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Host phylogeny and ecological associations best explain *Wolbachia* host shifts in scale insects

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Abstract

Wolbachia are among the most prevalent and widespread endosymbiotic bacteria on Earth. *Wolbachia*'s success in infecting an enormous number of arthropod species is attributed to two features: the range of phenotypes they induce in their hosts, and their ability to switch between host species. Whilst much progress has been made in elucidating their induced phenotypes, our understanding of *Wolbachia* host-shifting is still very limited: we lack answers to even fundamental questions concerning *Wolbachia*'s routes of transfer and the importance of factors influencing host shifts. Here, we investigate the diversity and host-shift patterns of *Wolbachia* in scale insects, a group of arthropods with intimate associations with other insects that make them well suited to studying host shifts. Using Illumina multitarget amplicon sequencing of *Wolbachia*-infected scale insects and their direct associates we determined the identity of all *Wolbachia* strains. We then fitted a generalized additive mixed model to our data to estimate the influence of host phylogeny and the geographical distribution on *Wolbachia* strain sharing among scale insect species. The model predicts no significant contribution of host geography but strong effects of host phylogeny, with high rates of *Wolbachia* sharing among closely related species and a sudden drop-off in sharing with increasing phylogenetic distance. We also detected the same *Wolbachia* strain in scale insects and several intimately associated species (ants, wasps and flies). This indicates putative host shifts and potential routes of transfers via these associates and highlights the importance of ecological connectivity in *Wolbachia* host-shifting.

KEYWORDS
endosymbionts, horizontal transfer, host switching, multiple infections, phylogenetic distance effect, route of transfer, strain diversity

1 | INTRODUCTION

Wolbachia is one of the best-known groups of heritable endosymbionts, widely distributed in arthropods and some nematodes (Hertig, 1936; Sironi et al., 1995; Werren, 1997). These bacteria form one of the most abundant and diverse groups of symbionts on Earth: an estimated 40%–60% of arthropod species are infected with *Wolbachia* strains (Weinert et al., 2015; Zug & Hammerstein, 2012).

Their ability to induce reproduction manipulations (Rousset et al., 1992; Werren et al., 2008) and their application in vector-borne disease control (Hoffmann et al., 2015; Kambris et al., 2009; Ross et al., 2019) are key aspects of *Wolbachia* that have been studied extensively in the last two decades.

Similar to many other symbionts, the current distribution of *Wolbachia* results from three major processes: codiversification with the host clade, shifting between host species and symbiont loss (Charleston & Perkins, 2006; Thompson, 1987). Although co-speciation is common among *Wolbachia* strains belonging to supergroups C and D in their nematode hosts (Bandi et al., 1998; Fenn & Blaxter, 2004) and certain strains of supergroup F infecting bed bugs (Balvín et al., 2018), many studies have failed to find evidence of codiversification between *Wolbachia* strains of supergroups A/B and arthropods, for example in fig wasps (Shoemaker et al., 2002), ants (Frost et al., 2010), butterflies (Ahmed et al., 2016), bees (Gerth et al., 2013) and collembolans (Ma et al., 2017). In the absence of codiversification, host-shifting is the alternative hypothesis to explain the current distribution of *Wolbachia* (reviewed in Sanaei, Charlat, et al., 2021). *Wolbachia* shift hosts when a given strain infects a novel arthropod species, mostly through horizontal transfer (Boyle et al., 1993; Heath et al., 1999) and possibly occasionally through hybridization (Cooper et al., 2019; Turelli et al., 2018). The possibility of host-shift events in *Wolbachia* has been confirmed through numerous transinfection studies when a strain is artificially introduced to an uninfected species (reviewed in Hughes & Rasgon, 2014), and the existence of "superspreader strains" that infect host species which are phylogenetically distantly related (e.g., ST41 strain type in Lepidoptera; Ilinsky & Kosterin, 2017). Physical transfer of *Wolbachia* from donor to recipient species is the first step of host-shifting, achieved via various "routes of transfer" and usually facilitated by a biological vector or a suitable environmental medium (Riegler et al., 2004; Vavre et al., 2003). Routes of transfer reported so far include prey–predator interactions (Le Clec'h et al., 2013), host–parasite interactions (Ahmed et al., 2015; Cook & Butcher, 1999; Vavre et al., 1999) and sharing a common food resource (Li et al., 2017).

Host phylogeny and ecological connectivity are thought to be the two main factors determining *Wolbachia* host-shifting. As phylogenetically closely related species are similar in many respects, including their intercellular environment and immunology (Perlman & Jaenike, 2003), it is expected that a given symbiont will shift more easily between them than between distantly related species (Charleston & Robertson, 2002). This assumption, referred to as the "phylogenetic distance effect" (PDE) (Engelstädter & Fortuna, 2019; Longdon et al., 2011), may partly explain host shifts of *Wolbachia* across closely related species. In spite of limited case studies which indicated the presence of PDE in part of the host phylogeny, such as in fig wasps (Shoemaker et al., 2002), fungus growing ants (Frost et al., 2010), bees (Gerth et al., 2013) and Collembolans (Ma et al., 2017), the influence of PDE on *Wolbachia* host-shifting is not clear. Overlapping geographical distributions of host species is another possible explanatory factor. Sharing a common habitat and consequently potential ecological interactions may lead to several direct and indirect physical contacts between a given donor and recipient host and, therefore, also increase the probability of *Wolbachia* host-shifting. Indeed, several case studies have documented host-shift events between host species that share the same habitat, for example in a rice field community (Kittayapong et al., 2003) and a mushroom habitat (Stahlhut et al., 2010).

