter gigasporarum, nonessential endosymbionts of arbuscular mycorrhizal fungi (Glomeromycota). We found that heritable facultative mutualisms can be both ancient and evolutionarily stable. We detected significant patterns of codivergence between the partners that we would only expect in obligate associations. Using codiverging partner pairs and the fungal fossil record, we established that the Glomeromycota–Glomeribacter symbiosis is at least 400 million years old. Despite clear signs of codivergence, we determined that the Glomeribacter endobacteria engage in recombination and host switching, which display patterns indicating that the association is not evolving toward reciprocal dependence. We postulate that low frequency of recombination in heritable endosymbionts together with host switching stabilize facultative mutualisms over extended evolutionary times.**

**KEY WORDS:** **Arbuscular mycorrhizal fungi, *Ca.* Glomeribacter gigasporarum, cospeciation, endosymbiosis, host switching, recombination.**

Most eukaryotes satisfy their various metabolic needs through interactions with bacteria (Sachs et al. 2011). Evolutionary theory predicts that reciprocal selection and close spatial association of the partners will over time lead to coupling of partner metabolic and reproductive interests through endosymbiont vertical transmission, eventually producing a relationship in which the partners cannot live without each other (Yamamura 1993; Frank 1995; Law and Dieckmann 1998). This prediction implies that countless facultative symbioses with heritable bacteria, in which one of the partners is not essential to the other, represent short-lived transitory evolution stages along the trajectory leading to reciprocal partner dependence (Fig. 1A). Support for the transitory nature of facultative endosymbioses comes from two observations: (1) many obligate endosymbioses, including symbiotic organelles in eukaryotic cells (Margulis 1981) and endobacteria essential to nutrition of insects (Moran et al. 2008), are of great evolutionary age, whereas (2) examples of ancient facultative endosymbioses are few (Baumann 2005; Moran et al. 2008; Degnan et al. 2010). However, evolutionary histories of facultative endosymbioses are exceedingly difficult to reconstruct because phylogenies of the partners are often incongruent with each other (Bright and Bulgheresi 2010). Consequently, the apparent shortage of old facultative endosymbioses may be related to impediments in estimating the evolutionary history and the age of these associations rather than to their ephemeral nature. Our goal was to test the hypothesis of the transitory nature of heritable


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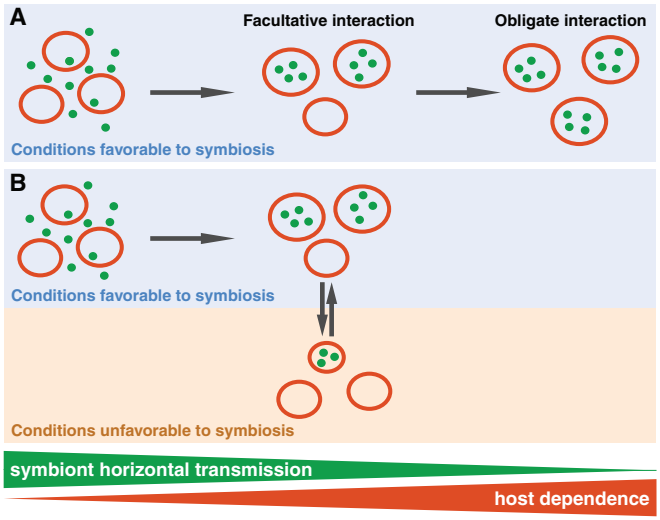


**Figure 1.** **Hypothetical evolutionary trajectories in heritable mutualisms. Hosts are depicted as red ovals; endosymbionts are shown as green dots. Relative host fitness is reflected by the size of ovals. (A) Evolutionary trajectory leading to obligate reciprocal partner dependence. (B) Shifting environmental conditions are expected to arrest an association at the facultative dependence stage. If conditions remain unfavorable for prolonged periods of time, host populations would be expected to completely lose endosymbionts.**

facultative endosymbioses. We identified several aspects of partner life histories and evolution that needed to be unraveled to achieve this goal, including (1) the extent of dependency between the partners, (2) the role of recombination and host switching in the history of the association, and (3) the time of evolutionary origin of the association. (1) The extent of the dependency between the partners defines whether the association is facultative or obligate. It can be uncovered by studying the distribution of symbionts among closely related hosts. (2) Recombination and host switching interfere with progressive coevolution between the partners (Frank 1994; Bright and Bulgheresi 2010). Evaluation of the role that these two processes played in the history of the association is critical to resolve whether the partners are in the process of evolving toward mutual dependence or whether the association is locked in a facultative mode. (3) The evolutionary origin of the association is needed to discern whether it is a recently formed relationship or an ancient symbiosis. The time of origin can be established based on fossil record. If fossil evidence is available for one partner only, a pattern of codivergence between the partners is required to infer the initial association event and date it.

Endosymbiont transmission mode is a key life-history feature that defines its population structure and evolutionary history. Strict vertical transmission of essential endosymbionts makes uncovering evolutionary origins and histories of their symbiotic associations relatively straightforward because hosts and symbionts develop clear patterns of codivergence (Bright and Bulgheresi 2010). In addition, vertical transmission has dramatic effects on genomic architecture of the endosymbiont, including increased fixation of slightly deleterious mutations that results in molecular evolution rate acceleration and genome size reduction relative to free-living taxa (Moran et al. 2008). These features can be attributed to the powerful role that genetic drift plays in endosymbiont populations (Moran 1996). Drift is enhanced in these populations because of their exceedingly small effective sizes brought about by clonal reproduction, population subdivision, and recurrent population bottlenecks. The patterns of molecular evolution associated with small effective sizes are witnessed nearly universally in essential endosymbionts of insects despite their divergent origins (Moran et al. 2008), revealing how strongly transmission mode influences evolution.

Although vertical transmission can be extensive in nonessential endobacteria, they do not share highly similar patterns of molecular evolution (Dale and Moran 2006; Moran et al. 2008). This is likely due to the fact that most nonessential endosymbionts maintain the capacity for both vertical and horizontal transmission as well as recombination (Toh et al. 2006; Degnan and Moran 2008; Degnan et al. 2010). A balance between these processes is expected to heavily contribute to the genomic variability observed across nonessential endosymbionts, as genetic drift is reduced through horizontal transmission and recombination, while enhanced during phases of vertical transmission. Through symbiont mixing and replacement, recombination and host switching also have the capacity to obscure codivergence patterns that could be used to make inferences about the age and history of an association.

