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ARTICLE

Distribution and population structure of endobacteria in arbuscular mycorrhizal fungi at North Atlantic dunes

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Abstract

Arbuscular mycorrhizal fungi (AMF, Glomeromycotina), in addition to forming symbioses with the majority of land plants, harbor vertically transmitted endosymbiotic bacteria '*Candidatus* Glomeribacter gigasporarum' (*Ca*Gg) and '*Candidatus* Moeniiplasma glomeromycotorum' (*Ca*Mg). *Ca*Gg is a nonessential mutualist of AMF, whereas the lifestyle of *Ca*Mg is unknown. To start unraveling the interactions between AMF and their endosymbionts in nature, we examined diversity and distribution of AMF-associated endobacteria in North Atlantic dunes at Cape Cod. Of nearly 500 foredune AMF isolates successfully genotyped during a systematic study, 94% were classified as Gigasporaceae. Two percent of all AMF spores harbored *Ca*Gg, and 88% contained *Ca*Mg. *Ca*Gg was found only in the Gigasporaceae, whereas *Ca*Mg was present in Gigasporaceae, Acaulosporaceae, and Diversisporaceae. Incidence of *Ca*Gg across AMF was not affected by any of the environmental parameters measured, whereas distribution of *Ca*Mg in one of the fungal hosts was impacted by plant density. *Ca*Mg populations associated with AMF individuals displayed high levels of genetic diversity but no evidence of gene flow, suggesting that host physical proximity is not sufficient to facilitate horizontal transmission of *Ca*Mg. Finally, in addition to a novel lineage of *Ca*Gg, we discovered that AMF likely harbor *Burkholderia*-related bacteria with close phylogenetic affinity to free-living *Burkholderia* and endobacteria of other Mucoromycota fungi.

Introduction

Arbuscular mycorrhizal fungi (AMF, subphylum Glomeromycotina) form mutualistic associations with roots of the majority of terrestrial plants [1]. They provision plants with mineral nutrients, such as phosphorus and nitrogen, in exchange for photosynthesis-derived carbon [1]. Consequently, AMF play important roles in functioning of terrestrial ecosystems and global nutrient cycling, and are of rising interest in sustainable agriculture as alternatives to non-renewable mineral fertilizers [2, 3].

For decades, AMF have been known to harbor morphologically diverse endosymbiotic bacteria in their hyphae and spores [4]. Among them, '*Candidatus* Glomeribacter gigasporarum' (*Ca*Gg, Betaproteobacteria) stands out as the most extensively studied endobacterium of AMF [5]. *Ca*Gg resides in fungus-derived vesicles inside hyphae and spores of AMF in the family Gigasporaceae. AMF spores that harbor *Ca*Gg have been shown to produce longer presymbiotic hyphae than spores that are *Ca*Gg-free [6], a phenomenon attributed to the ability of *Ca*Gg to prime energy metabolism of the fungus [7]. Despite the ancient origin of the *Ca*Gg–Gigasporaceae association, *Ca*Gg remains nonessential to its fungal host [6, 8]. In other words, AMF display facultative dependence on *Ca*Gg. In turn, *Ca*Gg cannot be cultivated in separation from AMF [9], and is obligately dependent on its host [10].

Accession numbers Sequences generated in this study are deposited at GenBank under accession numbers: MG493487–MG494246 (Table S5).

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Importantly, the *Ca*Gg–Gigasporaceae symbiosis does not appear to be in transition toward a reciprocally obligate state, in which *Ca*Gg is essential to AMF survival, as would be expected in associations where endosymbionts are vertically transmitted within host populations [8]. As a consequence, evolutionary stability of the *Ca*Gg–Gigasporaceae association in its current non-reciprocally obligate form was hypothesized to be the result of shifting environmental conditions, whereby only certain environments, such as those requiring extensive pre-symbiotic hyphal proliferation to contact the plant host, would favor *Ca*Gg presence in AMF [8]. However, the exact conditions that support *Ca*Gg incidence in AMF are unknown due to lack of ecological studies.

Another endobacterium of yet unknown lifestyle, which AMF harbor, has been recently named '*Candidatus* Moeniiplasma glomeromycotorum' (*Ca*Mg, Mollicutes) [11, 12]. Its metabolic profile and molecular evolution patterns suggest that *Ca*Mg might be a parasite of AMF [13, 14]. Importantly, *Ca*Mg and *Ca*Gg can co-exist and form an intracellular 'microbiome' of AMF [14, 15]. Similar to *Ca*Gg, *Ca*Mg is vertically transmitted via fungal spores [11]. However, in contrast to *Ca*Gg, which has only been found in the family Gigasporaceae, *Ca*Mg is widely distributed among phylogenetically distinct AMF lineages [11, 12]. Populations of *Ca*Mg in AMF isolates/spores/operational individuals exhibit unexpected intra-host genetic diversity [11–15]. Based on genomic and molecular evolution analyses, this genetic diversity is thought to be the result of symbiont horizontal transmission and recombination [13, 14, 16].

Our knowledge of the biology of AMF-associated endobacteria, their population structure, and distribution across hosts comes primarily from analyses of culture collection-derived fungal isolates, and little is known about these endobacteria in natural populations of AMF. In the present study, we set out to examine the natural distribution of *Ca*Gg and *Ca*Mg in AMF in the North Atlantic dune environment with a particular focus on: (1) surveying incidence of endobacteria in AMF, (2) exploring the effect of environmental factors on the association between AMF and endobacteria, and (3) assessing diversity of *Ca*Gg and *Ca*Mg.

The North Atlantic dune ecosystem is well suited for addressing questions concerning AMF and their endobacteria. Previous studies suggested that coastal foredune areas are dominated by AMF representing the family of Gigasporaceae [17–21], a group known to frequently harbor *Ca*Gg [8]. Abundance of AMF spores reaches maximum in late fall, when they can be isolated directly from field samples without the need for enrichment in trap cultures [17, 22]. Working directly with such field collected spores enables reliable quantification of endobacteria occurrence within AMF. As an early succession system, which is exposed to harsh environmental conditions, foredunes differ from many other natural terrestrial systems in having low plant diversity [23]. North Atlantic foredune plant communities are dominated by a native perennial pioneer species *Ammophila breviligulata*, American beachgrass [24, 25]. This near monoculture offers a convenient model in which the impact of plant community structure on AMF diversity is expected to be minimal, allowing to isolate effects of other variables. Specifically, steep environmental gradients, which extend from the ocean inland, permit for assessment of environmental impacts at small spatial scales. Typically, salinity and environmental disturbances, such as wind and substrate mobility, decrease inland from the ocean, whereas biotic pressures increase [23]. As a consequence, AMF closer to the ocean are expected to be exposed to higher abiotic pressure and disturbances relative to fungi farther away.

