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INVERTEBRATE MICROBIOLOGY

(My Microbiome) Would Walk 10,000 miles: Maintenance and Turnover of Microbial Communities in Introduced Dung Beetles

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Abstract

Host-associated microbes facilitate diverse biotic and abiotic interactions between hosts and their environments. Experimental alterations of host-associated microbial communities frequently decrease host fitness, yet much less is known about if and how host-microbiome interactions are altered by natural perturbations, such as introduction events. Here, we begin to assess this question in *Onthophagus* dung beetles, a species-rich and geographically widely distributed genus whose members rely on vertically transmitted microbiota to support normal development. Specifically, we investigated to what extent microbiome community membership shifts during host introduction events and the relative significance of ancestral associations and novel environmental conditions in the structuring of microbial communities of introduced host species. Our results demonstrate that both evolutionary history and local environmental forces structure the microbial communities of these animals, but that their relative importance is shaped by the specific circumstances that characterize individual introduction events. Furthermore, we identify microbial taxa such as *Dysgonomonas* that may constitute members of the core *Onthophagus* microbiome regardless of host population or species, but also *Wolbachia* which associates with *Onthophagus* beetles in a species or even population-specific manner. We discuss the implications of our results for our understanding of the evolutionary ecology of symbiosis in dung beetles and beyond.

Keywords Holobiont · Microbiota · Phylosymbiosis · Wolbachia · 16S rRNA · Invasion

Introduction

During ontogeny, all animals face the challenge of contending with and responding to a diverse array of environmental influences. For example, host-associated microbes play important roles in the instruction of host development (e.g., nematodes: [1]; mice: [2, 3]; and cephalopods: [4]), life-history traits and timing (like metamorphosis induction in marine invertebrates: [5–7]; reproductive timing in plants: [8]; and survival-reproduction trade-offs in invertebrates: [9]), learning [10, 11], and nutritional supplementation in a variety of taxa [12, 13]. In these and many other contexts, experimental alterations of host-associated microbial communities decrease host fitness and result in pathologies [3, 14–16]. Yet much less is known about if and how host-microbiome interactions are altered during natural perturbations, for example, when hosts colonize new geographic regions or habitats. Here, we begin to assess this question in *Onthophagus* beetles which have previously been shown to rely on a vertically transmitted microbiome throughout their development [17, 18]. Specifically, we ask to what extent microbiome community membership shifts during host introduction events and the relative significance of ancestral associations and novel environmental conditions in the structuring of microbial communities of introduced host species.

Onthophagus dung beetles are one of the most species-rich genera of animals, with over 2000 described species [19]. Yet this great species richness has arisen seemingly despite the inherent challenges dung beetles face in consuming dung as their sole food source throughout all stages of their life. Dung, particularly the ruminant dung on which the vast majority of *Onthophagus* species feed, is a nutritionally challenging food source deficient in amino acids and comprised primarily of

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recalcitrant carbon sources such as cellulose and lignin [20, 21]. Dung beetles have thus long been hypothesized to meet these dietary challenges through association with symbiotic microorganisms [22, 23], and recent findings have begun to provide experimental support for this prediction. Work in *Onthophagus taurus* and the closely related genus *Euoniticellus* has demonstrated that the gut microbial communities of mothers and their offspring are highly similar, but distinct from the dung they feed on and the soil they live in; and that these gut microbes are reliably passed from mother to offspring through a "pedestal"—a fecal secretion onto which mothers oviposit their eggs ([24]; "maternal gift" in [25]). Shortly after hatching, larvae consume the pedestal before continuing on to feed upon the remainder of the brood ball provisioned for them by their mother. Parallel work has further demonstrated that the microbes found within the pedestal are functionally significant, as (1) *Onthophagus* beetles reared without their pedestal microbiota are slower to develop and enclose to smaller adults compared with individuals given access to their pedestals as larvae [18]; (2) the negative growth consequences of pedestal-free development can be erased by re-inoculating larvae with pedestal derived bacteria cultivated in the laboratory [18]; and (3) the developmental benefits conferred by pedestals are host species-specific, i.e., *Onthophagus* beetles provided another species' pedestal during the egg stage suffer negative effects to their survival, and development—a subset of which continue to persist into the next generation [17]. Taken together, a growing body of evidence thus supports the notion that *Onthophagus* beetles engage in mutualistic and at least partly host-specific interactions with vertically transmitted gut microbiota.

At the same time, *Onthophagus* dung beetles present an excellent model to understand how host introductions may influence host-associated microbial communities. Diverse *Onthophagus* species have been subjected to recent introductions outside their native range as a result of both accidental releases as well as biocontrol programs intended to curb dung accumulation and the corresponding nuisance fly populations on pastureland. For example, *O. taurus* is native to the Mediterranean but became introduced into both Western and Eastern Australia as part of a biocontrol effort starting in the late 1960s [26, 27]. These introductions entailed a rigorous quarantine procedure which included the surface sterilizing of eggs and their subsequent rearing in artificial brood balls to exclude the possibility of co-introducing potentially harmful microorganisms as well as nematodes and mites commonly associated with dung beetles [26]. Upon introduction, exotic populations were then subject to repeated harvest and redistribution efforts to aid in their further range-wide establishment. In contrast, *O. taurus* was introduced into the Eastern United States by accident around 1971 from an unknown source population [28]. From its origination in a single location in Northern Florida, this population has since expanded as far west as Texas, and as far north as the Canadian border [27, 29] yet did so without the aid of deliberate redistribution efforts by people. These repeated introductions, coupled with the divergent circumstances surrounding them, therefore make *O. taurus* a promising candidate species to investigate the impact of introduction events on microbiome composition.