To test the hypothesis that facultative endosymbioses are transitive, we studied an association of arbuscular mycorrhizal (AM) fungi from the phylum Glomeromycota (Smith and Read 2008) and *Ca*. Glomeribacter gigasporarum (Bianciotto et al. 1996; Bianciotto et al. 2003) representing the *Burkholderia* lineage of beta-proteobacteria. Among diverse heritable symbioses, the Glomeromycota–Glomeribacter association provides an ideal system for testing the transitory nature of heritable facultative associations. Glomeromycota are one of the ancient fungal lineages (Heckman et al. 2001) with a rich fossil record (Remy et al. 1994; Redecker et al. 2000; Dotzler et al. 2006) that can be used to date their evolutionary history. The entire phylum appears to be asexual (Smith and Read 2008) and therefore, the evolutionary history of these fungi is unlikely to be confounded by frequent gene exchanges (Croll and Sanders 2009; Rosendahl et al. 2009; den Bakker et al. 2010). Glomeromycota colonize roots of the vast majority of terrestrial plants and improve plant mineral nutrient uptake from the soil in exchange for plant-assimilated

carbon (Smith and Read 2008). This energy dependence on plant hosts may be a source of significant selective pressure on Glomeribacter endosymbionts, which, in turn, may contribute to enhanced resolution of coevolution patterns in the Glomeromycota–Glomeribacter association. The Glomeribacter endobacteria improve hyphal growth of their fungal host after spore germination and prior to plant root colonization (Lumini et al. 2007). This developmental effect is related to Glomeribacter's ability to modify metabolism of lipids that are contained inside the fungal spores and fuel spore germination and presymbiotic hyphal proliferation (Salvioli et al. 2010). As Glomeromycota are horizontally transmitted obligate biotrophs, their success in root colonization and securing plant hosts depends on their ability to proliferate presymbiotically (Smith and Read 2008). The extent of hyphal proliferation needed to secure a plant host is largely dependent on environmental factors (Klironomos and Moutoglis 1999).

Glomeribacter endobacteria have been detected so far only in the Gigasporaceae family of Glomeromycota (Bianciotto et al. 2003). They are vertically transmitted by their AM fungal hosts and can be cleared from fungal cells through serial subculturing in vitro (Lumini et al. 2007). Although the endobacteria are not cultivable, they are capable of surviving for several weeks outside of the fungal host, albeit without replication (Jargeat et al. 2004). The genome sequence of Glomeribacter revealed that these endobacteria are energy dependent on their fungal host (Ghignone et al. 2011). It is not surprising, therefore, that Glomeribacter exhibit evolutionary patterns typical for vertically transmitted endobacteria, including an accelerated rate of molecular evolution relative to free-living taxa (Castillo and Pawlowska 2010) and a rather small 1.72 Mb genome (Ghignone et al. 2011). However, rRNA and protein-coding genes of Glomeribacter show very little accumulation of deleterious mutations (Castillo and Pawlowska 2010) that would be expected in heritable endosymbionts (Lambert and Moran 1998). The pattern of acceleration of the molecular evolution rate, combined with limited accumulation of deleterious mutations relative to free-living taxa, suggest that, even though vertical transmission has played a large role in the evolutionary history of this lineage, Glomeribacter have the capacity to at least partially mitigate the negative effects of genetic drift associated with vertical transmission.

In the present study, we reconstructed the evolutionary history of the symbiosis between *Ca.* Glomeribacter gigasporarum and Glomeromycota to resolve whether these bacteria are relatively new associates of Glomeromycota on the trajectory toward becoming essential endosymbionts, or whether this association is ancient and permanently locked in its current facultative state. Using the host fossil record and codiverging partner pairs, we inferred that the symbiosis of Glomeromycota with Glomeribacter is at least 400 million years old. We show that unlike many facultative endosymbioses, which have rapidly evolved into obligate associations, the association between AM fungi and Glomeribacter is maintained in a facultative state through a balance of vertical transmission, recombination in endosymbiont populations, and likely host switching.

## Materials and Methods

### DIVERSITY OF GLOMERIBACTER IN AM FUNGI

Globally distributed populations representing the taxonomic diversity of the Gigasporaceae family were obtained from the International Culture Collection of Vesicular AM Fungi (INVAM), West Virginia University, Morgantown, West Virginia, the International Bank for the Glomeromycota (BEG), INRA, Dijon, France, and Ylva Lekberg collection (Table S1). Rather than a single-spore isolate, each of the accessions represents an experimental population of morphologically similar fungi collected at a specific sampling location and maintained as a live culture. To understand how endobacterial diversity varied within and among fungal populations, we sampled individual fungal spores (isolates) from each accession.

### AM FUNGAL SPORE DECONTAMINATION

We developed a new spore surface decontamination technique. Spores were rinsed in 0.05% Tween 20 and subjected to two 15 min rinses in $H_2O_2$, the first at 1 mM concentration and the second at 50 mM (Imlay et al. 1988; Imlay and Linn 1988), followed by a 20 min soak in 4% chloramine T before final rinsing in nanopure water (three 20 min soaks). All spores were processed individually to minimize cross-contamination. Wash controls were set up for three spores per species by placing intact surface-decontaminated spores in illustra$^{TM}$ GenomiPhi-V2 Whole Genome Amplification (WGA) sample buffer (GE Healthcare, Piscataway, NJ), vigorous vortexing, and removing the spores. WGA of wash controls followed by polymerase chain reaction (PCR) with universal bacterial 16S rRNA gene primers 704f and 1495r (Bianciotto et al. 1996) confirmed the efficacy of spore decontamination.

### MOLECULAR MARKERS

Following surface decontamination, total DNA of individual spores was globally amplified using illustra$^{TM}$GenomiPhi-V2 kit (GE Healthcare). WGA products were diluted 1 to 20 in water for subsequent PCR reactions. Specific PCR primers were designed for bacterial loci encoding the 16S rRNA, 23S rRNA, FtsZ, and PstA (Tables 1 and 2). To ensure PCR amplification of divergent Glomeribacter genotypes, the 23S rRNA gene primers were designed and verified to successfully amplify not only

**Table 1. Molecular markers sampled in Glomeromycota and in Glomeribacter.**

| Gene | Taxon | Fragment length (bp) | Annotation |
|---|---|---|---|
| 18S rRNA | Glomeromycota | 516 | Small ribosomal subunit |
| 28S rRNA | Glomeromycota | 664 | Large ribosomal subunit |
| Beta-tubulin | Glomeromycota | 671 | Microtubule structural component |
| 16S rRNA | *Ca*. Glomeribacter gigasporarum | 930 | Small ribosomal subunit |
| 23S rRNA | *Ca*. Glomeribacter gigasporarum | 553 | Large ribosomal subunit |
| *ftsZ* | *Ca*. Glomeribacter gigasporarum | 483 | Structural binary fission protein |
| *pstA* | *Ca*. Glomeribacter gigasporarum | 695 | Phosphate transport system permease protein |