Our pilot survey of AMF at the Cape Cod National Seashore revealed presence of both *Ca*Gg and *Ca*Mg. Subsequent systematic sampling in the foredunes, which display the steepest environmental gradients and lowest plant diversity, indicated that *Ca*Gg and *Ca*Mg differed in their distribution patterns across AMF. Incidence of *Ca*Gg in spores of AMF was not affected by environmental parameters, whereas plant density significantly impacted distribution of *Ca*Mg. As in previous studies [12–15], individual isolates of dune AMF harbored diverse populations of *Ca*Mg. However, the lack of evidence of mixing between *Ca*Mg from different dune AMF suggested that host physical proximity is not sufficient for horizontal transmission of *Ca*Mg. Finally, in addition to a novel clade of *Ca*Gg, we discovered bacteria previously not known to be associated with Glomeromycotina, and likely hosted inside AMF hyphae and spores. These *Burkholderia*-related bacteria cluster phylogenetically with free-living *Burkholderia* and endobacteria of other Mucoromycota fungi.

Materials and methods

Pilot survey

To survey the diversity of AMF and their endobacteria at North Atlantic dunes, samples were collected haphazardly from diverse habitats in the Province Lands area of the Cape Cod National Seashore on 11 November 2010. Sampled habitats included foredunes (F, 10 samples), backdunes (B, 6 samples), transitionary dune-woodland areas (M, 10 samples), and woodlands (W, 7 samples). The Edelman auger ∅7 cm (Agrisearch Equipment) was used after removing the topmost layer of 20–40 cm of sand to reach the zone of

actively growing roots. A total of 33 soil samples were transported to the lab on ice, air dried, and stored at 4 °C until further processing.

Systematic sampling

To link diversity of AMF and their endobacteria with environmental variables, samples were collected in a systematic manner from a foredune area dominated by *A. breviligulata* (42°4'50.2''N 70°13'2.4''W) on 3 November 2013. Four transects were laid out starting from the edge of vegetation at the seaward side (corresponding to the end of the beach) and extending 100 m inland (Figure S1). Transects were placed 10, 20, and 40 m apart (10 m between Transect 1 and 2; 20 m between Transect 2 and 3; 40 m between Transect 3 and 4). A total of 44 samples were collected. Samples were taken every 10 m by collecting the soil around a plant nearest to each 10 m mark. These samples were used for AMF spore extractions and soil parameter analyses, as described further. At each sampling point, the following was recorded: (1) total number of individual plants in a 70 cm radius around the area of soil sampling, (2) distance to four nearest plants in the 70 cm radius around the area of soil sampling, later averaged to give average nearest neighbor distance (NND), and (3) total number of non-*A. breviligulata* plants. These parameters were used to estimate total plant density at each sampling point as well as dominance of *A. breviligulata* over other plants.

Spore extraction, collection, and decontamination

We used wet sieving and decanting followed by 2 M sucrose centrifugation to extract AMF spores from 50 g of air-dried soil suspended in 200 ml water as described by Daniels and Skipper [26]. Spores were collected on 0.45 µm gridded nitrate cellulose filters and selected for further processing at random under magnification following Moebius-Clune et al. [27]. In brief, the grid on the filter was used to define a transect line along which spores were sampled, collecting the spore closest to the grid intersection. To estimate the number of spores (isolates) to be genotyped per sample in order to obtain the total diversity of the population from that sample, we generated collector's curves in MOTHUR [28]. We found that successful genotyping of ±8 spores would encompass the AMF diversity in that sample at 0.05% cutoff. However, to be conservative, we aimed at surveying at least 10 spores per sample. Notably, this was not possible for all samples, as, for example, spores extracted from samples at 0 m away from the beach were difficult to genotype. Selected spores were decontaminated individually as in Mondo et al. [8]. In brief, spores were sequentially washed with 1 mM and 50 mM H_2O_2, then with 4% chloramine T, followed by a final wash with sterile nanopure water.

AMF spore identification

Following surface decontamination, each spore was crushed with a pipet tip to release its contents. Total spore DNA was whole-genome amplified using Illustra™GenomiPhi-V2 kit (GE Healthcare), and the 1/20 diluted product was used for PCR. A fragment of the fungal 28S ribosomal RNA (rRNA) gene was PCR amplified from individual spores with primers LR1 and NDL22 [29] (Table S1) using JumpStart RedTaq DNA Polymerase Master Mix (Sigma), as described in Mondo et al. [8]. PCR products were cycle sequenced with the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed at the Cornell University Biotechnology Resource Center on an ABI 3730xl DNA Analyzer (Applied Biosystems) after purification by Sephadex™ filtration, using the Performa® DTR Ultra 96-Well Plate Kit (EdgeBio, Gaithersburg, MD). DNA sequences were edited in Geneious 9.1.2 (Biomatters Ltd), aligned with MUSCLE [30], and grouped into operational taxonomic units (OTUs) at 95% similarity cutoff [27] using MOTHUR [28]. Because MOTHUR algorithms capture sequence diversity derived from degenerate bases, the majority of OTUs were singletons, that is, they were composed of a single sequence. To avoid overestimating the AMF diversity due to such singleton OTUs, we conducted further phylogenetic analyses in which we clustered the representative sequences from each OTU with reference AMF sequences to form statistically supported virtual taxonomic units (VTUs).

Screening for endobacteria

AMF spores were screened individually for incidence of *Ca*Gg by PCR with *Burkholderia*-specific primers amplifying a portion of the 23S rRNA gene [8, 31] (Table S1), followed by amplicon sequencing, as described above. *Ca*Mg was detected by gel electrophoresis of PCR products generated with *Ca*Mg-specific primers targeting a portion of the 16S rRNA gene [12] (Table S1). To dissect intra-host diversity of *Ca*Mg, its 16S rRNA sequences were subcloned for sequencing after PCR amplification with *Ca*Mg-specific primers [12] and Phusion High-Fidelity DNA polymerase (New England Biolabs) under conditions of 5-min initial denaturation at 98 °C followed by 15 cycles of 10 s at 98 °C, 30 s at 50 °C, and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. Amplicons were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Life Technologies). Plasmid DNA from recombinant bacterial colonies was amplified using the Illustra TempliPhi 100/500 DNA Amplification Kit (GE Healthcare Life Sciences).

Plasmid inserts were cycle-sequenced using T3 and T7 primers and analyzed as described above. Cloned *Ca*Mg sequences were clustered at 94% similarity level [11], followed by phylogeny reconstruction including the representative sequence from each OTU.

Phylogeny reconstructions

All DNA sequences were aligned using MUSCLE [30]. Phylogenies were reconstructed under the GTR+I+Γ nucleotide substitution model implemented in MrBayes 3.2.5 [32], with a 25% burn-in and the average standard deviation of split frequencies (<0.01) used as a convergence diagnostic.