In this study, we sought to compare and contrast the microbiome of *Onthophagus taurus* from native Mediterranean (MED) as well as exotic Eastern United States (EUS) and Eastern Australia (EA) ranges. Furthermore, we characterized the microbiota of three additional dung beetle species (*O. hecate*, *O. australis*, *Euoniticellus fulvus*) native to and syntopic (i.e., often occurring within the same dung pad) with *O. taurus* in each region to allow us to begin assessing the relative contributions of evolutionary history and local forces in driving microbiome assembly. Specifically, we aimed to test two hypotheses: (1) If dung beetle microbiota are structured primarily by evolutionary history, *O. taurus* microbial communities should remain similar regardless of region, and distinct from those of resident native species. (2) Alternatively, if dung beetle microbiota assembly is structured primarily by environmental factors, *O. taurus* microbial communities should be largely distinct between regions and instead more closely resemble the communities of respective native host species.

Materials and Methods

Sample Collection

Onthophagus taurus and native, sympatric, beetles were field collected from three different geographic regions and shipped to Bloomington, IN. In each region, beetle species pairs were collected on cow dung produced by cattle grazing on pastures subject to a temperate to Mediterranean-type climate. Specifically, *O. taurus* and *Euoniticellus fulvus* (final $n = 8$ each) representing the Mediterranean region (MED) were collected near Turin, Italy, while in the Eastern United States (EUS), *O. taurus* and *O. hecate* ($n = 3$ each) were collected near Morgantown, WV. Beetle abundances in this region were consistently low during the collection period, leading to a lower sample size for species collected from this region. Lastly, Eastern Australian (EA) *O. taurus* and *O. australis* ($n = 8$ each) were collected near Canberra, Australia. Immediately after arrival, all beetles were flash frozen in liquid nitrogen, and then stored at – 80 °C until used for nucleic acid extraction (Fig. 1).

RNA Extraction and Amplicon Library Preparation

We chose to analyze RNA for this study as it provides information about bacterial taxa that were alive and likely metabolically active at the time host beetles were frozen [30]. Before extraction of RNA from each sample, animals were first surface sterilized with 100 μL of 1% bleach and 0.1% Triton-X 100 solution followed by two rinses of 1 mL of deionized water. Samples were then ground in liquid nitrogen using a previously autoclaved, ceramic mortar and pestle washed with RNase away solution (Molecular BioProducts, San Diego, CA, USA). RNA was extracted from each sample using a modified RNeasy PowerSoil total RNA kit (Qiagen, Hilden, Germany) protocol after which residual genomic DNA contamination was subsequently removed using a DNase max kit (Qiagen). The quality and quantity of the cleaned, total RNA was then determined with a TapeStation 2200 (Agilent, Santa Clara, CA, USA). Samples determined to be of good quality were then converted to cDNA following the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) protocol.

Amplicon libraries of the V4 region of the 16S SSU rRNA were generated following the Earth Microbiome protocol (515F-806R primers; [31]), with some differences. HF Phusion polymerase mix (New England BioLabs, Ipswich, MA, USA) and 3% dimethylsulfoxide (DMSO) were used in PCR reactions which were cycled at 98 °C for 45 s, 60 °C for 60 s, and 72 °C for 90 s repeated 35 times in a Mastercycler gradient thermocycler (Eppendorf AG, Hamburg, Germany). Each sample was amplified in triplicate and then pooled before normalization based on concentration of DNA measured with Qubit 4 fluorometer (ThermoFisher, Waltham, MA). Final amplicon pool was cleaned following the standard QIAquick PCR purification kit (Qiagen) protocol before being sent to the Indiana University Center for Genomics and Bioinformatics (Bloomington, IN, USA) for sequencing.

Amplicon Sequencing and Processing

Pooled amplicons were sequenced using an Illumina MiSeq and 250 bp paired-end chemistry (Illumina, San Diego, CA, USA). Raw reads with primers and adapters removed were then processed using the software suite mothur v1.42.1 [32]. First, contigs were generated using the make.contigs() command. Sequences were then trimmed for length and ambiguous base pairs were removed using screen.seqs (maxambig = 0, maxlength = 275). Unique sequences were then aligned to v132 of the SILVA 16S reference alignment [33], trimmed to overlap only homologous regions, and preclustered based on a nucleotide difference of two. Chimeric sequences were identified and removed using the chimera.vsearch() command. OTUs identified as potential contaminants in the blank (all belonging to the Acinetobacter, Enterococcaceae_unclassified, Bacillales_unclassified, or Dysgonomonas lineages) were removed from all samples. Additionally, one sample (EA *australis* 2.1.19.2) identified as a likely sick animal, and all sequences classified as chloroplast, mitochondria, Archaea, Eukaryota, or unknown were removed from the dataset. Retained sequences were classified using a custom training set based on SILVA v132 at a confidence threshold of 80, and then clustered into OTUs (operational taxonomic units) with a 97% identity threshold. To generate the primary dataset used for our analyses, we then further removed all sequences classified as *Wolbachia* as this genus was so common in some samples (up to 71% of all reads) that it made analysis of other, less common, taxa difficult. All samples in this primary dataset were rarefied to about 23,000 sequences, roughly the size of the smallest sample. Finally, the get.oturep() command was used to obtain a representative DNA sequence for each OTU after which FastTree v2.1.10 [34] was used to generate an approximately maximum-likelihood phylogeny using the GTR-CAT model.

Data Analysis

Analysis of the final dataset was performed in R v3.5.3 [35] using the packages phyloseq [36], vegan [37], and ggplot2 [38]. Various alpha diversity estimates (Chao1, Shannon Index, Simpson Index) and between-Sample distances (Bray-Curtis, unweighted UniFrac, weighted UniFrac) were computed. Distance matrices were then used to cluster samples using non-metric multidimensional scaling (NMDS). Two-way ANOVA tests of the alpha diversity estimates, and PERMANOVAs and ANOSIMs on the distance matrices were used to test for statistically significant differences in microbiota composition and diversity between sample groups. Assumptions of ANOVA (normality and homoscedasticity) were validated visually (with Q-Q plots) and statistically (using Levene's test for equality of variance). Further statistical analyses of differentially abundant OTUs were performed using the mothur implementation of the Metastats program [39].