Here, we use scale insects as a model system to gain a better understanding of *Wolbachia* host-shifting. With more than 8200 described species and 24 families, the superfamily of scale insects (Coccoidea) is globally distributed (García Morales et al., 2016; Gullan & Cook, 2007). Like many other members of the suborder Sternorrhyncha, such as aphids, whiteflies and psyllids, scale insects exclusively feed on plants and some are considered serious agricultural pests (Kondo et al., 2008). Scale insects have been recorded in ecological associations with a range of arthropods species. In particular, many are usually observed in close interactions with ants through trophallaxis (where the honeydew produced by the scale insects is consumed by ants) (Buckley & Gullan, 1991; Gullan et al., 1993; Hölldobler et al., 1990). Despite several similarities with other hemipterans, Sanaei, Lin, et al. (2021) found that most species are predicted to have low to intermediate *Wolbachia* prevalence, in contrast to a u-shaped distribution (most species have a very low or very high prevalence) predicted for other groups (Hilgenboecker et al., 2008). Also, a positive correlation between *Wolbachia* infection in scale insects and their associate ants indirectly points to a plausible route of transfer (Sanaei, Lin, et al., 2021). These preliminary results provided a broad view of the *Wolbachia* infection dynamics in scale insects and thus inspired us to investigate *Wolbachia* strain diversity and consequently host-shifting in scale insects.

Study of *Wolbachia* host-shifting requires using and developing new methodologies. To overcome technical problems associated with Sanger sequencing (see Discussion), we adopted Illumina multi-target amplicon sequencing techniques to determine the *Wolbachia* strains in scale insects and their associated species. Using this effective methodology, we revealed the strain diversity and composition (including both single and multiple infections) in scale insects. Using phylogenetic trees of both scale insects and their *Wolbachia* strains, and the geographical distribution range of each scale insect species, we assessed which factors (phylogeny or geography) best explain host-shifting events. Finally, by determining *Wolbachia* strains in individual scale insects and directly associated individuals of other species, we identified plausible routes of horizontal transfer.

2 | MATERIALS AND METHODS

2.1 | Sampling

In a previous study, we screened 689 scale insect specimens for *Wolbachia* (Sanaei, Lin, et al., 2021). Among samples with positive *Wolbachia* infection, we selected those whose multilocus sequence typing (MLST) genes (Baldo et al., 2006) were successfully amplified. This included 59 specimens belonging to 29 species and four scale insect families (Monophlebidae, Pseudococcidae, Coccidae and Eriococcidae). Of 29 species, 15 are represented by single samples, two are represented by two samples from the same population and 12 species are represented by multiple samples from more than one population. Full details are given in Table S4. Sixteen of these scale insects were collected together with a directly associated insect (including ants, wasps, flies, beetles, moths), and these were also included. The tight ecological connection between the scale insect and an associate was established either by direct observation (e.g., ant–scale insect interactions) or by rearing both members of the pair in the laboratory conditions (e.g., rearing parasitoids from the scale insect sample). Based on observation, wasps and flies are mostly parasites and moth caterpillars are predators of scale insects. We selected 16 infected pairs: five scale insect–ant, seven scale insect–wasp, two scale insect-beetle, two scale insect–fly and one scale insect–moth pair. We were unable to determine the species for any of the associates, except for the ants (*Technomyrmex albipes*) and one of the beetles (*Neopocadius pilistriatus*); however, we determined their Cytochrome Oxidase I (*COI*) barcode.

2.2 | PCR and sequencing

To be able to detect all *Wolbachia* strains even in multiple infected host individuals, we implemented an approach of Illumina multitarget amplicon sequencing. For this, we used 16S and MLST genes, which included five housekeeping genes (*coxA*, *fbpA*, *ftsZ*, *gatb*, *hcpA*), as well as the *wsp* (*Wolbachia* Surface Protein) gene (Baldo et al., 2006). Despite some limitation in using MLST (Bleidorn & Gerth, 2018), it is still a reliable method in strain determination and evolutionary history analysis (Wang et al., 2020). For the host genes, we targeted *COI*, 18 S and 28 S rRNA genes. The host genes were used later to confirm both scale insect and associate species identity and to build the host phylogeny.

As a requirement for our Illumina sequencing platform, some of the primers were redesigned to yield products shorter than 500 bp in length (Table S1). We also added Illumina-specific overhang adapters at the start (5′) of the forward and reverse primers (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG and TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, respectively). First, each amplicon was amplified separately (see Table S1 for details on each primer). MLST, *Wolbachia* 16 S and host genes were amplified using the PCR (polymerase chain reaction) configurations suggested in the original papers (Table S1). Agarose gel electrophoresis was performed to verify the success of the PCR amplifications. In the next step, 2.5 μL of the produced PCR of each amplicon (seven *Wolbachia* and three host genes) were pooled per sample (total volume of 25 μL) and sent to the Australian Centre for Ecogenomics, ACE (The University of Queensland, Australia). At ACE, library preparation was performed by a dual indexing workflow elaborated by Teh et al. (2021). At first, PCR products were amplified by NEBNext Ultra II Q5 Mastermix (New England Biolabs #M0544). The generated PCR amplicons then were purified using Agencourt AMPure XP beads (Beckman Coulter). These products were then indexed with unique 8-bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1002) with NEBNext Ultra II Q5 Mastermix. All the PCRs were conducted in the standard condition. Indexed amplicons were pooled together in equimolar concentrations and sequenced on an Illumina MiSeq Sequencing System using paired-end sequencing with V3 300-bp chemistry. As part of the workflow, the following controls were applied: (i) negative amplification control from a similar processed reagent control to monitor for contamination in library construction; (ii) single-well empty chamber controls within processing plates to monitor for cross-contamination within the library preparation; and (iii) negative index positions between runs to monitor for run-to-run bleed through designated in-line controls. Passing quality control (QC) of the resulting sequence is determined as 10,000 raw reads per sample prior to data processing and passing QC metrics in line with Illumina-supplied reagent metrics of overall Q30 for 600-bp reads of >70%.