Soil chemistry analyses

Soil samples collected during systematic foredune sampling were air dried at room temperature and stored at 4 °C until analyzed at the Cornell Nutrient Analysis Laboratory (tests #1060 and #1880). Details of the procedures are described in Moebius-Clune et al. [33]. In brief, nutrients were extracted from soil by shaking with Modified Morgan's solution and filtered through a paper filter. The filtrate was analyzed on an inductively coupled plasma emission spectrometer (ICP, Spectro Arcos) for macro- and microelements. pH of a 2:1 suspension of water and soil was determined using a Lignin pH robot. Soluble salts were extracted in a 1:1 soil–water suspension, and the electrical conductivity of the supernatant measured with a calibrated conductivity meter. Three soil samples from each transect were analyzed corresponding to distances 0, 40, and 100 m from the beach.

Statistical analyses

Linear regression was used to examine the relationship between distance from the beach and different environmental variables, including vegetation and soil chemistry characteristics. To assess the impact of soil parameters on incidence endobacteria in AMF spores, soil chemistry data from samples collected at 0, 40, and 100 m from the beach were extrapolated, separately for each transect, to the remaining samples using two different methods: (i) linear interpolation values were computed for each missing distance from the beach based on soil chemistry parameters at distances 0, 40, and 100 m, and (ii) LOESS curve was fitted to the soil chemistry parameters at distances 0, 40, and 100 m versus distance relationship, and values were computed for the remaining missing distances.

Influence of environmental parameters on endobacteria incidence in AMF spores was analyzed using the lsmeans and lme4 packages in R. General linearized mixed models with binomial distribution were used to model *Ca*Gg and *Ca*Mg incidence in AMF spores. Environmental variables (plant density, dominance of *A. breviligulata*, soil salinity, and pH) were modeled as fixed effects. To account for variability between and within transects, transect was modeled as a random effect. To account for variability between and within each sampling point within a transect, distance was modeled as a random effect nested within transect. The resulting model accounted for potential correlation in endobacteria incidence between transects and between sampling points within transects. All environmental parameters, except distance, were modeled as continuous variables. Distance was modeled as a factor variable because log odds of endobacteria incidence did not appear to be a linear function of distance. Significance of distance was tested with a likelihood ratio test. Post-hoc pairwise comparisons between distances were performed using Tukey adjustments for multiple comparisons. Influence of soil chemistry parameters (soluble salts, sodium, calcium, and pH) on incidence of endobacteria within AMF spores was modeled three independent times, using either only the data that were obtained for distances 0, 40, 100 m, or the values from the two different extrapolations described above.

Analysis of molecular diversity

To quantify the extent of diversity among *Ca*Mg genotypes, we conducted hierarchical analysis of molecular variance (AMOVA) implemented in Arlequin 3.5 [34]. We tested the null hypothesis that any variation among *Ca*Mg is due to random sampling. To estimate variance components and Φ statistics, which are F statistic analogs and reflect the correlations of genotypic diversity at different levels of hierarchical subdivision, we used p-distances computed from the alignment of the 16S rRNA gene haplotypes found in *Ca*Mg associated with VTU *Gigaspora* GAR and VTU *Acaulospora* that co-occurred spatially. The specific Φ statistics were: (i) Φ_{ST}, the correlation of the molecular diversity of random *Ca*Mg genotypes within AMF isolates relative to the correlation of random pairs of genotypes drawn from the entire *Ca*Mg diversity, (ii) Φ_{SC}, the correlation of random *Ca*Mg genotypes among AMF isolates relative to the correlation of random pairs of *Ca*Mg genotypes drawn from a AMF VTU, and (iii) Φ_{CT}, the correlation of the molecular diversity of random *Ca*Mg genotypes within AMF VTUs relative to the correlation of random pairs of genotypes drawn from the entire *Ca*Mg diversity. Statistical significance of the null hypothesis was tested by permutational analysis: 90,000 permuted matrices were generated to obtain the null distribution and to test for the significance of the variance components and the Φ statistics.

Detection of recombination

To detect recombination among *Ca*Mg associated with VTU *Gigaspora* GAR and VTU *Acaulospora* that co-occurred spatially, we used the genetic algorithm for recombination detection (GARD) method [35] available through a web interface [36]. GARD searches the sequence alignment for evidence of segment-specific phylogenies and assesses goodness of fit using the small sample AIC (AIC_C) criterion derived from a maximum likelihood model fit to each segment. To verify whether the segment-specific topologies are significantly different, the Kishino–Hasegawa test [37] is conducted. The Kishino–Hasegawa test estimates the variance of the difference between log likelihood scores of two phylogenetic trees.

Results

AMF and their endobacteria across dune habitats at Cape Cod

A pilot survey of AMF and their endobacteria across diverse dune habitats at Cape Cod, including foredunes, backdunes, dune-woodland transition, and woodland, yielded 325 AMF isolates (spores). Only ~40% of these AMF could be genotyped successfully, revealing 10 VTUs (see Materials and methods section) (Fig. 1, Table S2). In terms of abundance, over 75% of isolates were classified in the family Gigasporaceae, followed by Acaulosporaceae, which accounted for 16% of isolates. The foredunes were dominated by VTU *Gigaspora* GAR, which comprised AMF clustering with *Gi. gigantea, Gi. albida* and *Gi. rosea* (Fig. 1). Other Gigasporaceae VTUs stood out as more abundant in the remaining areas of backdunes, dune-woodland transition, and woodland (Table S2). In addition to Gigasporaceae and Acaulosporaceae, we also detected representatives of Diversisporaceae and Glomeraceae, as well as a couple of isolates whose relationship with Paraglomeraceae, Archaeosporaceae, and Ambisporaceae remained unresolved.

*Ca*Gg was found in 7% of the Gigasporaceae isolates, including VTU *Gigaspora* GAR, VTU *Scutellospora*, and VTU *Cetraspora* (Fig. 2, Table S2). In contrast, *Ca*Mg was found in nearly 50% of all AMF isolates, including representatives of Gigasporaceae, Acaulosporaceae, and Diversisporaceae (Table S2). Several isolates of Glomeraceae were consistently free of *Ca*Mg (Table S2).