Glomeribacter, but their sister lineage *Burkholderia rhizoxinica* as well (data not shown). The 16S and 23S rRNA genes are present in one copy in the Glomeribacter genome (Ghignone et al. 2011). The rRNA, *ftsZ*, and *pstA* genes are all located in different contigs in the Glomeribacter draft genome assembly. To amplify fungal loci encoding 18S rRNA, 28S rRNA, and beta-tubulin, we used a combination of published and our own primers (Table 2). PCR amplifications targeting bacterial loci were carried out in 50-μl reactions containing 25 μl REDTaq PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO), 21.8 μl water, 0.6 μl of each 10 μM primer, and 2 μl of diluted WGA product under the following cycling conditions: initial denaturation of 2 min at 94°C, 10 touchdown cycles of 30 sec at 94°C, 30 sec at 59–1°C per cycle, and 1 min at 72°C followed by 25 cycles of 30 sec at 94°C, 30 sec at 49°C, and 1 min at 72°C with final extension of 7 min at 72°C. For the fungal 18S rRNA and beta-tubulin genes, we conducted touchdown PCR with target annealing temp of 49 and 52°C, respectively. Conventional PCR was done for the fungal 28S rRNA genes at an annealing temp of 57°C. All other conditions were identical to those in bacterial PCR amplifications. Amplicons were purified using the QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA), cycle sequenced with BigDye version 3.1 (Applied Biosystems, Carlsbad, CA), purified using Performa Ultra 96-well plates (Edge BioSystems, Gaithersburg, MD), and sequenced on the Automated 3730 DNA Analyzer (Applied Biosystems). For the *pstA* gene in endobacteria from *S. pellucida*, amplicons were cloned using TOPO-TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Recombinant plasmid DNAs were amplified with illustra$^{TM}$TempliPhi kit (GE Healthcare), and sequenced using the T3 and T7 primers.

Marker sequences were edited in Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI) and aligned with MUSCLE

**Table 2. Primers for PCR amplification of molecular markers in Glomeromycota and in Glomeribacter.**

| Gene | Taxon | Primer[1] | Oligonucleotide sequence | *Tm*[2] | Reference |
|---|---|---|---|---|---|
| 18S rRNA | Glomeromycota | NS1 | GTAGTCATATGCTTGTCTC | 47.2 | White et al. (1990) |
| 18S rRNA | Glomeromycota | NS2 | GGCTGCTGGCACCAGACTTGC | 63.9 | White et al. (1990) |
| 28S rRNA | Glomeromycota | LR1 | GCATATCAATAAGCGGAGGA | 46.7 | Van Tuinen et al. (1998) |
| 28S rRNA | Glomeromycota | NDL22 | TGGTCCGTGTTTCAAGACG | 55.2 | Van Tuinen et al. (1998) |
| Beta-tubulin | Glomeromycota | Btub16f | ATGATGTGCACGTTTTCTGT | 52.9 | This study |
| Beta-tubulin | Glomeromycota | 2iR | GTGAAGACGTGGGAAAGGAAC | 55.9 | Msiska and Morton (2009) |
| 16S rRNA | Glomeribacter | SSU534f | GGTAAATAATCGGAGTGGAT | 49.2 | This study |
| 16S rRNA | Glomeribacter | SSU709f* | GCATACGTGACTGGCAGACT | 57.2 | This study |
| 16S rRNA | Glomeribacter | SSU1520r* | TCTGGTAAAACTCACTCCC | 51.3 | This study |
| 23S rRNA | Glomeribacter | GlomGiGf* | GGGTCCATTGCGGATTACTTC | 55.9 | Bianciotto et al. (2004) |
| 23S rRNA | Glomeribacter | GlomGiGr | GGGACCAGGACTTCCATCCCCC | 63.8 | Bianciotto et al. (2004) |
| 23S rRNA | Glomeribacter | LSU483r* | GGTGCAGGAATATTAACC | 47.6 | This study |
| *ftsZ* | Glomeribacter | FtsZ354f | GTGGTCTCAAAGCCGTTT | 53.1 | This study |
| *ftsZ* | Glomeribacter | FtsZ857r | CCATCGCATCATCGTAGA | 51.5 | This study |
| *ftsZ* | Glomeribacter | FtsZ16fPell* | CTTGAGGAGCATGTGGATTC | 53.3 | This study |
| *ftsZ* | Glomeribacter | FtsZ944rPell* | GTGTGGAGCAAGGTCATGG | 56.1 | This study |
| *pstA* | Glomeribacter | PstA13f | CTGCTATGGCTCGCGTGGAT | 60.0 | This study |
| *pstA* | Glomeribacter | PstA727r | GCGAGCGTATTCAGGCCCAG | 60.9 | This study |

[1]In the case of the bacterial 16S rRNA, 23S rRNA, and *ftsZ* genes, primer combinations used to amplify *S. pellucida* endobacteria are denoted with an asterisk.
[2]*Tm* values in °C were determined by Integrated DNA Technologies, Inc. (Coralville, IA).

(Edgar 2004). Bayesian and maximum likelihood (ML) multigene phylogenies were constructed in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) and RAxML version 7.0.4 (Stamatakis 2006), respectively, using the Generalized Time Reversible (GTR) nucleotide substitution model (Tavaré 1986) plus invariant sites (I) and Γ rate heterogeneity. Genes were partitioned separately, allowing for independent models of sequence evolution across loci. The Markov chain Monte Carlo (MCMC) analysis was run for 10 million generations. A total of 1000 bootstrap replicates were performed for trees constructed with RAxML (Stamatakis 2006). Placement of the *Ca*. Glomeribacter gigasporarum lineage in the bacterial phylogeny was examined using reference organisms with complete genomes available at GenBank (accession numbers in Fig. 2). To root the fungal tree, we used *Glomus versiforme* BEG47 (AJ132666, FJ461852, FJ174286). Sequences generated in this study were deposited in GenBank under accession numbers JF816707–JF817217.