2.3 | Wolbachia strain determination

To determine the identity of the *Wolbachia* strain in our sample, we developed an R-based (R Core Team, 2013) bioinformatics pipeline based on the DADA2 pipeline (Callahan et al., 2016), which includes a series of quality controls, trimming and mapping to the references. In addition, we blasted all generated operational taxonomic units (OTUs) against GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the *Wolbachia* MLST database (https://pubmlst.org/organisms/Wolbachia-spp). Strain determination was first conducted on single infected samples. In the next step, these strains as well as registered strains in the MLST database were utilized as references to determine all the strains in multiple infected samples. Despite our methodology being powerful enough to identify coinfecting strains, it is limited in its ability to detect intrahost recombination. Details of the pipeline are explained in Appendix S1 and Figure S1.

Wolbachia strains are commonly determined by their MLST allele. Based on data available on the MLST database, any genetic variation from one or more MLST alleles of a given strain (which can be a single nucleotide base) is defined as a distinct strain (Baldo et al., 2006). This definition of *Wolbachia* strains is controversial (Bleidorn & Gerth, 2018). Based on MLST genes, the lowest estimated and suggested pairwise distance among *Wolbachia* strains is 0.001 (Ilinsky & Kosterin, 2017). Therefore, to avoid promoting subtle variations in MLST as a new strain, we grouped strains with up to five bases difference across all seven genes (a total of ~3065 bp) into "strain groups." (e.g., grouping *w*Api1.1 and *w*Api1.2 into *w*Api1). To construct *Wolbachia* phylogeny, conduct host–parasite congruency tests, detect potential host shift events and finally run the generalized additive mixed model (GAMM), we always used strain groups instead of strains.

2.4 | Reconstruction of phylogenies

Wolbachia and host genes were aligned in GENEIOUS (version 11.0.5; Biomatters) using the MAFFT algorithm (Katoh et al., 2002). Each gene was then trimmed to have identical lengths across samples. PARTITIONFINDER2 (Lanfear et al., 2017) was used to find the best-fit partitioning scheme and substitution model for phylogeny estimation using default parameters. The results were then used as an input for estimating the maximum likelihood (ML) tree using RAXML (Stamatakis, 2014) with "Rapid Bootstrapping and Search for the Best scoring ML" and 1000 bootstrap replicates. As recombination is common among *Wolbachia* genes, the branch lengths of the *Wolbachia* phylogenetic tree were corrected with CLONALFRAMEML (Didelot & Wilson, 2015) to account for recombination events.

The MLST profile of all registered strains in the *Wolbachia* MLST depository (https://pubmlst.org/organisms/Wolbachia-spp) was downloaded (on November 5, 2020). As most of the original *Wolbachia* MLST gene fragments were longer than the gene fragments in our study, the imported database was trimmed in GENEIOUS to match the current study. The phylogenetic tree of all strains, including the reported strains in the MLST database and those from the current study, was estimated as above. This tree was used to determine the position of strains from scale insects within the various *Wolbachia* supergroups.

The phylogenetic trees of all hosts and *Wolbachia* strains, as well as the *Wolbachia*–host association network, were plotted in R by using the PHYTOOLS package (Revell, 2012). A 3D interactive bipartite graph (Appendix S3) was also created using the BIPARTITED3 R package (Terry, 2019). To test the phylogenetic congruence between *Wolbachia* and their hosts, we ran two tests. First, we performed a Parafit test (Legendre et al., 2002), which assesses the genetic distance similarity of host and parasite phylogenies. To this end, we used the parafit function implemented within the APE R-package (Paradis & Schliep, 2019) with the lingoes correction method for negative eigenvalues and 100,000 permutations. Second, we adopted the Procrustean approach (known as PACo) which assesses the similarity between host and parasite trees by estimation of Euclidean embeddings derived from distance matrices (Balbuena et al., 2013). This test, which is implemented in the PACO R package (Balbuena et al., 2013), was performed with 100,000 permutations. These two tests provide statistics to assess the independence of phylogenies by either rejecting or accepting the null hypothesis that the similarity between the trees is not higher than expected by chance.

2.5 | Factors determining host shifts

An expanded version of a GAMM, originally developed for viral sharing across mammal species (Albery et al., 2020), was applied by using the MGCV package in R (Wood, 2011). This GAMM allowed us to model a nonlinear fit between our explanatory and response variables and allowed us to more readily account for their uneven distributions. Specifically, this model examined the probability of a given pair of scale insect species sharing one or more *Wolbachia* symbionts, as a function of their phylogenetic and geographical similarity, with a logistic link function:

$$\text{Wolbachia}\ (0/1) \sim \text{phylogenetic distance} + \text{home range overlap} + \text{geographical distance}$$

Phylogenetic distance was inferred from the Australian scale insect phylogenetic tree as explained above. To quantify habitat sharing between scale insect species, we constructed each species' geographical range using their observed locations. For all species with five or more samples, we constructed a minimum convex polygon (MCP) in R. The coordinates for the MCP (Table S6) were collected from various sources, mainly including the LGC collection (Cook Lab, School of Biological Science, The University of Queensland), ScaleNet (García Morales et al., 2016), the Atlas of Living Australia website (https://www.ala.org.au/) and several published articles (Table S6). For each pair of species, we calculated the overlap of these polygons as a proportion of both species' total range size. We also derived Euclidean distances between species' sampling locations by calculating pairwise distances between species' centroids. Species with fewer than five geographical observations were not included in the model. We also excluded *Coccus formicarii* which was collected from Taiwan and therefore difficult to fit in the model. A total of 22 species were included in the GAMM.