Results

Our 250 bp paired-end MiSeq run resulted in a total of 15,430,451 reads. Of these, 23%, or 3,604,277 reads, passed all quality control and cleaning steps (including the removal of highly prevalent *Wolbachia* reads), resulting in a final range of 22,646 to 173,634 reads per sample in our primary dataset. The dataset was then rarefied to the size of the smallest sample (22,646 reads). A total of 7109 bacterial OTUs were identified in the rarefied dataset at 97% identity. Rarefaction curves (Fig. S1) show that our chosen rarefaction cutoff point

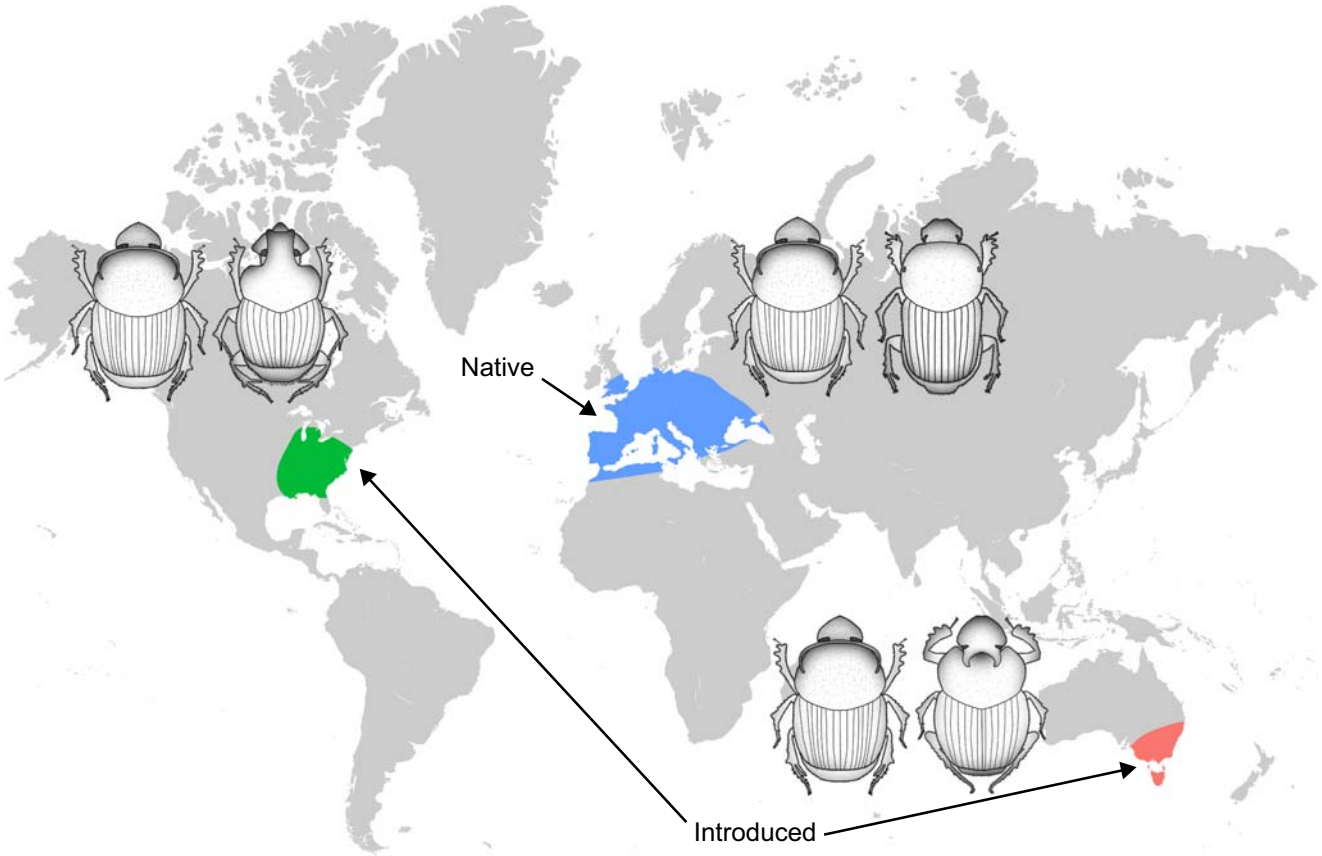

Fig. 1 Native and introduced ranges of *Onthophagus taurus* used in this study. Animals pictured are *O. taurus* (on the left) and corresponding native, syntopic, beetles selected from each region (on the right). Native species paired with *O. taurus* at each location are *O. hecate* in the Eastern US, *Euoniticellus fulvus* in the Mediterranean, and *O. australis* in Eastern Australia

captures the vast majority of microbial diversity in most samples. This conclusion was supported by estimates of Good's coverage (1 − (number of individuals in species / total number of individuals)) which ranged from 97.8 to 99.8% for all samples in the rarefied dataset. To facilitate comparisons across individuals and taxa, we generated a filtered dataset with representing common OTUs—defined as those found in at least one sample, at least 5% total relative abundance. These criteria identified 42 common OTUs (Fig. 2), 41 of which were classified to four bacterial phyla (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria), and one which was unclassified at the phylum level.