## CODIVERGENCE ANALYSES

The Permutation test (Hommola et al. 2009) and the ParaFit test (Legendre et al. 2002) were used to determine the overall signal of codivergence within the dataset. Both these methods test the null hypothesis of independent host and symbiont evolution. The Permutation test is based on correlation between host and parasite genetic distances (Hommola et al. 2009). The ParaFit method uses a similar approach but transforms host and symbiont patristic distances derived from their phylogenetic trees into matrices of principal coordinates (Legendre et al. 2002). In addition to testing the global null hypothesis, ParaFit can be also used to examine the contribution of each individual host–symbiont pair to the overall codivergence signal. As these tests require that only partner pairs are included in the analysis, no outgroups or host fungi without endosymbionts were analyzed. To generate patristic distances for ParaFit, unrooted Bayesian multigene phylogenies were constructed for hosts and symbionts only (Fig. S1) using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). The MCMC analysis was run for 10 million generations using the GTR + I + Γ nucleotide substitution model. Principal coordinates were generated from patristic distances using the DistPcoA program (Legendre and Anderson 2002). At least 10,000 permutations were conducted for each codivergence test. For each host–endosymbiont pair, *P*-values were determined by ParaFitLink1 (Legendre et al. 2002). Tanglegrams in Figure S1 were generated with Dendroscope 3 (Huson et al. 2007). For consistency, we use the term "codivergence" to describe patterns of significant parallel diversification at the level of host and symbiont populations. "Cospeciation" is reserved for patterns detected within a single host genus, whereas "cophyly" refers to patterns apparent across genera.

## DETECTION OF RECOMBINATION IN GLOMERIBACTER

The Genetic Algorithm for Recombination Detection (GARD) (Kosakovsky Pond et al. 2006) available through a web interface (Delport et al. 2010) was used to identify recombination breakpoints in both fungal and Glomeribacter genes. GARD is a likelihood-based model selection procedure that uses a genetic algorithm to search multiple sequence alignments for evidence of recombination breakpoints (Kosakovsky Pond et al. 2006). The alignments of concatenated sequences, including the rRNA genes, were searched for evidence of segment-specific phylogenies under the GTR+Γ nucleotide substitution model (Tavaré 1986). Goodness of fit was assessed using the small sample corrected AIC ($AIC_C$) derived from an ML model fit to each segment. The Kishino–Hasegawa (KH) method (Kishino and Hasegawa 1989) was applied to evaluate congruence of the segment-specific topologies.

To measure the extent of recombination in the Glomeribacter population, concatenated sequences of *ftsZ* and *pstA* genes were examined with ClonalFrame (Didelot and Falush 2007). ClonalFrame reconstructs the clonal genealogy of a sample of individuals, as well as the mutation and recombination events that took place on the branches of this genealogy, based on a coalescent model (Vos and Didelot 2009). Ribosomal RNA genes were excluded from this analysis because recombination signal could be confounded by nucleotide substitution rate heterogeneity often observed in rRNA gene evolution (Vos and Didelot 2009). Run specifications followed those of Vos and Didelot (2009) with the MCMC run of 200,000 generations after an initial 200,000 burn-in. Convergence was assessed using the Gelman–Rubin statistic (Gelman and Rubin 1992) with a cutoff of 1.1.

Bacterial individuals for which all genes could not be PCR-amplified were excluded from both GARD (Kosakovsky Pond et al. 2006) and ClonalFrame (Didelot and Falush 2007) analyses. Only one representative per accession was used for both tests as endobacterial diversity did not vary within an accession.

## GLOMERIBACTER HETEROGENEITY IN AM FUNGAL INDIVIDUALS

To assess genetic heterogeneity of Glomeribacter in AM fungal individuals, the 23S rRNA gene fragments were PCR amplified from individual spores using primers GlomGiGf (Bianciotto et al. 2004) and LSUb-483r (Table 2) in 50-μl reactions with RED-Taq (Sigma) under cycling conditions of initial denaturation of 2 min at 94°C, 25 cycles of 30 sec at 94°C, 30 sec at 49°C, and 1 min at 72°C followed by final extension of 7 min at 72°C. Amplicons were cloned and sequenced as described above. At least 16 recombinant colonies were analyzed per spore.