Foredune AMF

To assess whether environmental factors affect the incidence of endobacteria across AMF, we conducted systematic sampling along four transects spanning a foredune area dominated by *A. breviligulata* (Figure S1). Of over 1000 AMF spores sampled, 499 could be genotyped and grouped into five VTUs: VTU *Gigaspora* GAR (68.2%), VTU *Racocetra* (22.5%), VTU *Acaulospora* (5.4%), VTU *Dentiscutata* (3.6%), and VTU *Corymbiglomus* (0.2%) (Fig. 1, Dataset S1). At the edge of the vegetation line (0 m away from the beach), AMF spores were sparse, and the ones that were isolated oftentimes failed to yield PCR amplicons of an AMF 28S rRNA gene sequence. As a result, there was a low number of AMF sequences obtained at 0 m away from the beach. VTU *Gigaspora* GAR dominated the AMF community at all sampling points except at 0 and 70 m where VTU *Acaulospora* was dominant (Fig. 3). VTU *Racocetra* increased in abundance beyond 10 m from the beach.

Foredune environmental parameters

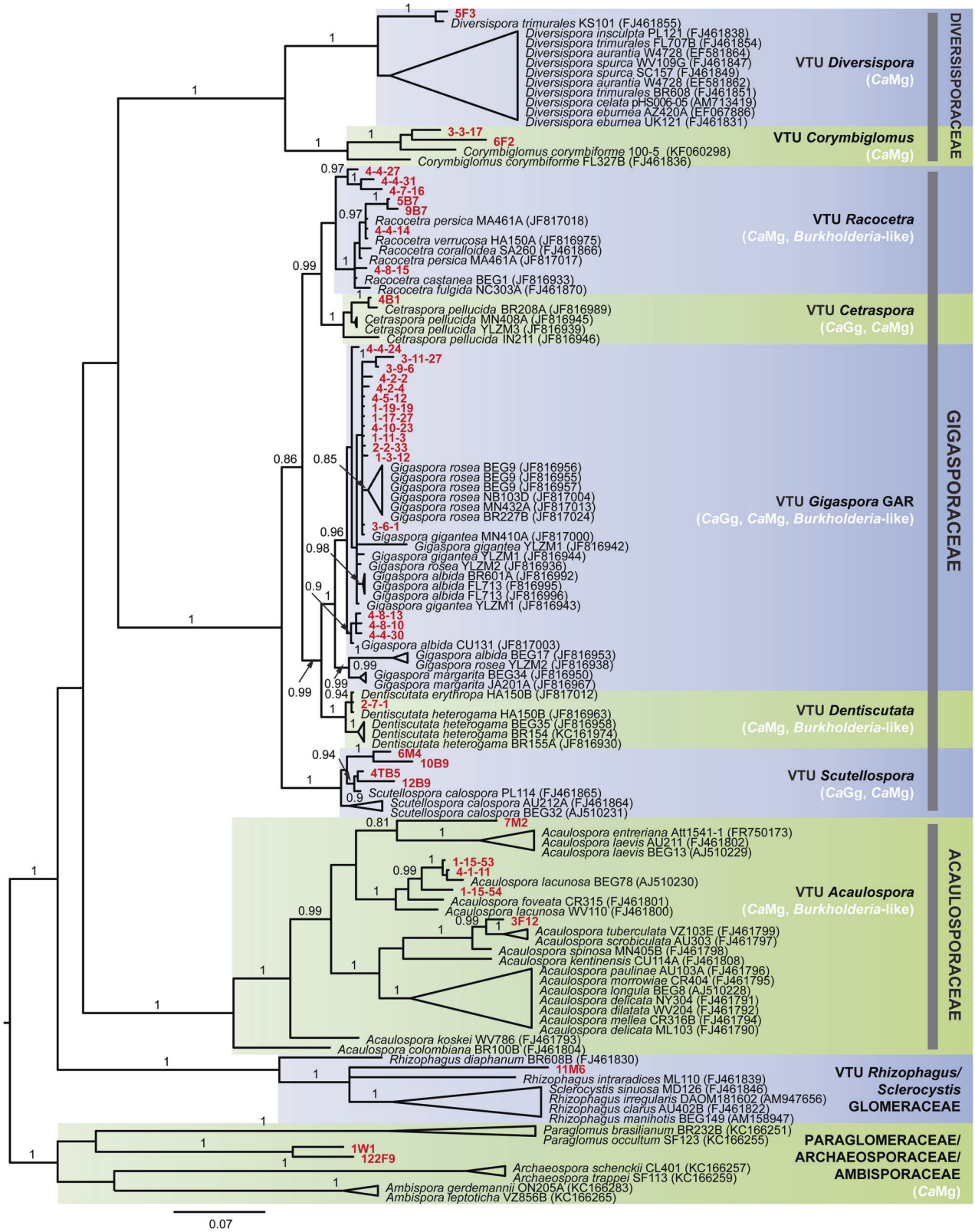
Total plant number at sampling points across the foredune study site was inversely proportional to average NND. In other words, as the number of plants at a sampling point increased, distance between them decreased ($r^2 = 0.75$, $P < 0.001$, Fig. 4a). Because total number of plants and average distance between them were so tightly correlated, we focused on total plant number as the measure of plant density. We found that plant density decreased slightly with increasing distance from the beach ($r^2 = 0.04$, $P < 0.001$, Fig. 4b), and that dominance of *A. breviligulata* also declined with increasing distance from the beach ($r^2 = 0.1$, $P < 0.001$, Fig. 4c).

The pH of soil along the foredune transects ranged from 5.5 to 7.5, with the average of 6.21 (Table S3). These values, although consistent with what has been previously reported by the National Park Service [38], did not correlate with distance from the beach. Soil salinity, measured as soluble salts (conductivity, mmhos cm^{-1}) declined with increasing distance from the beach ($r^2 = 0.37$, $P = 0.03$), as did the calcium ($r^2 = 0.65$, $P = 0.001$) and sodium ($r^2 = 0.45$, $P = 0.01$) ion concentrations (Table S3). These patterns were largely expected, because the dune environment is typically characterized by decreasing levels of soil salinity with increasing distance from the shore due to decreased exposure to sea spray [23].

Influence of environmental factors on endobacteria incidence in foredune AMF

An examination of endobacteria incidence in foredune AMF revealed a striking difference in the abundance of *Ca*Cg and *Ca*Mg. *Ca*Gg was found in only 2% of all AMF spores, and was limited to VTU *Gigaspora* GAR (Fig. 2, Dataset S1). *Ca*Mg, on the other hand, was very abundant,

with 88% of all AMF spores harboring it, including representatives of the family Gigasporaceae, as well as VTU *Acaulospora* and VTU *Corymbiglomus* (Dataset S1). All spores that supported *Ca*Gg also contained *Ca*Mg (Fig. 2), which was not always the case in AMF examined during the pilot survey (Table S2) or in culture collections [14, 15]. In



◀ **Fig. 1** AMF phylogeny based on the 28S rRNA gene sequence. Taxa in red are representatives of Cape Cod OTUs constructed by clustering at 95% similarity of sequences retrieved during the 2010 pilot survey and 2013 systematic study; the remaining are reference taxa. 2010 taxon identifiers include spore number, sampling location (F, foredune; B, backdune; M, transition area; W, woodland), and sample number; 2013 taxon identifiers denote transect number, sample number, and spore number. Numbers above branches represent Bayesian posterior probability; values above 0.8 are displayed. The complete phylogeny and sequence alignment are included in Dataset S2

particular, *Gigaspora margarita* BEG34, a model for the *Ca*Gg–Gigasporaceae symbiosis, harbors *Ca*Gg but not *Ca*Mg [12, 14].