Alpha and Beta Diversity

The most abundant bacterial phylum in our primary dataset was Proteobacteria with an average abundance of 45.5% (15.4–75.3% per sample), followed by Bacteroidetes (31.3% average, 9.5–68.6% per sample), Firmicutes (15.6%, 1.02–52.1%), and Actinobacteria (5.9%, 0.327–20.9%). Reads which were unclassified, or belonged to other, rarer, phyla, accounted for the remaining 1.7%. To investigate differences in alpha diversity between samples, we calculated estimates for the Chao1, Shannon, and Simpson diversity indices (Fig. S2). We did not detect any significant differences in Shannon and Simpson alpha diversity metrics between sample groups (ANOVA, $p = 0.575$ and $p = 0.45$, respectively). In contrast, Chao1 diversity estimates were significantly different between sample groups (ANOVA, $p = 0.00792$); however, this result may have been influenced by the unusually large estimated microbial diversity of the three EUS *O. taurus*. Consistent with this interpretation, the removal of these three samples from the dataset brought the Chao1 test results in line with the other two (ANOVA, $p = 0.426$). Furthermore, no statistically significant difference was found in the within-sample group variation for any of the alpha diversity estimates for either the full (Levene's test: Shannon, $p = 0.4539$; Simpson, 0.8172; Chao1, 0.6617) or *O. taurus* only (Levene's test: Shannon, $p = 0.2671$; Simpson, 0.1658; Chao1, 0.7301) datasets.

To investigate potential differences in microbial community membership between sample groups, we performed permutational multivariate analysis of variance (PERMANOVA), analysis of similarities (ANOSIM), clustering analysis, and ordination using non-metric multidimensional scaling (NMDS). Microbial communities of samples tested were

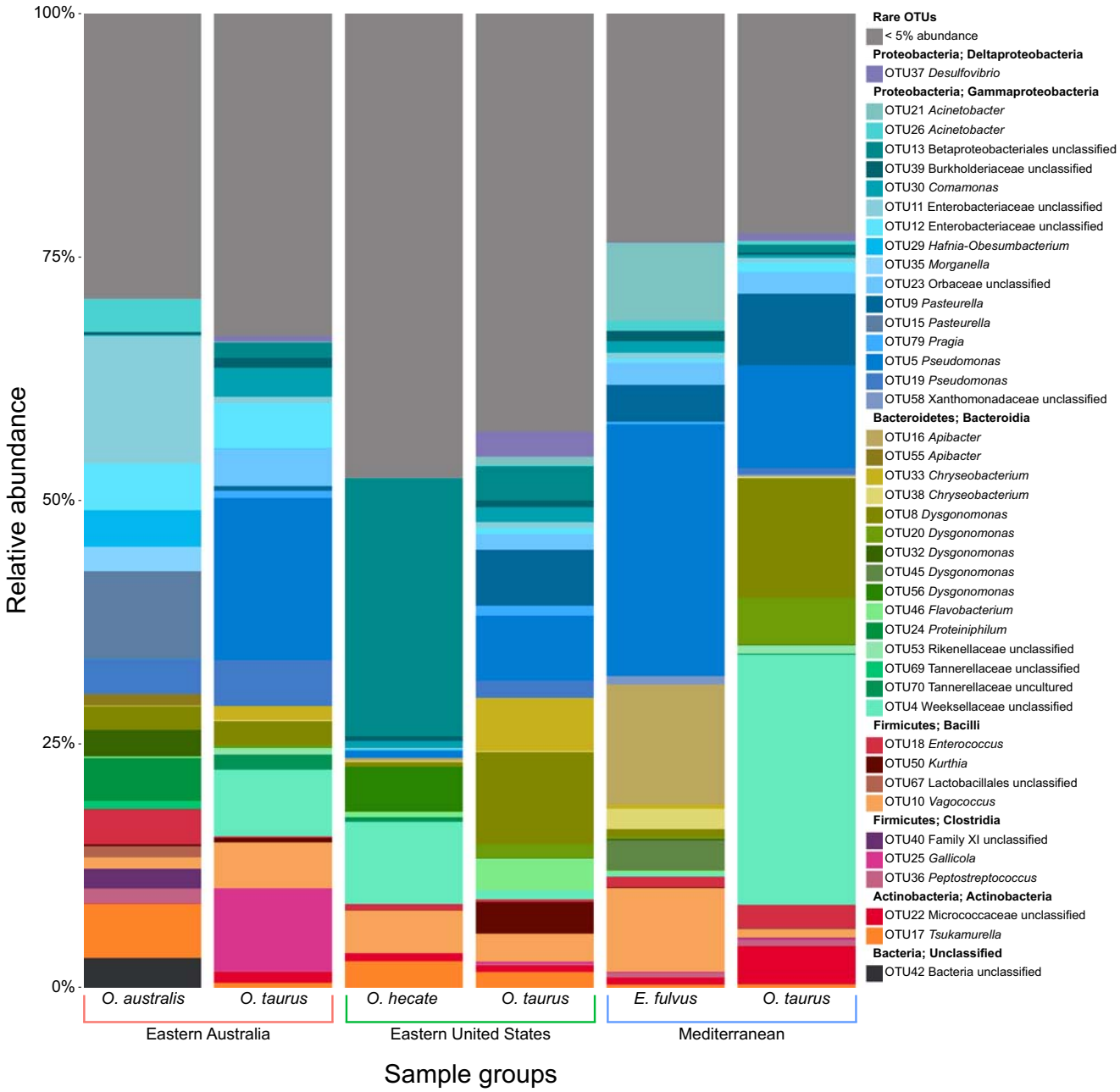

Fig. 2 Bar plot of relative average abundances of microbial taxa for each sample group. Colored blocks represent bacterial OTUs found at ≥ 5% relative abundance in at least one sample. All other rare OTUs not making this cutoff are binned into the gray bars for < 5% relative abundance. The key is sorted by phylum and then class (bolded), followed by each OTU number and the genus of that OTU

found to be significantly different based on both the beetle species they were extracted from (weighted UNIFRAC, PERMANOVA; $p < 0.001$) and the region a given beetle originated from ($p < 0.001$). Specifically, in a model that considers sequentially the region from which a given beetle was obtained and a given beetle species identity, the amount of variance explained by these two factors was 16.7% and 26%, respectively (weighted UNIFRAC; both factors were significant and explained similar amounts of variance when unweighted UNIFRAC and Bray-Curtis distances were considered). Additionally, microbial communities largely clustered in a manner that reflected both host species identity and region—save for *O. taurus* from Eastern Australia (Fig. 3). While the microbiomes of the Mediterranean samples clustered tightly both within and between species (weighted UniFrac, ANOSIM; $R = 0.687$, $p < 0.001$), those of *O. taurus* introduced to Eastern Australia were split between clustering primarily with the Mediterranean group (weighted UniFrac, ANOSIM; $R = 0.646$, $p < 0.001$) and the native Australian representative species, *O. australis* (weighted UniFrac,