We fitted phylogenetic distance, home range overlap and geographical distance as explanatory variables, and we fitted paired species' identities as multimembership random effects to account for variation in richness and sampling frequency between species (Albery et al., 2020). To quantify their impact on model fit we examined the change in deviance information criterion (DIC), where a change in 2 DIC was taken to represent an improved model. To avoid fitting too many variables in the model, we sequentially added each pairwise term, retaining the one that most improved model fit, and then repeating the process with the remaining variables, until no remaining variables improved the model. All R scripts developed and used in this study are provided in Appendix S2.

3 | RESULTS

The output of our bioinformatics pipeline successfully recovered 94.03% of all *Wolbachia* amplicons and yielded read count numbers between 4658 and 36,548 per sample (summed over all seven *Wolbachia* genes; for details see Appendix S1, Tables S2–S4). This high coverage enabled us to identify a diversity of *Wolbachia* strains within and across hosts. Among 75 *Wolbachia*-positive samples (59 scale insect samples and 16 associates), 68% were infected with a single *Wolbachia* strain and 32% were infected with more than one *Wolbachia* strain (20 double and four triple infected). Among nine scale insect species with samples from more than one population, intraspecies infection polymorphism was observed in *Cryptes baccatus*, *Eriococcus confusus*, *Icerya purchasi*, *Parasaissetia nigra* and *Sphaerococcus ferrugineus* (Table S2). Among 29 scale insect species that were screened in this study, six species were always found co-infected (Table S2, Figure S3, Appendix S3). For example, two screened samples of *Akermes scrobiculatus* were co-infected with strains belonging to Supergroup A (*w*Ake1) and Supergroup B (*w*Ake2) and two samples of *Cystococcus echiniformis* were co-infected with strains belonging to Supergroup A (*w*Sph1) and Supergroup F (*w*Cys1).

A total of 62 strains were identified and clustered into 31 strain groups belonging to three *Wolbachia*-supergroups (Figures 1, Figure S2, Table S3). Most of the strains belong to Supergroups A (38) and B (21), but we also identified three strains groups from Supergroup F: *w*Cys1 and *w*Sph5 (respectively infecting *Cy. echiniformis* and *Sp. ferrugineus*), and *w*Sph3 (infecting two specimens of *Sp. ferrugineus*). Based on the MLST database, these are the first Supergroup F strains reported in Australia. Although *w*Cys1 is placed within Supergroup F, it forms a unique clade compared to other reported Supergroup F strains (Figure S2).

The most diverse and abundant *Wolbachia* strain group in our data set is *w*Sph1, which includes 12 closely related strains and was detected in 23 samples belonging to eight scale insect, four wasp and one ant species (Figures 1, S3, Appendix S3). Similar strains which are grouped within *w*Sph1 were reported before in several Australian ant species (MLST sequence type [ST] =54, 19, 478 and 112) (Russell, 2012). Based on the MLST database, it appears that this strain group has a cosmopolitan distribution (Oceania, North America, Europe, Asia and South Africa) and has already been reported in various insect groups (e.g., ST 19 in Coleoptera, Hymenoptera, Lepidoptera and Orthoptera) (Table S5). By contrast, some of the scale insect species are infected with unique *Wolbachia* strains that were not observed in other scale insects or reported in any other insects (by searching both MLST and GenBank on October 1, 2021), including infection of *Apiomorpha variabilis* with *w*Aphi1 and co-infection of *Coccus hesperidium* with *w*Coc1 and *w*Coc2.