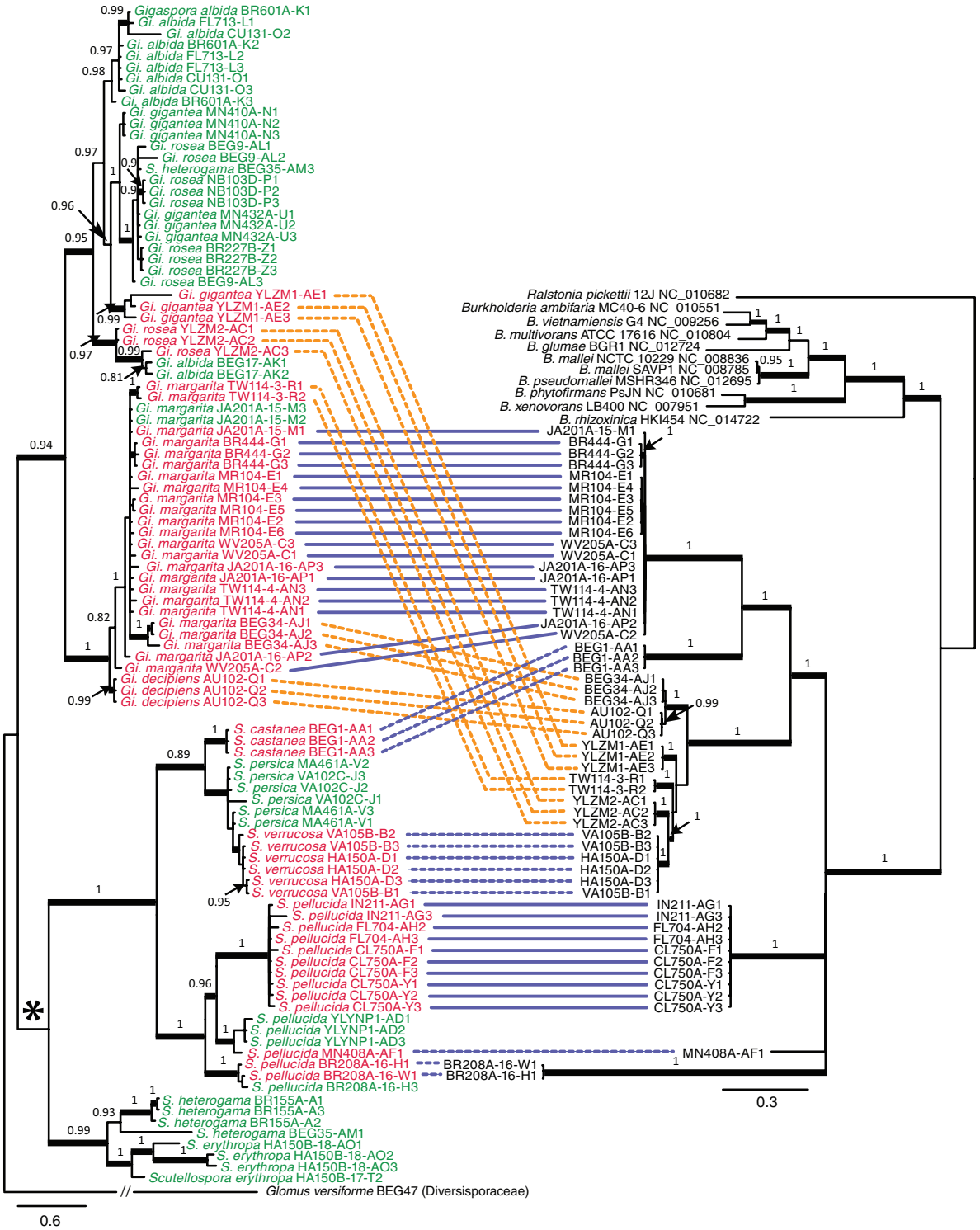


**Figure 2.** Patterns of coevolution between the Gigasporaceae fungal hosts (left) and the *Ca*. Glomeribacter gigasporarum bacterial endosymbionts (right). The fungal phylogeny was reconstructed using 18S rRNA, 28S rRNA, and beta-tubulin gene sequences; the bacterial phylogeny is based on 16S rRNA, 23S rRNA, *ftsZ*, and *pstA* genes. Bayesian posterior probabilities greater than 0.80 are shown above branches. Branches with ML bootstrap support over 70% are thickened. Solid blue lines connecting host and symbiont pairs indicate significant evidence of codivergence detected by ParaFit in concatenated datasets of fungi and bacteria (Tables 3 and S2; Fig. S1). Dashed blue lines link partners showing evidence of codivergence after recombination was accounted for in the bacterial dataset. Dashed orange lines indicate partner pairs with no evidence of codivergence, likely due to host switch events. Fungal isolates harboring endobacteria are colored red; individuals with no endobacteria are shown in green. The node likely associated with the *Scutellospora* Rhynie chert fossil record (Dotzler et al. 2006) is marked by an asterisk.

# Results

## DIVERSITY OF GLOMERIBACTER ENDOBACTERIA IN AM FUNGI

The association between Glomeromycota and *Ca*. Glomeribacter gigasporarum appears to be facultative for the fungus, as it can survive and reproduce after removal of the endobacteria under laboratory conditions (Lumini et al. 2007). However, it has been unclear whether the association between Glomeribacter and Glomeromycota is also nonessential for the fungus in nature, given the significance of presymbiotic growth to the fitness of Glomeromycota. To test the hypothesis that the endobacteria are nonessential for the fungus in nature, we examined the distribution of Glomeribacter among isolates of Glomeromycota. We surveyed 115 isolates from 34 globally distributed experimental populations representing the Gigasporaceae family of Glomeromycota (Table S1). Fungal individuals were characterized by PCR amplification and sequencing of the 18S rRNA, 28S rRNA, and beta-tubulin genes, whereas bacterial endosymbionts were identified by PCR amplification and sequencing of the 16S rRNA, 23S rRNA, *ftsZ*, and *pstA* genes (Tables 1 and 2). Only 18 of the surveyed populations harbored Glomeribacter (Fig. 2). Endobacteria presence varied even within closely related fungal groups, indicating that very similar fungal genotypes can survive with or without endobacteria (Fig. 2). For example, although most *Scutellospora pellucida* populations harbored Glomeribacter, endobacteria were not detected in any individual sampled from the YLYNP1 population. Moreover, presence of endobacteria was not consistent within populations. For instance, only two of the three spores sampled from *S. pellucida* BR208A contained Glomeribacter. Similarly, endosymbionts were only present in four of the six spores sampled from the JA201A population of *Gigaspora margarita*. Altogether, these data support the hypothesis that the Glomeribacter endobacteria are not essential for the survival of their fungal hosts.

To understand the evolution of the Glomeribacter bacteria, we reconstructed their phylogeny using 16S rRNA, 23S rRNA, *ftsZ*, and *pstA* gene sequences. We found that Glomeribacter form a monophyletic clade nested within the *Burkholderia* genus, with endobacteria associated with *S. pellucida* being the most similar to free-living *Burkholderia* (Fig. 2). This pattern suggests that the ability to associate with Glomeromycota evolved in the *Burkholderia* lineage only once.

## GLOMERIBACTER CODIVERGENCE WITH GLOMEROMYCOTA

To explore the history of the association between Glomeromycota and the Glomeribacter endobacteria and examine how closely Glomeribacter have coevolved with their host, we measured the degree of phylogenetic codivergence of the partners using the Permutation test (Hommola et al. 2009) and the ParaFit test (Legendre et al. 2002) on sequences of concatenated fungal genes encoding 18S rRNA, 28S rRNA, and beta-tubulin and concatenated endobacterial genes encoding 16S rRNA, 23S rRNA, FtsZ, and PstA (Tables 1 and 2). Using these markers, the phylogenies of both host and endosymbiont were well resolved and strongly supported (Figs. 2 and S1), and thus the codivergence patterns are unlikely to be obscured by topological uncertainties. However, internal conflict generated by recombination (if present) may still affect codivergence patterns. Both tests rejected the null hypothesis of independent evolution of the Gigasporaceae hosts and the Glomeribacter endobacteria (the Permutation test $P < 0.0001$ and the ParaFit test $P = 0.0001$). The ParaFit test, which uses phylogenetic distances to detect the overall codivergence signal within the dataset, also uses these distances to identify which individual host–endosymbiont associations significantly contribute to the codivergence signal. In the case of Gigasporaceae and Glomeribacter, the test revealed that the global signal of codivergence was not due to uniformly codiverging host and endosymbiont populations, but instead was generated mostly by several significant contributions from relatively few lineages (*Gi. margarita* and *S. pellucida*; Tables 3 and S2; Figs. 2 and S1). This finding indicates that other processes, such as recombination and host switching, are likely interfering with codivergence between these partners.

## GLOMEROMYCOTA ARE LOCKED IN FACULTATIVE DEPENDENCE ON GLOMERIBACTER

Recombination and host switching can interfere with progressive coevolution between the partners by disrupting reciprocally adapted suites of alleles (Frank 1994). Consequently, evaluating the contributions of recombination and host switching in the history of a symbiosis is likely to reveal whether the association is in the process of transitioning toward reciprocal dependence. These two processes introduce incongruence between host and symbiont phylogenies and disrupt patterns of codivergence.