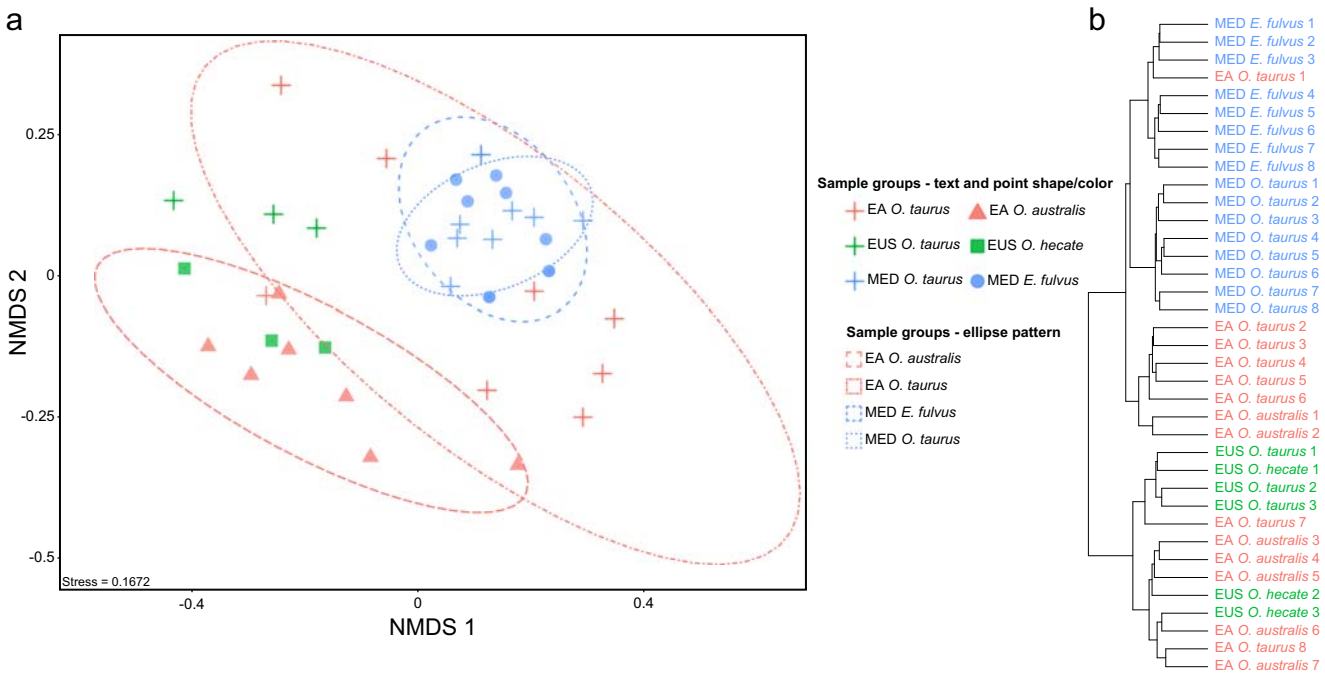

Fig. 3 (A) Non-metric multidimensional scaling (NMDS) plot of unweighted UniFrac distances for each sample. Points are colored by region, with different shapes for each species. Colored ellipses with confidence levels of 90% were generated for the EA and MED samples, but not for EUS as there were too few datapoints. (B) Cluster diagram of all samples based on unweighted UniFrac and the Ward.D2 clustering algorithm. Sample names colored by sample region

ANOSIM; $R = 0.307$, $p = 0.006$). *Onthophagus australis* showed a similar but less pronounced spread between samples, and clustered largely separately from the other groups and most closely to Australian *O. taurus* (weighted UniFrac, ANOSIM; $R = 0.307$, $p = 0.005$), likely due to the large within-sample variance seen in each group.

Common OTUs Associated with Dung Beetles

Of the 42 most common OTUs in our primary dataset (which excluded OTUs identified as contaminants and *Wolbachia*), two (OTUs 16 and 55) were classified as *Apibacter*. Reads from these OTUs were found in high abundance primarily in non-*taurus* samples collected in the Mediterranean and Eastern Australia (Fig. 2, Fig. S3). In fact, these OTUs were either completely absent from or at exceedingly low abundances in all *Onthophagus taurus* samples analyzed. Indeed, OTU 16 was found to be significantly more abundant in MED *E. fulvus* compared with MED *O. taurus* (using Metastats, average abundance of 12.3 vs < 0.01%, $p < 0.001$).

All beetle species examined, across all three regions, were found to be associated with OTUs classified as *Dysgonomonas*. Five different OTUs (8, 20, 32, 45, and 56) were found in the common dataset, with multiple OTUs often appearing in the same sample. In the larger, rarefied, dataset, 855 OTUs in total were classified to the genus *Dysgonomonas*—roughly 12% of all OTUs identified. Further, four OTUs (5, 9, 15, and 37) classified as "bacterium endosymbiont of *Onthophagus taurus*" using a training set with data from a previous study of the *O. taurus* microbiome [24]. Two of these, OTUs 9 and 15, fell within the genus *Pasteurella* while the other two, 5 and 37, were classified as *Pseudomonas* and *Desulfovibrio,* respectively. While the study which originally identified these bacteria was performed on *O. taurus* beetles only, our data suggest that these taxa may contribute to the microbiota of multiple dung beetle species. This appears particularly true for *Pseudomonas* which was seen at high abundance in nearly every sample, and also for *Pasteurella* which was common in three species: Mediterranean *O. taurus* and *Euoniticellus fulvus*, and Eastern Australian *O. australis*. However, a subset of these OTUs did exhibit marked differences in relative abundances across samples: for example, *Pasteurella* OTU 9 was significantly more common in the native, source MED *O. taurus* population as compared with the introduced EA *O. taurus* (7.3 vs 0.047% average abundance, $p < 0.001$), while the other common *Pasteurella* OTU (15) was found almost exclusively in EA *O. australis* but rare in any other sample group including EA *O. taurus* (9% vs < 0.01% average abundance, $p < 0.001$).