*Ca*Mg distribution varied among the different foredune AMF VTUs. *Ca*Mg was abundant in spores of VTU *Gigaspora* GAR (90%), VTU *Racocetra* (93%), and VTU *Dentiscutata* (83%), and found in less than half of spores of VTU *Acaulospora* (46%).

Distribution of *Ca*Gg and *Ca*Mg in foredune AMF was not significantly affected by distance from the beach. Moreover, none of the environmental parameters that we measured (plant density, *A. breviligulata* dominance, salinity, pH, Ca, and Na soil content) had an impact on *Ca*Mg or *Ca*Gg distribution across all AMF isolates. However, when we modeled effect of environmental parameters on endobacteria distribution in individual VTUs, we found that in VTU *Acaulospora*, *Ca*Mg incidence was correlated with plant density ($P = 0.01$, Fig. 5). These patterns suggest that fungal host identity has an important role in how *Ca*Mg distribution is affected by environmental variables.

*Ca*Mg diversity in Cape Cod AMF

*Ca*Mg is known to exhibit high genetic diversity in culture collection isolates of AMF, often with higher levels of diversity within than among host individuals [11–15]. To determine whether this pattern was also apparent in natural populations of *Ca*Mg from sand dunes, we analyzed *Ca*Mg population structure from two distantly related AMF identified in our samples, VTU *Gigaspora* GAR and VTU *Acaulospora* (Fig. 6, Figure S2). Besides being distantly related phylogenetically, these AMF differed in *Ca*Mg incidence, with VTU *Gigaspora* GAR displaying a much higher *Ca*Mg incidence than VTU *Acaulospora* (Dataset S1).

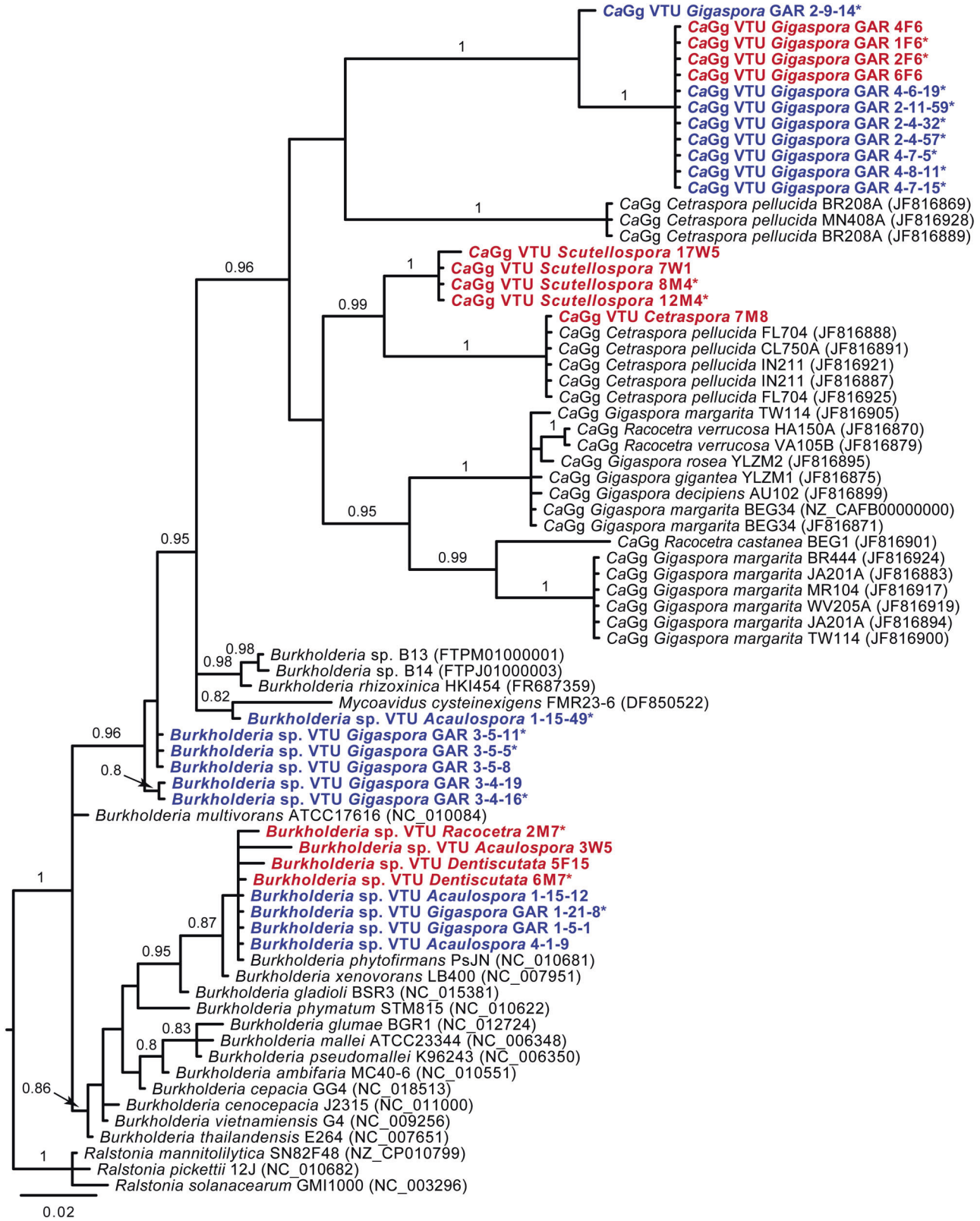
To examine *Ca*Mg diversity, we assessed unique cloned 16S rRNA gene sequences (haplotypes) from different spores of VTU *Gigaspora* GAR and VTU *Acaulospora* co-occurring within three soil samples (Fig. 6 and Figure S2). We found that individuals of VTU *Gigaspora* GAR harbored several deeply divergent *Ca*Mg haplotype clusters, which were interspersed across the phylogeny of *Ca*Mg associated with Acaulosporaceae, Diversisporaceae, and Gigasporaceae (Fig. 6). Conversely, VTU *Acaulospora* harbored two divergent haplotypes of *Ca*Mg (Fig. 6). AMOVA [34] revealed that the variance component of *Ca*Mg diversity between these two VTUs of AMF was small and, instead, high levels of diversity were apparent within *Ca*Mg populations associated with individual AMF isolates (over 70% of variance), as well as among isolates within AMF VTUs (over 27% of variance) (Table S4). The latter pattern resembles partitioning of *Ca*Mg diversity within and among AMF isolates within a geographic region [14].