To evaluate the contributions of recombination and host switching to partner coevolution, we searched for breakpoints in the fungal host and in the Glomeribacter sequence data and then reassessed the patterns of codivergence by comparing the evolutionary histories of partner gene sequences partitioned into segments defined by the recombination breakpoints. We used the GARD (Kosakovsky Pond et al. 2006), followed by the KH test (Kishino and Hasegawa 1989) to identify recombination breakpoints. No evidence of recombination was detected in concatenated 18S rRNA, 28S rRNA, and beta-tubulin gene sequences of fungal hosts harboring endosymbionts. This finding is consistent with the expectation that Gigasporaceae are asexual. In contrast, the GARD detected three possible recombination breakpoints in a set of concatenated bacterial 16S rRNA, 23S rRNA, *ftsZ*, and

**Table 3. Population-level evidence of codivergence between Glomeromycota and Glomeribacter represented as a majority-rule consensus of the ParaFit test results for each fungal isolate (complete data are in Table S2). Patristic distances were based on phylogenetic trees generated from fungal and bacterial sequence data without partitions as well as bacterial data partitioned into segments defined by recombination breakpoints (Fig. S1). MS = marginally significant ($P \leq 0.07$); *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; **** $P \leq 0.0001$; — = not significant ($P > 0.07$).**

| Fungal population | Bacterial concatenated gene sequence alignment positions | | | |
|---|---|---|---|---|
| | All | 1–1085 | 1086–1905 | 1906–2693 |
| *Gi. decipiens* AU102-23 | – | – | – | – |
| *Gi. gigantea* YLZM1 | – | – | – | – |
| *Gi. margarita* BEG34 | – | – | – | – |
| *Gi. margarita* BR444-22 | *** | *** | ** | * |
| *Gi. margarita* JA201A-15 | *** | *** | ** | MS |
| *Gi. margarita* JA201A-16 | *** | ** | *** | MS |
| *Gi. margarita* MR104-5 | *** | *** | ** | MS |
| *Gi. margarita* TW114-3 | – | – | – | – |
| *Gi. margarita* TW114-4 | *** | ** | ** | MS |
| *Gi. margarita* WV205A-24 | *** | *** | *** | * |
| *Gi. rosea* YLZM2 | – | – | – | – |
| *S. castanea* BEG1 | – | *** | – | – |
| *S. pellucida* BR208A-16-H | **** | – | **** | **** |
| *S. pellucida* BR208A-16-W | **** | – | **** | *** |
| *S. pellucida* CL750A-13-F | **** | *** | **** | **** |
| *S. pellucida* CL750A-13-Y | **** | **** | **** | **** |
| *S. pellucida* FL704-14 | **** | *** | **** | **** |
| *S. pellucida* IN21-7 | **** | *** | **** | **** |
| *S. pellucida* MN408A-16 | **** | – | **** | **** |
| *S. verrucosa* HA150A-16 | – | – | * | – |
| *S. verrucosa* VA105B-16 | – | – | * | – |

*pstA* gene sequences at alignment positions 1085 (the 5′-end of the 23S rRNA gene), 1617 (the 5′-end of the *ftsZ* gene), and 1905 (the 3′-end of the *ftsZ* gene). The $AIC_C$ score of 13,851.6 for the best-fitting model allowing for different topologies of the alignment segments defined by these breakpoints was lower than the $AIC_C$ score of 13,937.5 for the model that assumed the same topology for all segments, indicating that a multiple tree model is preferable over a single tree model. Using the KH test, only the 1085 ($P < 0.05$) and 1905 ($P < 0.01$) breakpoints were identified as resulting in significant topological incongruence between segments.

The GARD results suggested that overall codivergence patterns based on the concatenated bacterial dataset may be obscured by multiple recombination events. Therefore, based on these recombination breakpoints, we partitioned the Glomeribacter dataset into three alignment segments (Fig. S1) and conducted the ParaFit test for each of these partitions separately. We found that the endobacteria were engaging in recombination and likely in host switching (Tables 3 and S2). Recombination was evidenced by disappearance of the codivergence signal in some of the partitions of data that showed codivergence without partitioning (*S. pellucida* BR208A and *S. pellucida* MN408A), and by appearance of codivergence signal in partitions of data that did not show codivergence without partitioning (*S. castanea* and *S. verrucosa*; Figs. 2 and S1). A lack of codivergence across all data partitions observed in *Gi. decipiens*, *Gi. gigantea*, *Gi. margarita* BEG34, *Gi. margarita* TW114–3, and *Gi. rosea* was interpreted as an indication of host switching. Although this is the most parsimonious interpretation (Brooks and McLennan 1991), it is also possible, although unlikely, that the lack of codivergence across all data partitions was caused exclusively by recombination. Interestingly, after recombination breakpoints were considered, it became apparent that all members of the *Scutellospora* genus that harbor endosymbionts (*S. pellucida*, *S. castanea*, and *S. verrucosa*) show evidence of cospeciation. However, patterns of recombination and host switching were evident even within *Gi. margarita* and *S. pellucida*, which, for the most part, harbored perfectly codiverging endosymbionts. The fact that recombination and host switching occur even in predominantly codiverging clades indicates that the partners still maintain the flexibility to associate with different genotypes of each other despite the establishment of long-term associations. Therefore, we conclude that

the Gigasporaceae-Glomeribacter association is not in transition toward reciprocal dependence.

The finding of recombination and host switching throughout the Glomeribacter population further implies that genetically different strains have similar effects on their hosts. Such functional interchangeability of Glomeribacter genotypes suggests that retaining the capacity for horizontal transmission in this highly coevolved association is likely to have an adaptive role. We speculate that if the host costs of maintaining the association outweigh the benefits of improved presymbiotic hyphal growth imparted by endobacteria, the endobacteria can be lost from the hosts. If conditions change, the endobacteria can be reacquired. Horizontal transmission offers a mechanism that could restore the association, stabilize it in fluctuating environments, and thus contribute to its evolutionary antiquity.

## RECOMBINATION AND MIXING IN GLOMERIBACTER POPULATION

Genetic drift associated with small effective population sizes in vertically transmitted endobacteria is the underlying cause of their genomic degeneration (Moran et al. 2008, 2009). Recombination can mitigate the negative effects of vertical transmission. To determine whether the extent of recombination in the Glomeribacter population is sufficient to protect their genomes from accumulation of deleterious mutations, we examined concatenated sequences of *ftsZ* and *pstA* genes using the ClonalFrame software (Didelot and Falush 2007), which quantifies recombination frequency in a population. We modeled Glomeribacter genealogy and estimated the rate of recombination relative to the rate of mutation in the population ($\rho/\theta$). We also computed the per-site effect of recombination relative to mutation, that is, the ratio of rates at which nucleotides become substituted as a result of recombination versus mutation ($r/m$). The values measured for Glomeribacter were $\rho/\theta = 0.1$ with a 95% confidence interval of 0.01–0.39 and $r/m = 2.5$ with a 95% confidence interval of 0.44–6.30. The $\rho/\theta$ value suggests that recombination events were less frequent than mutations in the history of Glomeribacter. However, because each recombination event is likely to introduce several nucleotide polymorphisms, the per-site effect of recombination $r/m$ was on average over twofold larger than the effect of mutation. Such impact of recombination on nucleotide diversity is considered to be moderately high and is comparable to that found in many free-living bacteria (Vos and Didelot 2009). Therefore, it appears that the recombination frequency in the Glomeribacter population is sufficient to moderate accumulation of deleterious mutations in its genome (Castillo and Pawlowska 2010). This level of gene exchange is also consistent with the impact that recombination has on codivergence between Glomeribacter and Glomeromycota.