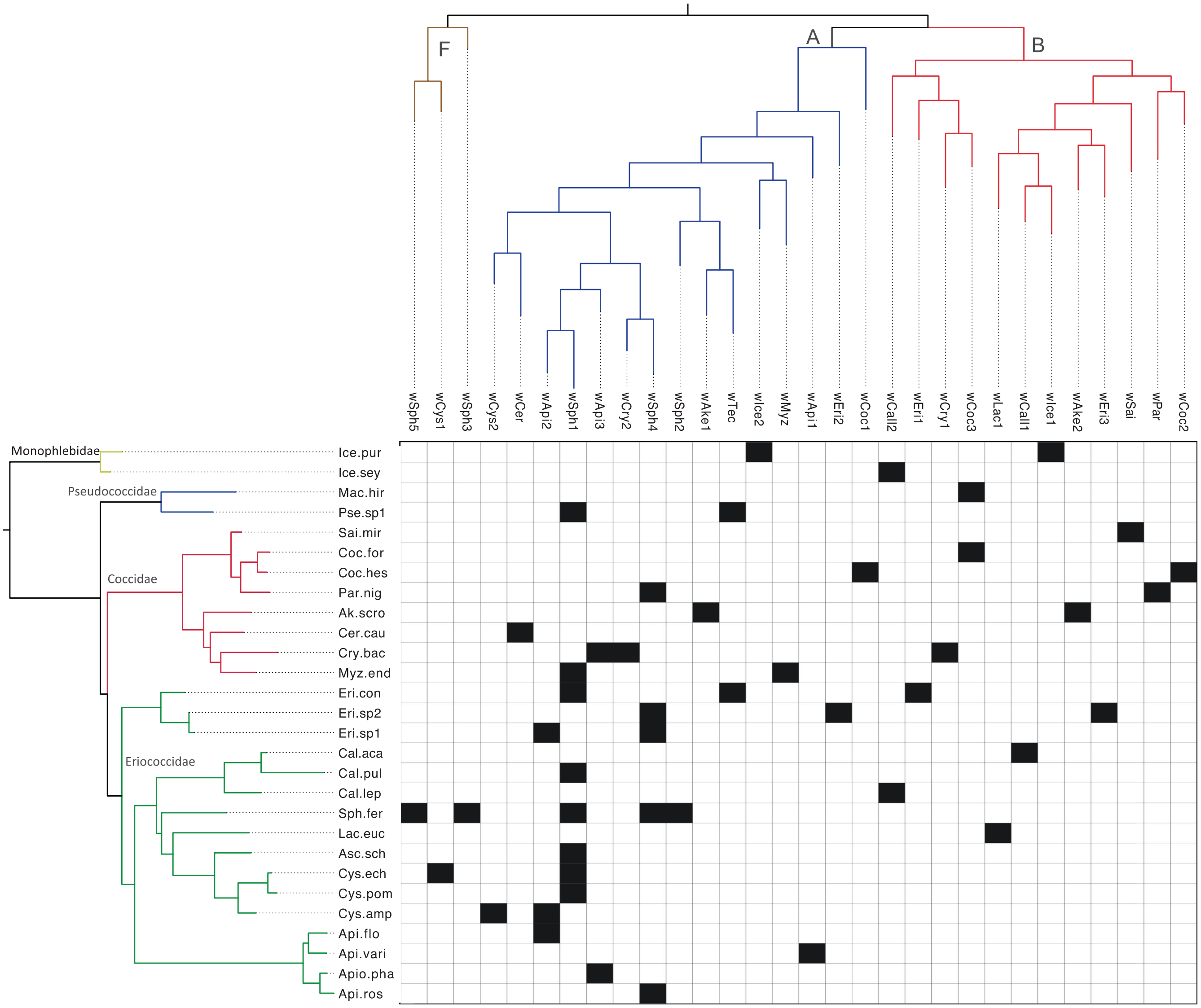

FIGURE 1 Phylogenetic tree of *Wolbachia* strain groups (top) and their scale insect hosts (left), with the black squares in the matrix indicating which host species are infected with which *Wolbachia* strain group(s). Colours in the phylogenies represent supergroups in *Wolbachia* and host families, respectively. Host species are represented by codes (for full species names see Table S4).

ML trees based on a 947-bp alignment of scale insect genes (including *COI*, 28S and 18S), and based on 3065-bp aligned *Wolbachia* genes (including MLST, *wsp* and 16S) are shown in Figure 1. In addition, an interactive figure of *Wolbachia* sharing among all host species, including associates, is provided in Appendix S3. Evidence of numerous host-shifting events can be seen in these figures. Both the Parafit test (ParaFitGlobal = 0.0008, $p = .27$) and the PACo test ($m^2_{XY} = 49.3897$, $p = .2056$) were nonsignificant. Therefore, there is no evidence of phylogenetic congruence between *Wolbachia* and their scale insect hosts, in the sense that nonindependent evolution of the two groups is not supported by these tests.

Our *Wolbachia* sharing models revealed that incorporating phylogenetic distance substantially improved model fit (change in DIC = −5.88) and had a significant effect in the model ($p < .0001$). The effect was highly nonlinear, with high sharing probabilities at high relatedness that quickly dropped to near zero at greater phylogenetic distances (Figure 2). In contrast, incorporating geographical home range overlap slightly improved model fit (change in DIC = −2.76), but had no significant effect in the model ($p = .199$). Inspecting the shape of the effect was not revealing. Furthermore, there was no significant effect of geographical distance between sampling locations (change in DIC > −2). Therefore, we do not interpret this effect as representing strong support for geographical effects on *Wolbachia* sharing.

In searching for *Wolbachia* strains in pairs consisting of a scale insect and its direct associate, we found the same *Wolbachia* strain groups in one out of five ants, one out of two flies and three out of seven wasps (Figure S4, Appendix S3). In the ant–*Eriococcus* sp1 pair that is co-infected with *w*Sph4 and *w*Apio2 strain groups, both the ant and the scale insect are infected with the same *w*Sph4 strain (*w*Sph4.4), but different *w*Apio2 strains (*w*Apio2.4 in the ant and *w*Apio2.5 in the scale insect, which differ by 4 bp in the *gatB* gene). The three wasp–scale insect pairs with similar *Wolbachia* strain groups have a single infection (wasp–*Ascelis schraderi* pair with *w*Sph1.11, wasp–*Apiomorpha floralis* pair with *w*Api2.1 and wasp–*Lachnodius eucalypti* pair with *w*Lac). The fly–*Icerya seychellarum* pair share identical strains of *w*Cal2.2. We did not find the same strain group in the single tested moth–scale insect and two beetle–scale insect pairs. As in scale insects overall, the most common strain group shared between scale insects and associates is the *w*Sph1 strain group (Table S2).