*Ca*Mg transmission within AMF is predominantly vertical [11, 12]. However, *Ca*Mg molecular evolution patterns indicate a low level of horizontal transmission [14]. We tested the hypothesis of horizontal transmission in *Ca*Mg by examining the patterns of 16S rRNA gene diversity among *Ca*Mg associated with VTU *Gigaspora* GAR and VTU *Acaulospora* co-occurring within the same soil samples (Figure S2), with a particular focus on genetic recombination. GARD [35] revealed no evidence of gene exchanges among *Ca*Mg from these two distantly related hosts that co-existed spatially, suggesting that physical proximity between AMF species is not sufficient for horizontal transmission of *Ca*Mg to occur.

Analysis of *Ca*Gg diversity at Cape Cod reveals previously uncharacterized bacteria

To date, our knowledge of *Ca*Gg and *Ca*Mg population structure comes primarily from analyses of culture collection isolates of AMF [8, 13–15, 39], and little is known about these endobacteria in nature. Using 23S rRNA gene sequences PCR amplified with *Burkholderia*-specific primers, we reconstructed a phylogeny of *Ca*Gg detected at Cape Cod, and discovered a new clade that was distinct from endobacteria in culture collection isolates (Fig. 2). These *Ca*Gg sequences were recovered during both the 2010 pilot survey and the 2013 systematic study (Fig. 2, Table S2, Dataset S1). Interestingly, they shared 99.9% identity with each other, regardless of the sampling year, indicating a temporally stable population.

In addition to *Ca*Gg, *Burkholderia*-specific PCR primers revealed novel Burkholderiaceae sequences in spores of dune AMF collected during the pilot survey and systematic sampling. These sequences were repeatedly recovered from VTU *Gigaspora* GAR and VTU *Acaulospora* spores, and grouped away from the *Ca*Gg (Fig. 2, Table S2, Dataset S2). One of these *Burkholderia*-related sequences clustered with *Mycoavidus cysteinexigens*, an endosymbiont of another Mucoromycota fungus, *Mortierella elongata* [40]. The remaining sequences fell into two clusters, one grouping with free-living *Burkholderia* and the other



clustering away from both free living and other known endofungal *Burkholderia*. Considering that we surface decontaminated all spores examined, these bacteria were likely present inside the fungal cells and could represent a new group of endobacteria previously not known to live in AMF. However, fluorescence in situ hybridization

◀ **Fig. 2** Relationships between *Ca*Gg, *Burkholderia*-related endobacteria of *Rhizopus microsporus* and *Mortierella elongata*, as well as free-living *Burkholderia* based on the 23S rRNA gene sequence. Endobacteria marked with asterisks co-existed with *Ca*Mg. Taxon identifiers in red represent bacterial sequences obtained from AMF during the 2010 pilot survey, and denote AMF VTU, spore number, sampling location (F, fordune; B, backdune; M, transition area; W, woodland), and sample number. Taxon identifiers in blue represent bacteria found in AMF in 2013, and indicate AMF VTU, transect, and sample number followed by spore number. Numbers above branches represent Bayesian posterior probability, values above 0.8 are shown

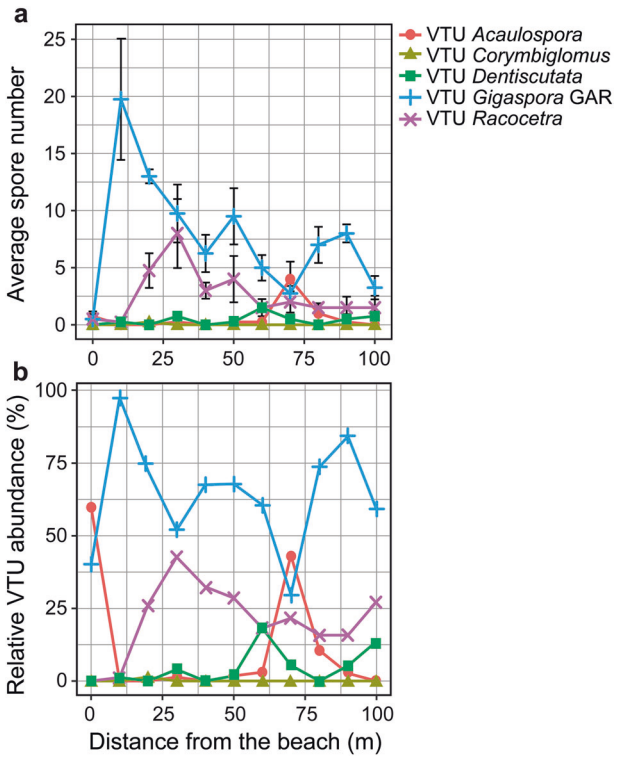


Fig. 3 Abundance and distribution of AMF at the Cape Cod National Seashore foredune study site at varying distances from the beach. **a** Abundance; error bars represent 1 SEM. **b** Relative abundance

experiments with *Burkholderia*-specific probes are needed to confirm this hypothesis.

Discussion

We found that the foredune North Atlantic study system at Cape Cod, with its nearly monospecific cover of *A. breviligulata* and steep environmental gradients, was dominated by AMF classified in the families Gigasporaceae and Acaulosporaceae. As these families were reported to dominate the AMF community at Cape Cod in the 1990s [21], our findings indicate that Gigasporaceae and Acaulosporaceae form