Finally, one OTU (4) classified as Weeksellaceae showed a largely *O. taurus*–biased association. This OTU was present in all *O. taurus* samples, often at high relative abundance (average of 15%, ranging from 0.02 to 42.5%) though it was largely absent from samples of other species. This observation of differential abundance was further supported by the results

of the Metastats program which showed that OTU 4 was significantly enriched in Mediterranean *O. taurus* (average abundance of 25.7 vs 0.29%, $p < 0.001$), and nearly so in EA *O. taurus* (average abundance of 6.8 vs 0.03%, $p = 0.117$) when compared with the corresponding native species of that region.

Wolbachia in Dung Beetles

Earlier studies have failed to identify *Wolbachia* as a member of the *O. taurus* microbiota ([24]; John (Jack) Werren, personal communications). We identified *Wolbachia* is indeed present, at times at high abundance, in a subset of populations and species (Fig. 4, Fig. S4). Specifically, four different OTUs were classified as *Wolbachia* in our primary, rarefied dataset, and 33 in the full, un-rarefied data set. Two of these (OTUs 1 and 27) were common enough to be included in our cutoff of at least 5% relative abundance in at least one sample, while the two others (OTUs 175 and 387) exhibited ≤ 10 reads in most samples.

Specifically, *Wolbachia* was most prevalent in Eastern Australian *O. taurus* and Mediterranean *Euoniticellus fulvus*. In EA *O. taurus*, OTU 1 dominated and was found at an average relative abundance of 47% (ranging from 0.01 to 71%; 95% confidence interval of 26.7–67.3%). The MED *E. fulvus* samples also carried heavy *Wolbachia* infection

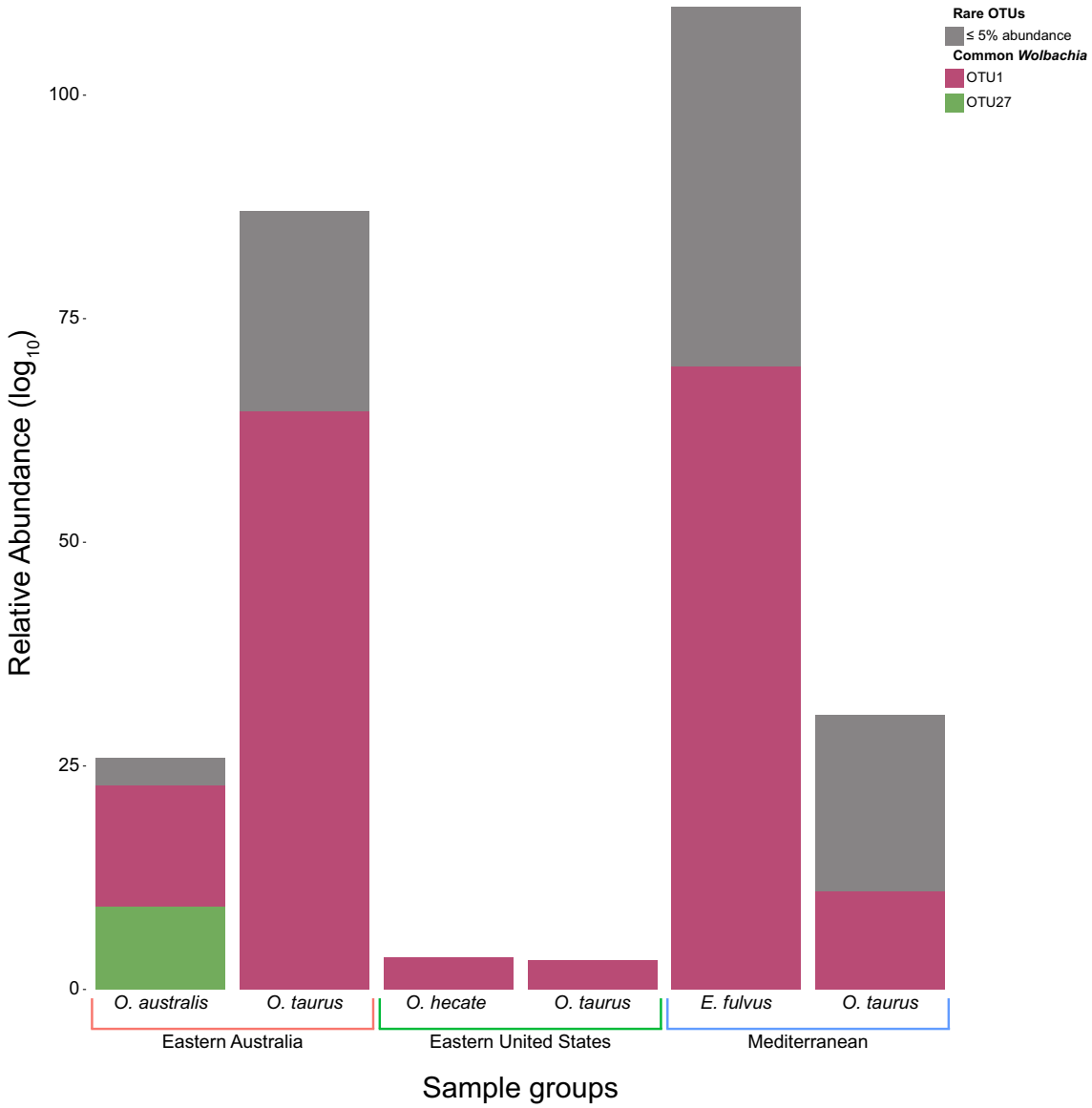

Fig. 4 Log_{10} transformed bar plot showing the relative abundance of common *Wolbachia* OTUs found in each sample group. Commonness criteria chosen was ≥ 5% relative abundance in at least one sample—all other OTUs falling below this cutoff were binned into the gray bars. Colored brackets below species names illustrate region of origin for the animals. Log_{10} transformation was used to increase legibility by correcting for large differences in abundance between samples