4 | DISCUSSION

4.1 | Amplicon sequencing as a powerful method of *Wolbachia* strain determination

Strain determination is a key step in studying *Wolbachia* distribution and host-shifting among a given host group that needs to be performed using an efficient method. Given that infection with more than one *Wolbachia* strain is common in various arthropod groups (Hiroki et al., 2004; Hou et al., 2020; Narita et al., 2007; Perrot-Minnot et al., 1996; Werren et al., 1995), strain determination methods should be able to distinguish and identify strains in both singly and multiply infected samples. The traditional method of using Sanger sequencing is not effective in dealing with co-infected arthropod samples, and improvements such as using different primers and cloning (Schuster, 2008; Vo & Jedlicka, 2014) are costlier and more labour-intensive, and also have limitations (Schuler et al., 2011; Van Borm et al., 2003). High-throughput whole genome sequencing (WGS) would seem to be the most accurate available methodology for strain identification, but this approach has its own difficulties (Bleidorn & Gerth, 2018). First, given that *Wolbachia* is not culturable, it is challenging to obtain genetic material enriched for *Wolbachia* relative to host DNA, possibly resulting in low sequencing depth. Second, even with high sequencing depth, assembling *Wolbachia* genomes can be difficult due to a high density of mobile elements (Wang et al., 2019) and thus only draft genomes can be recovered. Finally, the still relatively high costs of WGS make this approach less applicable in large *Wolbachia* surveys. Due to these limitations, only 33 *Wolbachia* annotated whole genomes have been publicly available on GenBank so far (as of October 2021). As suggested by Bleidorn and Gerth (2018), instead of whole genomes, sequencing and assembling a *Wolbachia* draft genome is sufficient for strain determination. However, many more draft genomes should be publicly available first to provide a reliable reference bank for strain determination. Although a draft genome can indeed be mapped to the selective marker amplicons (e.g., MLST), generating such data for large surveys is still time- and cost-intensive. To overcome these technical obstacles, we suggest Illumina multitarget amplicon sequencing as a middle-ground, efficient and affordable method that can be applied to large surveys and is also capable of dealing with multiple infections. In particular, the five *Wolbachia* MLST genes along with *wsp* and 16 S used in our study appear to be well suited to distinguish between strains, as has also been shown in a recent comparative study of available whole genomes of *Wolbachia* (Wang et al., 2020).

4.2 | Wolbachia diversity in scale insects

This study revealed that a substantial portion of tested scale insects are infected with more than one strain of *Wolbachia* (27% double and 5% triple infected). We also found *Wolbachia* multiple infections in associate species (including wasps and ants), indicating co-infection might be a common phenomenon in most of these insect groups. However, it is important to caution that detecting a given *Wolbachia* strain in a given host is not conclusive evidence of a stable infection, and laboratory assays should be conducted to ascertain *Wolbachia* maternal transmission and establishment within the host population (Chrostek et al., 2017). Moreover, in the case of parasitoids and predators, a detected strain may derive from their undigested prey rather than the screened insect itself (Ross et al., 2020). Unfortunately, laboratory rearing of collected samples is not feasible for large *Wolbachia* surveys such as the current study. Therefore, any interpretation from this type of data should be treated with caution.

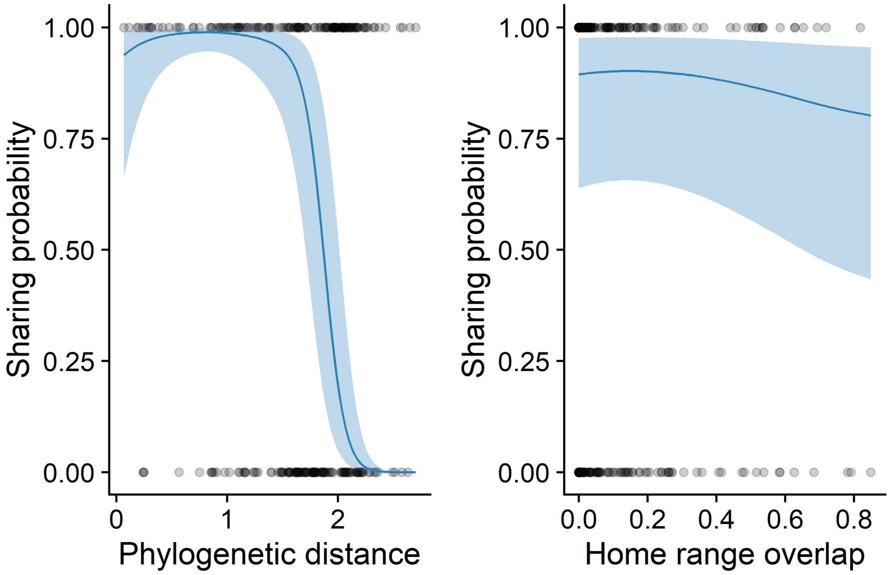

FIGURE 2 The effect of host phylogenetic distance (left) and home range overlap (right) on *Wolbachia* sharing probability. Points represent pairs of host species that either share (1) or do not share (0) the same *Wolbachia* strain; the thick blue line represents the mean predicted effect from our *Wolbachia* sharing GAMMs. The light blue ribbon represents the 95% confidence intervals of that effect. Phylogenetic distance is the sum of the branch lengths (i.e., the estimated numbers of substitutions derived from the maximum likelihood tree). Sharing decreased with both phylogenetic and geographical distance, but the phylogenetic effect was significant and much steeper.