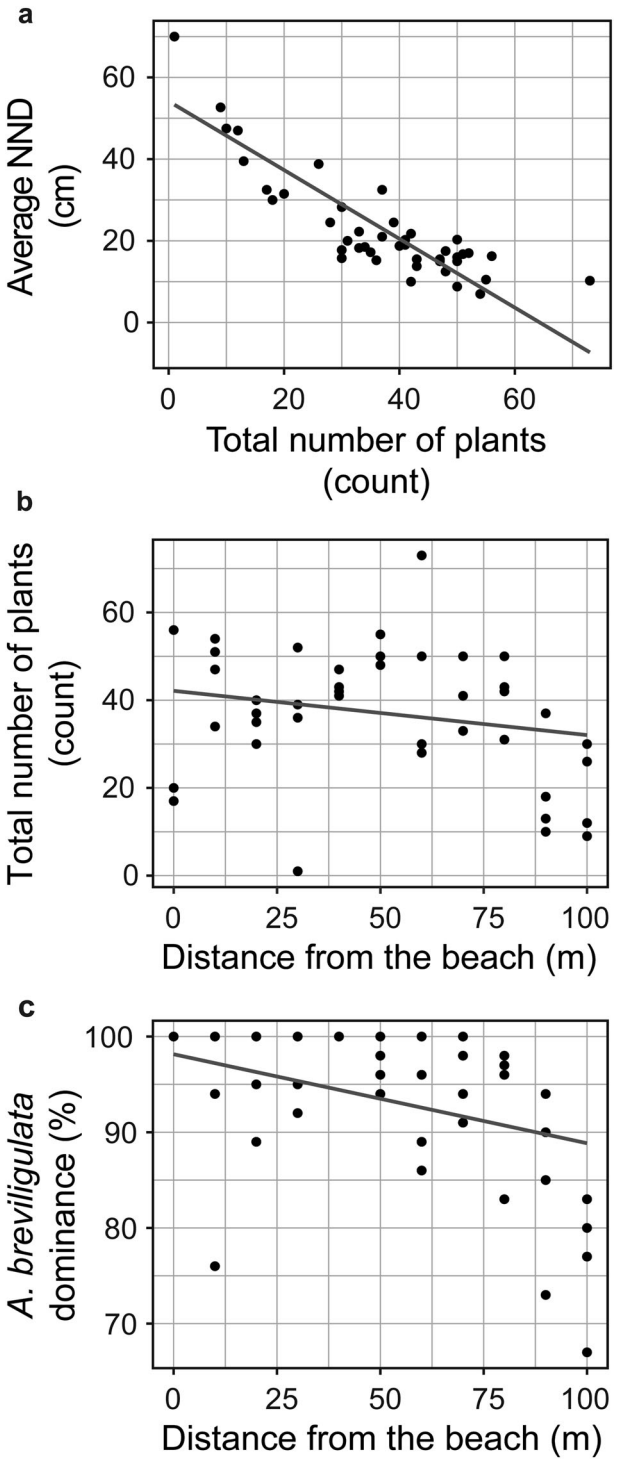


Fig. 4 Vegetation characteristics at the Cape Cod National Seashore foredune study site. **a** Relationship between average nearest neighbor distance (NND), i.e., average distance to four nearest plants in the 70 cm radius around the area of soil sampling, and total number of plants in a 70 cm radius from the sampling point. **b** Relationship between total number of plants and distance from the beach. **c** Relationship between dominance of *A. breviligulata* and distance from the beach. Linear regression was used to model the relationships in these graphs

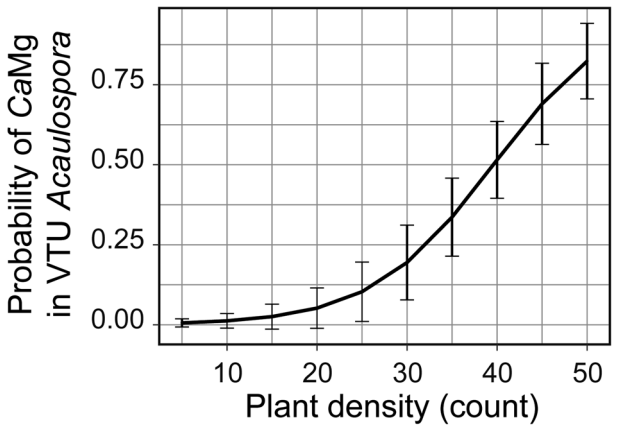


Fig. 5 Distribution of *Ca*Mg in VTU *Acaulospora* at the Cape Cod foredune study site. Probability of VTU *Acaulospora* harboring *Ca*Mg at different plant density. Error bars represent 1 SEM

temporally stable associations with dune vegetation. Despite a high relative abundance of the Gigasporaceae fungi, which have been reported to harbor *Ca*Gg in >50% of globally distributed populations [8], this endobacterium turned out to be surprisingly rare, occurring in only 2% of the Gigasporaceae isolates. In contrast, *Ca*Mg was substantially more evenly distributed across AMF spores.

Evolutionary stability of the *Ca*Gg–Gigasporaceae association in its current non-reciprocally obligate state was hypothesized to be the result of shifting environmental pressures, whereby harboring *Ca*Gg is only advantageous under certain circumstances [8]. Given experimental evidence indicating that *Ca*Gg contributes to increased proliferation of AMF pre-symbiotic hyphae that seek out and contact the plant host for symbiosis establishment [6], *Ca*Gg could be expected to be beneficial only under conditions that require extensive pre-symbiotic hyphal growth.