To assess whether endosymbiont mixing, suggested by the recombination and host switching data, can also be detected in the Glomeribacter populations contained within host individuals, we cloned and sequenced 23S rRNA gene fragments from endobacteria within single AM fungal spores representing populations CL750A-13, VA105B-16, WV205A-24, and YLZM2. Although several single nucleotide polymorphisms were present within all but the VA105B-16 dataset, the observed polymorphism level was minimal and not different from that generated by PCR error alone, and thus, it did not provide evidence to reject Glomeribacter clonality within any AM fungal individual. Consequently, we found that, despite the signature of recombination and evidence of host switching, Glomeribacter populations associated with individual hosts remain genetically uniform, which, in turn, suggests a powerful role of transmission bottlenecks in the life history of these endosymbionts. Population bottlenecks are responsible for reducing effective sizes of endosymbiont populations and exacerbating the effects of genetic drift, which are evidenced in Glomeribacter by the accelerated rate of molecular evolution relative to free-living taxa (Castillo and Pawlowska 2010).

## THE ANTIQUITY OF ASSOCIATION BETWEEN GLOMERIBACTER AND GLOMEROMYCOTA

As fossil information is available for the Gigasporaceae lineage of Glomeromycota (Dotzler et al. 2006), evidence of codivergence can be used to estimate the age of the association between AM fungi and the Glomeribacter endobacteria. Because the ParaFit (Legendre et al. 2002) approach utilizes phylogenetic distances to assess whether the host and the symbiont are diverging from their relatives at similar rates, the codivergent host–symbiont pairs can be used to infer the phylogenetic node where the initial codivergence event must have occurred. When the confounding effects of recombination were accounted for (Fig. S1), the ParaFit test revealed that all members of the *Scutellospora* lineage harboring endosymbionts (*S. castanea*, *S. pellucida*, *S. verrucosa*) show evidence of cospeciation with their bacterial partners (Tables 3 and S2; Figs. 2 and S1). Moreover, cophyly with endobacteria is apparent in the *Scutellospora* and *Gigaspora* hosts, as several isolates of *Gi. margarita* show significant codivergence with Glomeribacter. This pattern places the first association event between AM fungi and Glomeribacter at the base of the Gigasporaceae family tree (Fig. 2), indicating that the Glomeribacter endobacteria were present in the Glomeromycota lineage prior to the Gigasporaceae speciation.

As no true cospeciation was detected within the *Gigaspora* genus (Tables 3 and S2; Figs. 2 and S1), the hypothesis that endobacteria codivergent with *Gi. margarita* are the result of a more recent host switch from *Scutellospora* is worth considering. However, we believe that this scenario is unlikely for several reasons. First, other host switches including those involving a few *Gi. margarita* isolates were readily detected within our dataset. Second, the pattern of cophyly that we observed indicates that

divergence levels across host genera are similar to those apparent across the endosymbiont phylogeny. Third, the endosymbiont evolutionary history, reconstructed using both concatenated data and data partitioned at recombination breakpoints, shows differentiation into few deeply divergent clades, including a clade associated with *Gi. margarita* (Figs. 2 and S1). Some of these deep clades expand into shallow terminal branches. Such a divergence pattern is expected in endosymbionts with a lengthy history of codivergence and occasional host switches, and would not be likely in a young symbiosis (Ronquist 2003). Instead, a recent association characterized by a series of host switches would be evidenced by a more contemporaneous proliferation of endosymbiont clades.

The origin of the Glomeromycota–Glomeribacter symbiosis prior to the Gigasporaceae radiation allows us to place a lower bound on the age of this association. The oldest Gigasporaceae fossils, which closely resemble present-day spore structures of the genus *Scutellospora*, were found in the Rhynie chert dated to 396 $\pm$ 12 Mya (Dotzler et al. 2006). In conjunction with our codivergence results, the existence of this fossil record establishes that the association between Gigasporaceae and Glomeribacter is at least 400 million years old. However, as the radiation of the Gigasporaceae family likely predates the fossil record, the association is expected to be even older. This ancient origin suggests that the symbiosis between Glomeromycota and Glomeribacter existed in the mycorrhizal associations that AM fungi formed with the Early Devonian land plants (Remy et al. 1994), which might have significantly contributed to plants' ability to colonize land.

## Discussion

Our analyses revealed that Glomeromycota are facultatively associated with *Ca*. Glomeribacter gigasporarum endobacteria and have been locked in this state for at least 400 million years. Of the 34 fungal populations surveyed, only 18 harbored endobacteria and showed strong overall evidence of codivergence. This global signal was due to large contributions from two fungal species, *S. pellucida* and *Gi. margarita*. However, when recombination breakpoints in the endosymbiont sequences were considered in the analyses (Fig. S1), cospeciation signals were detected across all members of the *Scutellospora* genus harboring endosymbionts, and codivergence was consistently observed in one member of the *Gigaspora* (*Gi. margarita)*. These codivergence patterns indicate that the association between Glomeromycota and Glomeribacter is maintained through a balance between vertical transmission, recombination, and host switching. Given the great evolutionary age of this association, it is likely that the rate of horizontal transmission in Glomeribacter exceeds the critical level needed to preserve endosymbiont fitness over time (O'Fallon 2008). Even though the means of horizontal transmission in Glomeribacter are unknown, the partner codivergence patterns are not the only indication of its incidence. Recombination and host switching are also supported by the observation that, in a highly reduced genome, the Glomeribacter endobacteria retain and express genes encoding most of the recombination machinery as well as genes required for the type III secretion system, which in other bacteria is involved in invasion of and persistence in eukaryotic hosts (Ghignone et al. 2011). The patterns of symbiont mixing apparent in the Glomeribacter population combined with the antiquity of Glomeribacter's association with Glomeromycota provide evidence that not all facultative endosymbioses represent transitory stages along the trajectory toward obligate reciprocal dependence predicted by the evolutionary theory (Yamamura 1993; Frank 1995; Law and Dieckmann 1998). Instead, the Glomeromycota–Glomeribacter symbiosis shows that long-term associations can exist in the absence of strict vertical transmission and host dependence. This finding offers an incentive for identification and critical evaluation of conditions that keep facultative symbioses from evolving toward reciprocal partner dependence.