loads, where OTU 1 also predominated, with an average relative abundance of 26% (range 5–45%, 95% confidence interval of 17.2–35.5%). In contrast, the corresponding sympatric EA and MED populations (EA *O. australis* and MED *O. taurus*) did not show high prevalence of *Wolbachia* infection. Aside from one EA *O. australis* individual in which OTU 27 accounted for 20% of the total relative abundance, no other *O. australis* or MED *O. taurus* sample exhibited a *Wolbachia* OTU with over 0.01% relative abundance. Lastly, only a single individual (EUS *O. hecate*) was found to be completely *Wolbachia* free aside from the blank.

Discussion

Host-associated microbes influence host fitness and health by shaping diverse aspects of host biology [14], and hosts are thus predicted to evolve microbial relationships that maximize their fitness in a given environment [40]. However, when hosts colonize novel environments, microbial partnerships may shift, for instance because original modes of microbial acquisition become disrupted, the relevance of specific microbiota for host fitness is altered, or novel microbial partners become available. Yet relatively few studies have examined how microbiome composition changes in natural populations when hosts colonize novel geographic regions. In this study, we leveraged the *Onthophagus* dung beetle system to determine to what extent microbiome assemblies shift during host introduction events and the significance of ancestral associations and geography in the structuring of microbial communities of introduced species. Below we discuss our results and their most important implications.

***Onthophagus taurus* Microbiota Are Structured by both Evolutionary History and Local Environmental Forces** We find that microbiota associated with native Mediterranean *Onthophagus taurus* cluster most closely to those of native Mediterranean *Euoniticellus fulvus*. That is, even though there is relatively little microbial community variation within these populations—seen both graphically (in Fig. 3) and statistically—they emerge as each other's nearest neighbor in our analyses. Likewise, microbiota associated with exotic Eastern US *O. taurus* cluster more closely to those of *O. hecate*, a species native to the same region, than to *O. taurus* microbiota from other regions (Fig. 3). These observations suggest that local environmental conditions contribute to structuring the microbial compositions of our focal host taxa.

At the same time, we also observe patterns consistent with an influence of ancestral host-microbiome relationships on host beetles collected in non-native environments. For example, six of the eight microbiota samples derived from non-native EA *O. taurus* cluster with the clade containing microbiota associated with native MED (*O. taurus* and *E. fulvus*) beetles, yet are more distinct from the majority of syntopic EA *O. australis* (Fig. 3). This result is particularly interesting given the artificial introduction program employed to introduce exotic beetles into Australia [26]. This effort included the surface sterilization of eggs and their subsequent rearing in artificial brood balls, two measures intended to eliminate or at least disrupt microbial transfer from field collected to quarantined and field-released individuals. Yet these measures notwithstanding, the majority of EA *O. taurus* microbial communities continue to most resemble the communities seen in their native Mediterranean range. This suggests that the quarantine procedures put in place either failed or alternatively that EA *O. taurus* were able to reassemble microbial partners similar to those also utilized in their native MED region. The ability of host species to reliably guide the assembly of specific microbial communities has recently been noted in a number of study systems (e.g., insects: [41, 42]; rodents: [41, 43]; other mammals: [44]), yet the mechanisms underlying this ability remain to be determined in most instances.

Onthophagus taurus' introduction to the Eastern US in contrast did not involve any quarantine measures and instead is believed to have resulted from the accidental release of a single and small founding population. Remarkably, it was this accidental introduction that was followed by a rapid post-introduction range expansion far exceeding that observed following the deliberate releases of *O. taurus* in Eastern Australia (as well as Western Australia and California; [27]). Importantly, climatic conditions now inhabited by *O. taurus* in the Eastern US are significantly different than those observed for its native Mediterranean distribution [27]. This raises the possibility that adoption of a Eastern US range-specific microbiome could have contributed to the successful spread of *O. taurus* in this, but not other exotic ranges, similar to what has been suggested for other taxa (e.g., wasps: [45, 46]; ants: [47, 48]; and pine trees: [49]). This explanation is consistent with our observation of a shift in the microbial communities of EUS *O. taurus* animals away from the ancestral MED population, and toward a closer resemblance to the EUS *O. hecate* population (Fig. 3), even though the sample sizes of our EUS populations limit the conclusions we can reach on this front. At the same time, we presently do not know how uniform the microbial communities associated with *O. taurus* throughout its native Mediterranean range are, and therefore cannot exclude the possibility that founder effects could be contributing to the microbiome divergences observed between native and exotic *O. taurus* populations,

Microbial communities associated with native Eastern Australian *O. australis* showed a disjunct clustering, with two samples clustering with non-native Eastern Australian *O. taurus*, while the remaining five samples clustered with *O. hecate* native to the Eastern US. *Onthophagus australis* is

unusual in that it is the only native species that can be found reliably and in appreciable numbers in cow dung, whereas the remaining > 200 native Australian *Onthophagus* species are largely restricted to marsupial dung [50]. It is interesting to speculate that the composition of the *O. australis* microbiome may be reflecting this resource shift toward microbiome members more typical of bona fide cow dung specialists, though future work on other native Australian *Onthophagus* is needed to assess this possibility.