Based on the MLST database (as of August 31, 2021), 24 strains with complete MLST gene sequences had previously been reported from the Australian fauna (https://pubmlst.org/organisms/wolbachia-spp). Here, we report 62 new strains (belonging to 31 strain groups) for Australia, including the first three Supergroup F strains in Australasia (Table S3). Apart from two strains (*w*Sph4.1 = ST 289, and *w*Cal = ST 357), none of the strains in the current study were 100% identical to any registered in the MLST database. As our sequenced regions were slightly (~5%) smaller than the MLST amplicons available on the MLST online database, there is a possibility that the two strains that were identical to the MLST profiles were different in the remaining part of the gene fragments. We found *w*Sph1 to be the most common and widely distributed strain group in Australia (detected in seven scale insects, four wasps and one ant species). Based on the phylogenetic tree of all reported strains in the MLST database and the current study strains, there are six registered strains (STs) within the *w*Sph1 strain group (Figure S2). These strains seem to be globally distributed across various insect orders. For example, one of the strains in this group, registered as ST = 19, has been reported in 16 different host species belonging to four insect orders. This broad host range may be an indicator of an extraordinary host-shifting ability of *w*Sph1. Mostly based on the number of infected host species, several *Wolbachia* strains have been reported with a similar ability, for example HVR-2 in ants (Tolley et al., 2019), ST41 in Lepidoptera (Ilinsky & Kosterin, 2017) and *w*Hypera in weevils (Sanaei et al., 2019). Among all the superspreaders, *w*Ri is one of the best-studied *Wolbachia* strain groups that has rapidly (within 14,000 years) naturally infected five *Drosophila* species (Turelli et al., 2018). *w*Ri can also be introduced to mosquitoes by transinfection, corroborating this strain's potential to infect new host species (Fraser et al., 2017). Compared to *w*Ri, it seems that *w*Sph1 has been reported in a higher number of host species that are taxonomically more diversified (belonging to various insect orders). Although the *w*Ri group has an extensive genomic diversity (Ishmael et al., 2009; Turelli et al., 2018), low variation has been observed within its MLST profiles (https://pubmlst.org/organisms/wolbachia-spp). Four strains have been reported in the *w*Ri group and only one strain (ST = 17) has been reported in more than one species of *Drosophila* (based on the MLST website as of August 31, 2021). Therefore, *w*Sph1 might have a higher diversity than *w*Ri and may therefore have the potential to be artificially introduced to other insects for human applications (e.g., controlling vector-borne disease). However, transinfection studies are necessary to ascertain the host-shifting ability of *w*Sph1 in laboratory conditions.

4.3 | Phylogenetic distance effect can explain host-shifting

There was no signal of congruence between *Wolbachia* and scale insect phylogenetic trees, as is typical of *Wolbachia* infection in an arthropod family. Instead, the current distribution of *Wolbachia* in scale insects was probably shaped by host-shifting. Among many potential factors determining host shifts, it seems that host phylogeny and geographical distributions are two major players (Sanaei, Charlat, et al., 2021). Combining data from 25 transinfection studies, Russell et al. (2009) showed that there is a positive correlation between host phylogenetic relatedness and success of the *Wolbachia* transinfection. In addition, by focusing only on a part of the host phylogenetic tree, several studies uncovered a pattern of host-shifting among closely related species (Guz et al., 2012; Haine et al., 2005; Turelli et al., 2018). On the other hand, the observation of identical *Wolbachia* strains in species that live in the same area points to a role of geography in host-shifting (Gupta et al., 2021; Kittayapong et al., 2003; Morrow et al., 2014; Stahlhut et al., 2010). The relative contributions of the host phylogenetic and geographical distance effect on *Wolbachia* host shifts are poorly understood. Here, we tried to evaluate these two factors in *Wolbachia* host-shifting by using a powerful statistical method. The results of our GAMM indicate that host shifts in scale insects can be mainly explained by the phylogenetic distance effect (host-shifting is more feasible between closely related species compared to distantly related species) (Figure 2). This result is in line with numerous examples of finding the same *Wolbachia* strain group in congeneric species, for example *wHypera1* in the genus *Hypera* (Coleoptera) (Sanaei et al., 2019), *w*Lev in the genus *Lutzomyia* (Diptera) (Vivero et al., 2017) and ST19 in the genus *Bicyclus* (Lepidoptera) (Duplouy & Brattström, 2018).

Horizontal transfer of parasites/symbionts among closely related species can generate a phylogenetic signal similar to host–parasite cospeciation (De Vienne et al., 2007). However, there is indirect evidence advocating *Wolbachia* sharing patterns in scale insects that can be explained best by recent host-shifting. In contrast to horizontal transmission which occurs rapidly, cospeciation happens in an evolutionary time frame which allows *Wolbachia* genes to be mutated. By investigating *Wolbachia* infection in the *Nasonia* species complex, it is estimated that the mutation rate of *Wolbachia* MLST genes is one third that of their host nuclear genes (from nine single-copy nuclear regions) (Raychoudhury et al., 2009). Although this ratio can be slightly different among various host species and *Wolbachia* strains (see also Conner et al., 2017), it can be adopted as a tool to distinguish codiversifications from recent host-shifting. Given that the lowest pairwise distance between host species nuclear genes that we have in our data set is 2%, in the case of *Wolbachia* cospeciation, at least 17-bp differences (out of 2608 bp) should be observed between two closely related strains. We infer host-shift events based on sharing either identical strains or identical strain groups (which includes strains with up to only 5-bp differences across all *Wolbachia* amplicons) (Appendix S1). In addition, in 73% of determined host-shift events in scale insects, shared *Wolbachia* strains have identical *wsp* genes, which is less conserved compared to the MLST genes.

Interestingly, the significant impact of host phylogenetic distance on *Wolbachia* strain group sharing was not mirrored by statistically significant tests for host and *Wolbachia* phylogenetic tree nonindependence (Parafit and Paco), as might have been expected under host-shifting with a PDE. We speculate that these tests for tree independence are not sufficiently powerful to detect minor departures from tree independence as caused by host-shifting under a PDE. It would be useful to verify this using computer simulations of host-shifting with PDE (e.g., De Vienne et al., 2007; Engelstädter & Fortuna, 2019).