Unlike associations in which endosymbionts provision their hosts with essential nutrients required by the host for survival, such as amino acids needed by phloem sap-feeding insects (Baumann 2005), nonnutritional endosymbioses are often not consistently beneficial (Russell and Moran 2005). This is almost certainly true for facultative endosymbioses that insects form with *Ca*. Hamiltonella defensa and *Ca*. Regiella insecticola, in which endosymbionts protect their hosts from parasite pressure (Russell and Moran 2005). Given that the cost of supporting Glomeribacter may be a significant part of the fungal host carbon budget and that the extent of hyphal proliferation needed to secure a plant host is largely dependent on environmental factors (Klironomos and Moutoglis 1999), we speculate that Glomeribacter endobacteria resemble these protective insect endosymbionts by benefiting their fungal hosts only under conditions where extensive presymbiotic hyphal development is needed to colonize plant roots. In all these symbioses, the fluctuation of costs and benefits across environments would make obligate host dependence less likely (Genkai-Kato and Yamamura 1999). Nevertheless, long periods of vertical transmission and resulting genetic drift will still propel endosymbionts toward obligate dependence on their hosts, making these interactions superficially appear to be transitioning between associative states. However, with hosts occasionally losing their endosymbionts, the benefits gained by endobacteria that maintain the capacity for horizontal transmission and recombination would remain high. Thus, we speculate that under conditions of high environmental variability, facultative endosymbioses will be maintained in their present state and are less likely to evolve toward obligate reciprocal dependency (Fig. 1B).

Patterns of molecular evolution in *Ca*. Hamiltonella defensa and *Ca*. Regiella insecticola may support the hypothesis that environmental variability locks endosymbioses in a facultative state. Similar to Glomeribacter, Hamiltonella and Regiella harbor largely reduced genomes of 2.1 and 2.07 Mb, respectively (Degnan et al. 2009; Degnan et al. 2010). In addition, Hamiltonella shows evidence of recombination (Degnan and Moran 2008; Degnan et al. 2010). However, with the possible exception of a subclade of aphids where Hamiltonella appear universally (Degnan and Moran 2008), both systems show no cospeciation between partners (Russell et al. 2003). The overall lack of cospeciation could potentially be due to a recent origin of these associations, enhanced horizontal transmission rates, or high recombination. However, divergence levels between Hamiltonella and Regiella indicate that these lineages have likely been around for as long as *Buchnera aphidicola*, an essential endosymbiont of aphids that is at least 60–200 million years old (Degnan et al. 2010). Consequently, the low cospeciation levels in these associations are most likely due to horizontal transmission in Regiella and horizontal transmission combined with recombination in Hamiltonella (Degnan and Moran 2008). This observation suggests that the protective associations of insects formed with Hamiltonella and Regiella, in addition to being evolutionarily old (Degnan and Moran 2008; Degnan et al. 2010), may also be stable in their facultative state.

Although the majority of completely obligate interactions are of great age, such interactions may transition toward reciprocal dependence over relatively short time spans. Notably, *Ca*. Tremblaya princeps, an essential endosymbiont of mealybugs (Pseudococcidae) (Baumann 2005) and close relative of Glomeribacter, was postulated to be as young as 40 million years (Moran et al. 2008). Even though these two symbioses are markedly different, development and generation times are comparable between mealybugs (Chong et al. 2008) and AM fungi (Smith and Read 2008). It therefore seems that sufficient time must have elapsed for the Gigasporaceae-Glomeribacter association to become obligate, yet it still maintains its facultative nature. This observation is concordant with estimates of the relative rates of 16S rRNA gene evolution in Glomeribacter versus other *Burkholderia* (Castillo and Pawlowska 2010). Although evolving 1.5 times faster than free-living relatives, Glomeribacter is evolving 2.3 times slower than Tremblaya (Castillo and Pawlowska 2010). This disparity in evolution rates between Glomeribacter and other *Burkholderia* can now be explained by our finding of recombination and host switching within the Glomeribacter population. Both of these processes are expected to result in larger effective population sizes ($N_e$) than those in the essential endosymbionts such as Tremblaya, whereas prolonged periods of vertical transmission are expected to force $N_e$ to remain smaller than in free-living bacteria.

## Conclusion

Our analysis of the association between *Ca*. Glomeribacter gigasporarum and Glomeromycota revealed that not all facultative associations with heritable endobacteria represent transitory stages along the trajectory toward obligate reciprocal dependence predicted by evolutionary theory (Yamamura 1993; Frank 1995; Law and Dieckmann 1998). The patterns of molecular evolution in the Gigasporaceae-Glomeribacter association indicate that this facultative symbiosis has been maintained over 400 million years through a balance of vertical transmission, recombination, and host switching and it is not evolving toward a mutually obligate relationship. We speculate that associations in which host costs and benefits fluctuate with shifting environmental pressures are less likely to evolve toward reciprocal obligate dependence and endosymbiont strict vertical transmission in comparison to associations exposed to relatively stable forms of selection generated, for example, by specialized nutritional demands of the host (Fig. 1). Preserving the capacity for horizontal transmission in endosymbionts under variable selective conditions permits recolonization of host lineages that may have lost their partners due to environmental change. Similarly, retaining the ability to exchange genes is adaptive in such environments, as it protects endosymbionts from losing the capacity for horizontal transmission due to genomic degeneration caused by accumulation of deleterious mutations in populations of small effective sizes. Low frequency of recombination in heritable endosymbionts together with host switching may stabilize facultative mutualisms over extended evolutionary times.

### ACKNOWLEDGMENTS

We thank V. Gianinazzi-Pearson for fungal isolates from the International Bank for the Glomeromycota, and E. Angert, P. Bonfante, and N. VanKuren for comments on the manuscript. We are grateful to two anonymous reviewers for their insightful suggestions. This work was supported by the National Science Foundation (DEB-0918880).

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Associate Editor: P. Turner

## *Supporting Information*

The following supporting information is available for this article:

**Table S1.** Arbuscular mycorrhizal fungal isolates surveyed for association with Glomeribacter.

**Table S2.** Evidence of codivergence between Glomeromycota and Glomeribacter detected by the ParaFit test using a set of bacterial gene sequences with no partitions as well as bacterial sequence data partitioned into segments defined by recombination breakpoints.

**Figure S1.** Patterns of coevolution between the Gigasporaceae fungal hosts and the *Ca*. Glomeribacter gigasporarum bacterial endosymbionts.

Supporting Information may be found in the online version of this article.

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