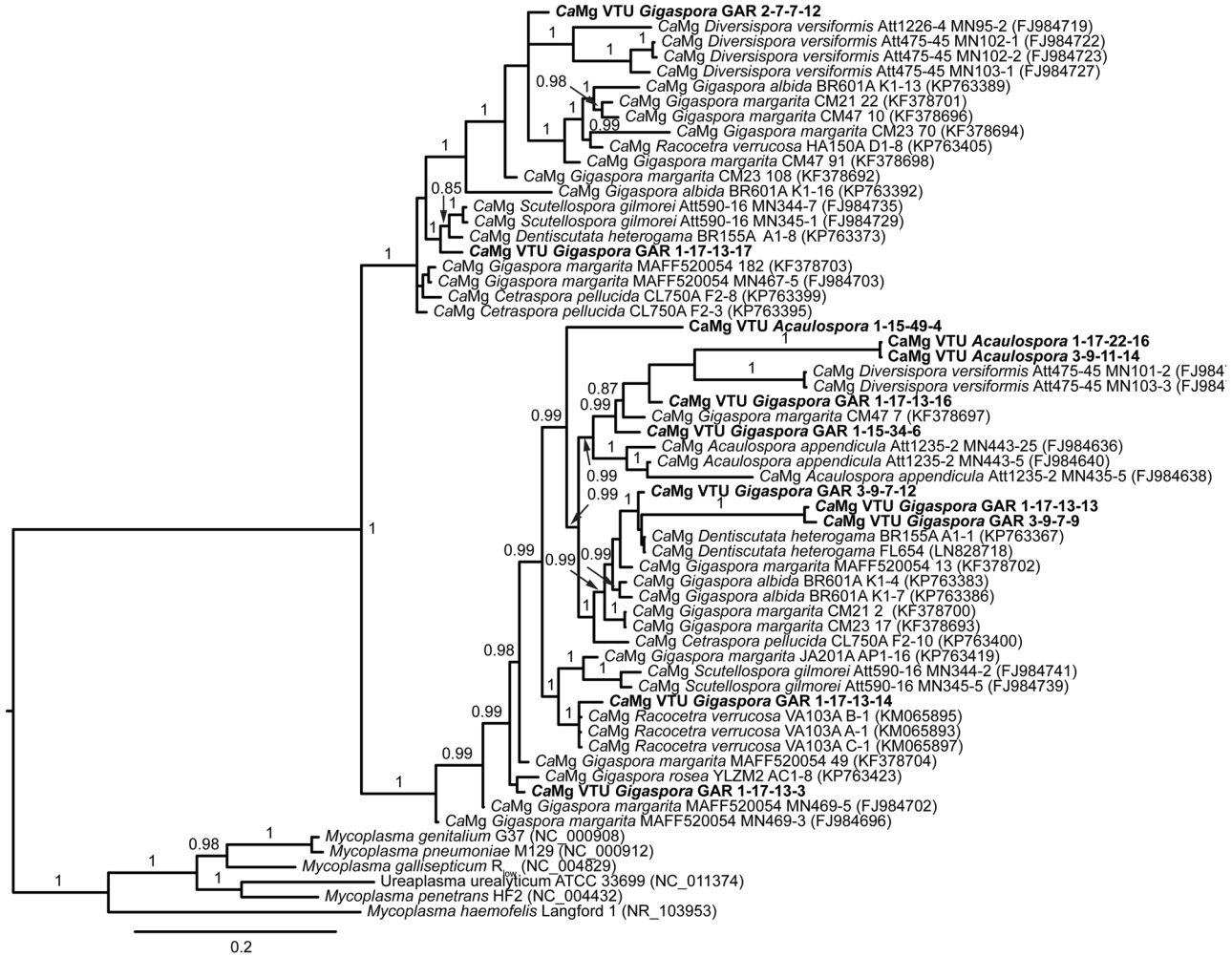


Fig. 6 Phylogeny of *Ca*Mg associated with Gigasporaceae, Acaulosporaceae, and Diversisporaceae based on the 16S rRNA gene sequence. OTUs detected at the Cape Cod foredune study site are in bold. 16S rRNA gene clones were obtained from individual spores of VTU *Acaulospora* and VTU *Gigaspora* GAR, and grouped into OTUs at 94% sequence similarity. Taxon identifiers indicate AMF VTU, sample location (transect, sample number), spore number and clone number. Numbers above branches represent Bayesian posterior probability; values above 0.8 are shown

Such conditions are likely to occur when chances of a germinating fungal spore to contact its plant host are lowered due to low plant density typified by larger distances between potential plant hosts. In Cape Cod foredunes, plant density declined with increasing distance from the ocean, with larger distances recorded between individual plants. However, we detected no correlation between *Ca*Gg presence in AMF and plant density, suggesting that plant density may not be a factor in determining *Ca*Gg incidence in AMF.

In contrast to *Ca*Gg, *Ca*Mg was extremely abundant in dune AMF. This was largely unexpected, as molecular evolution patterns suggest that *Ca*Mg may be a parasite of AMF [13, 14]. Moreover, there is experimental evidence indicating that endobacteria closely related to *Ca*Mg are conditional parasites of Mortierellomycotina, another group of Mucoromycota fungi [41]. Although the exact role of *Ca*Mg in the biology of AMF has not been established, two evolutionary scenarios could account for the persistence of a heritable parasite in AMF populations: (i) occasional *horizontal transmission* [42, 43], and (ii) *conditional mutualism* [44–46].

According to evolutionary theory, some degree of *horizontal transmission* is important for vertically transmitted symbionts that lower host fitness in antagonistic interactions [42, 43]. Otherwise, such heritable parasites are unlikely to persist in a host population. Horizontal transmission has been hypothesized to occur in *Ca*Mg [13, 14, 16], however, it was never demonstrated experimentally. At Cape Cod we found no evidence of *Ca*Mg mixing between two distantly related AMF that co-occurred within the same soil samples, suggesting that physical proximity is not sufficient to facilitate horizontal transmission of *Ca*Mg. Such absence of *Ca*Mg transfer between neighboring hosts suggests that partner genetic factors may have a role in horizontal transmission of *Ca*Mg, a hypothesis that remains to be tested.

In the absence of horizontal transmission, persistence of heritable parasites is predicted to depend on their ability to act as *conditional mutualists* that improve host fitness under specific conditions [44–46]. These specific conditions may be related to resources available to the host [44, 46]. In habitats with patchy distribution of resources, antagonistic symbionts are expected to persist even if their vertical transmission is imperfect, that is, they are not inherited by all host progeny [46]. In coastal dunes, we found that in VTU *Acaulospora*, incidence of *Ca*Mg was variable and correlated positively with plant density. If horizontal transmission of *Ca*Mg is indeed absent among dune AMF, this pattern may suggest that *Ca*Mg is a conditional mutualist presenting its AMF hosts with variable fitness outcomes that depend on resource availability represented by host plant density.

Protecting the host from another, more virulent, horizontally transmitted antagonist is another form of *conditional mutualism* expected to keep heritable parasites from extinction [45]. Although no known horizontally transmitted parasites have been characterized in AMF to date, AMF associate with soil bacteria in the mycorrhizosphere environment, and these bacteria can have both mutualistic and antagonistic/parasitic effects on the fungus [47]. In this context, testing the hypothesis that *Ca*Mg is a conditional defensive mutualist of AMF is likely to unravel a complex network of functionally uncharacterized interactions that AMF form with rhizospheric bacteria. In particular, at Cape Cod, we identified multiple AMF isolates from which we recovered DNA sequences of *Ca*Mg together with DNA of bacteria related to free-living *Burkholderia* and endobacteria of another Mucoromycota fungus. Future work is needed to confirm the intracellular location of these bacteria, examine their mode of transmission, measure impact on AMF fitness, and characterize interactions with *Ca*Mg.

Overall, the effects of environmental factors on incidence of endobacteria across dune AMF differed between the two endosymbionts and were related to the identity of host fungi. *Ca*Gg was rare despite a high relative abundance of its Gigasporaceae hosts, and its distribution was not explained by any environmental variables. *Ca*Mg was common in VTU *Gigaspora* GAR and less frequent in VTU *Acaulospora*. Importantly, incidence of *Ca*Mg in VTU *Gigaspora* GAR was not affected by environmental factors, whereas in VTU *Acaulospora*, it was favored by increasing plant density. These patterns suggest that fungal host identity is a notable determinant of how *Ca*Mg distribution across AMF responds to environmental variability.

Conclusion

Our study showed that associations between AMF and their heritable endobacteria are not easily perturbed by shifting environmental conditions that typify costal dunes. We confirmed the existence of heterogeneous populations of *Ca*Mg in AMF in nature, analogous to what has been reported in culture collection isolates of AMF. However, contrary to our expectation, we found no evidence that *Ca*Mg is horizontally transmitted between distantly related hosts occupying the same habitat. In addition, assessment of endobacteria diversity revealed a novel group of *Ca*Gg, as well as a previously unreported group of *Burkholderia*-related endobacteria in AMF. Collectively, we conducted the first ecological study of AMF-associated endobacteria and assessed their diversity and population structure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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