Hybridization between closely related species may lead to introgression of *Wolbachia* into a new species by vertical transmission. This has been demonstrated in the *Nasonia* species complex (Raychoudhury et al., 2009) and some species of *Drosophila* (Cooper et al., 2019; Turelli et al., 2018). Introgression can be considered a special case of host-shifting (referred to as 'hybridisation-mediated host shifts' by Sanaei, Charlat, et al., 2021), but also has similarities to cospeciation. Since this type of host-shifting can only occur between closely related species, it is expected to produce a PDE signal. We believe, however, that hybridization is unlikely to explain the observed patterns of strain group sharing in our data set. There are only two congeneric species in our data set that share the same *Wolbachia* strain (*Cystococcus pomiformis*/*Cy. echiniformis* and *Eriococcus* sp1/*E.* sp2), and hybridization between species from different genera seems unlikely. Given that *Wolbachia* and mitochondria are cotransmitted, introgression of *Wolbachia* would lead to mitochondrial DNA (mtDNA) hitchhiking and hence be expected to leave a signature of similar mtDNA sequences (Cooper et al., 2019; Jiggins, 2003; Miyata et al., 2020). By contrast, none of our congeneric species have very similar *COI* sequences. (The difference between *Cy. pomiformis* and *Cy. echiniformis* is 6.5%, and the difference between *E.* sp1 and *E.* sp2 is 3%).

4.4 | No signal of geographical home range on *Wolbachia* strain sharing

As a first step in the host-shift process, *Wolbachia* need to physically reach the recipient host species, which requires direct or indirect species interactions. Therefore, it is expected that one should observe host-shifting among species with an overlap in geographical distribution. However, our model indicates that the geographical home range of scale insect species has no significant contribution to *Wolbachia* sharing (Figure 2). This finding may first relate to the age of infection. Estimation of the *Wolbachia* infection age and consequently the intervals of host-shift events is controversial, with vastly different estimates across different case studies being reported, from a few thousand (Cooper et al., 2019; Turelli et al., 2018) to nine million years (Bailly-Bechet et al., 2017; see also Sanaei, Charlat, et al., 2021). If the changes in the host geographical distribution occur faster than *Wolbachia* host-shift events, the current geographical distribution may not be able to explain host-shift events (and thus we would need to reconstruct the historical home range). In addition, the geographical distribution of a given species is not necessarily representative of the realized niche of that species, including ecological connectivity (Kearney, 2006; Peterson & Soberón, 2012; Pulliam, 2000). Therefore, two species may have the same geographical distribution but have no direct or even indirect physical interactions (e.g., via sharing foods or other resources). In that case, host ecological niche may be a better tool to explain *Wolbachia* host-shifting. However, ecological niches are technically harder to measure, especially when trying to account for ecological interactions.

4.5 | Role of scale insect associate species in *Wolbachia* host shifts

Another possible reason why the host geographical distance effect has such a weak effect on *Wolbachia* sharing is host-shifting via ecological vectors. Such vectors can carry on the infection, either temporarily or permanently, and transmit it to a recipient species at a distant geographical location from the donor species. From prey–predator (Johanowicz & Hoy, 1996; Le Clec'h et al., 2013) to host–parasitoid (Kageyama et al., 2010; Tzuri et al., 2020; Vavre et al., 1999) and trophallaxis interactions (Ramalho & Moreau, 2020), there are several direct and indirect ecological pathways which can be routes of *Wolbachia* transfer. Intimacy of direct physical interactions between ants and scale insects may provide a route for microbial exchange, as seen in other hemipteran groups (Ivens et al., 2018; Pringle & Moreau, 2017). Moreover, Gruwell et al. (2009) found plausible routes of *Cardinium* horizontal transmission between armoured scale insects and their associate parasitoids. *Wolbachia* may also utilize these ecological routes to spread within scale insect communities.

In a previous study, a positive correlation between *Wolbachia* infection in scale insects and their associates indicated that ants may play a role in host-shifting (Sanaei, Lin, et al., 2021). Here, we found that only one out of five cases of infected ant–scale insect pairs shares the same *Wolbachia* strain (Table S2, Figure S4). Although positive correlations were not previously observed between infection of scale insects and their associates (Sanaei, Lin, et al., 2021), here we observed sharing of similar *Wolbachia* strains between pairs of scale insect and not only ants but also wasps (three out of seven cases), and flies (one out of two cases) (Figure S4). In addition, infection by the superspreader strain "*w*Sph1" of several species of scale insects, ants and wasps is another source of evidence for a substantial contribution of associate species in *Wolbachia* host-shifting in scale insects. While we do not have enough statistical power to test which route of transfer is the most common and in which directions these transfers take place, our data support the hypothesis that the associates tested in the current study may play a role in host-shifting.

This study has provided the first insight into *Wolbachia* strain diversity in scale insects, revealed a high portion of co-infected samples and detected *w*Sph1 as one of the most common strains of *Wolbachia* in scale insects. We also found that the host phylogenetic distance effect plays a critical role in host-shifting in scale insects. In future studies, the methodology suggested by this study could be applied to a larger data set to detect the factors influencing host-shifting at a the global perspective.

AUTHOR CONTRIBUTIONS

E.S., Y.P.L. and L.G.C. collected scale insects, their associates as well as the information of the geographical distribution of species and performed DNA extractions. E.S. designed the primers, performed the PCRs and prepared the samples for Illumina multitarget amplicon sequencing. E.S., Y.K.Y. and J.E. developed the bioinformatics pipeline. E.S. built the phylogeny and conducted the analysis regarding strain determination. G.F.A. developed the mathematical model. E.S. wrote the manuscript under the supervision of L.G.C. and J.E.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at Appendix S1 and Table S7.

DATA AVAILABILITY STATEMENT

All the raw sequences are available on public databases (GenBank BioSample: SAMN21874044–SAMN21874118 and SRA: SRR16095177–SRR16095251) as described in Appendix S1 and Table S7. In addition, we have provided all generated metadata in the form of supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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