Lastly, it is important to note that our understanding of the extent to which *Onthophagus* beetles rely on vertical transmission of their microbiota as compared with horizontal transmission from the environment remains incomplete. Previous work suggests that some fraction of the microbiome is indeed vertically inherited from mother to larvae, resulting in concordance between maternal microbial OTUs and those of larval offspring [24]. Recent work also showed, however, that at least under benign laboratory conditions, several *Onthophagus* species are able to horizontally assemble functionally competent microbial communities even when their normally vertically transmitted microbiota is experimentally disrupted [17, 18]. Yet the microbial community found in cow manure (the most common food source for *O. taurus*, and the other species used in this study) is rather distinct from that inhabiting the gut of *O. taurus* beetles feeding on that same manure [24]. Evidence available to date thus suggests that *Onthophagus* beetles rely on a mix of both vertical transmission and environmental filtering to construct their microbial communities, but more work is clearly needed to determine the relative contributions of horizontal and vertical transmission to microbiota assembly in this genus.

Putatively Beneficial Dysgonomonas Symbionts Are Common Among Dung Beetle Species Even though each of the host populations we examined was associated with several unique microbial taxa, some putatively beneficial symbionts were shared across all samples, such as the numerous OTUs classified as *Dysgonomonas*. *Dysgonomonas* bacteria were seen at overall similar abundances in every sample (Fig. 2, Fig. S3), and this genus also exhibited the greatest overall diversity in the dataset (12% of all classified OTUs). Insights into the biological significance of this genus outside the context of human health are limited, but common OTUs identified in this study (8, 20, 32, 45, and 56) closely matched sequences previously identified as associated with guts of not only dung beetles (*O. taurus*: [24]; *Euoniticellus intermedius*: [25]) but also fungus-farming *Odontotermes* termites [51, 52]. Because *Dysgonomonas* is only found in *Odontotermes* fungal farms when termites are present, it has been suggested that these bacteria play a role in controlling the spread of pathogenic fungus [52]. Likewise, *Onthophagus* beetles must contend with attacks from entomopathogenic fungi such as *Metarhizium* ssp. throughout development and into adult life, and preliminary data support the hypothesis that maternally transmitted microbiota protect developing larvae from *Metarhizium* infections (Schwab et al. in prep.). Our results thus raise the possibility that *Dysgonomonas* may constitute a candidate bacterial genus for the possible synthesis of antifungal compounds able to protect their dung beetle hosts from fungal attacks. If correct, this might explain the maintenance of *Dysgonomonas* across diverse dung beetle species as well as native and recently established exotic *O. taurus* populations. Future work must now focus on directly examining the precise functional significance of this microbial taxon, and address whether strong diversifying selection for antifungal compounds may be responsible for the great OTU diversity observed for this genus within and across *Onthophagus* species and populations.

Wolbachia Infections Are Common, but Differentially Abundant, Among Dung Beetle Populations and Species *Wolbachia* are intracellular symbionts that are estimated to infect 20–66% of all insect species [53, 54]. These infections have diverse effects on host insects, ranging from beneficial (nutrient supplementation: [55] and virus protection: [56]) to conditionally deleterious (feminization, and killing of males: [57]). Despite the widespread distribution of *Wolbachia* infection among insects, only one study has so far detected *Wolbachia* in a dung beetle endemic to Thailand (*Onthophagus vaulogeri*: [58]). Our results demonstrate that *Wolbachia* may be a common member of the dung beetle microbiota, though its abundance differs greatly between species and populations (Fig. 4, Fig. S4). Interestingly, the two populations in which *Wolbachia* infections are most prevalent belong to different species and derive from different geographic regions (EA *O. taurus* and MED *Euoniticellus fulvus*). In contrast, *Wolbachia* exhibited low abundances in both native MED and introduced EUS *O. taurus.* Population-specific differences in *Wolbachia* infection rates may, as already highlighted above, reflect founder effects: the MED *O. taurus* population examined in this study may not be reflective of the populations used to fuel EA and EUS introductions of this species. Alternatively, population-specific differences in *Wolbachia* infection rates may be a consequence of the divergent circumstances associated with both introductions. Recall that *O. taurus* introduced into Australia were surface sterilized as eggs, and then reared in artificial brood balls to avoid co-introducing exotic microbes [26]. As *Wolbachia* is an intracellular endosymbiont which aggregates in female ovaries and eggs, it likely escaped this sterilization procedure. Research in mosquitos has demonstrated that the native microbiome is able to contain the spread of *Wolbachia* infection, but that when the microbiome is disrupted by antibiotics, *Wolbachia* is more readily able to infect hosts and spread [59]. This raises the possibility that *Wolbachia* bacteria may be held at low levels by the native microbiome of MED

O. taurus, but able to opportunistically proliferate in EA individuals. However, if correct, it remains unclear how EUS *O. taurus* were able to undergo a major divergence in their microbiome composition yet retain low *Wolbachia* infection rates. Possibly, this difference in outcomes might be related to the sudden versus gradual microbiome disruption seen in EA and EUS introductions, respectively. Work is underway to address these and related questions, as well as to assess the potential phenotypic consequences of *Wolbachia* infections in Onthophagus beetles.

Conclusion

The data presented here offer a first glimpse into how the *Onthophagus taurus* microbiome is shaped by host-derived and local environmental forces. We find that both factors structure the microbial communities of these animals, but that their relative importance is closely related to the unique introduction history of each population. Our results are thus compatible with both host-mediated maintenance of microbiomes across environments (i.e., phylosymbiosis, in EA *O. taurus*: [41]), but also highlight the possibility of microbiome-mediated rapid local adaptation (in EUS *O. taurus*: [60]). Even though more work is needed to further assess these implications, alongside the putative functional significance of key microbial taxa, our results underscore the promise of *Onthophagus* dung beetles as an exciting study system with which to explore the evolutionary ecology of symbiosis.

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Data Availability All 16S rRNA sequences are accessible on the NIH Sequence Read Archive (SRA) under the accession number PRJNA599403.

Compliance with Ethical Standards

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

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