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Millennia-old coral holobiont DNA provides insight into future adaptive trajectories

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Abstract

Ancient DNA (aDNA) has been applied to evolutionary questions across a wide variety of taxa. Here, for the first time, we utilized aDNA from millennia-old fossil coral fragments to gain new insights into a rapidly declining western Atlantic reef ecosystem. We sampled four *Acropora palmata* fragments (dated 4215 BCE to 1099 CE) obtained from two Florida Keys reef cores. From these samples, we established that it is possible both to sequence aDNA from reef cores and place the data in the context of modern-day genetic variation. We recovered varying amounts of nuclear DNA exhibiting the characteristic signatures of aDNA from the *A. palmata* fragments. To describe the holobiont sensu *lato*, which plays a crucial role in reef health, we utilized metagenome-assembled genomes as a reference to identify a large additional proportion of ancient microbial DNA from the samples. The samples shared many common microbes with modern-day coral holobionts from the same region, suggesting remarkable holobiont stability over time. Despite efforts, we were unable to recover ancient Symbiodiniaceae reads from the samples. Comparing the ancient *A. palmata* data to whole-genome sequencing data from living acroporids, we found that while slightly distinct, ancient samples were most closely related to individuals of their own species. Together, these results provide a proof-of-principle showing that it is possible to carry out direct analysis of coral holobiont change over time, which lays a foundation for studying the impacts of environmental stress and evolutionary constraints.

KEYWORDS
ancient DNA, coral, genomics, holobiont evolution, microbial diversity, reef palaeoecology

1 | INTRODUCTION

Applications of ancient DNA (aDNA) to ecological and evolutionary questions have been conducted across a variety of taxa (Der Sarkissian et al., 2020; Padró et al., 2020)—from horses (Orlando et al., 2013) to humans (Narasimhan et al., 2019). One of the most successful marine applications of aDNA has been to the carbonate shells of marine molluscs, where nuclear DNA over 100,000 years old was recovered and compared to extant molluscs (Der Sarkissian et al., 2020). Here, we apply this technology to scleractinian corals whose populations are declining on a global scale as a result of climate change and other anthropogenic disturbances (Gardner et al., 2003; Kuffner et al., 2017). Although the coral animals themselves die and degrade over time, the calcium carbonate skeleton

corals produce remains on the reef, providing a fossil record of long-term reef evolution. This creates the potential for skeletons of long-dead corals extracted from reef cores to preserve DNA suitable and sufficient for aDNA analysis.

Beyond the coral themselves, microbes and dinoflagellate Symbiodiniaceae associated with corals constitute a dynamic system which forms the coral holobiont or metaorganism that, together, determines coral resilience to environmental change (Glasl et al., 2019). Several projects (Hugerth et al., 2015; Parks et al., 2017; Tully et al., 2018; Wilkins et al., 2019) have aimed to capture microbial diversity through the construction of metagenome-assembled genomes (MAGs; Cárdenas et al., 2018; Robbins et al., 2019; Shibl et al., 2020). MAGs are assembled using shotgun sequence data from environmental or host-centric samples, in contrast to genomes assembled from laboratory cultures, reducing bias towards highly studied (or cultivable) microbes (Tully et al., 2018). Further, MAGs have successfully been constructed from ancient material (Wibowo et al., 2021), suggesting we may be able to recover coral-associated microbes from the past.

There have been several studies that have examined the population genetics of corals from modern-day reefs, including from the Florida Keys, where our study is based. These large-scale sampling experiments involved dozens of individuals collected from multiple reef sites and have documented population structure (Ayre & Hughes, 2000; Baums et al., 2005; Cunning et al., 2021; Kitchen et al., 2019) and hybridization (Richards et al., 2008), and elucidated the genomic basis of thermal tolerance (Cunning et al., 2021; Fuller et al., 2020). These reef- and basin-wide genetic studies have informed predictions of which extant coral populations are most vulnerable to climate change (Matz et al., 2018). While present-day genetic diversity and distribution have been assessed in coral, to date, no aDNA-based studies have been carried out to examine long-term demographic trends or to understand how corals have responded to global change that has occurred over the past few thousand years.

In this study, we had three related scientific goals. First, we aimed to extend the genetic analysis of coral to ancient time periods by establishing that we could successfully recover nuclear DNA from corals in reef cores that were thousands of years old. Second, we wanted to compare the data from ancient corals to those from extant populations to place both coral and microbial aDNA in the context of present-day genetic variation. Third, we wanted to utilize MAGs in order to assess microbial species richness within ancient coral holobionts and to examine if there have been changes in the distribution of these holobionts.

2 | MATERIALS AND METHODS

2.1 | Sampling

The ancient coral samples were obtained from reef cores in the U.S. Geological Survey (USGS) Core Archive housed at the USGS Coastal and Marine Science Center in St. Petersburg, Florida. Cores were initially collected using the USGS wireline hydraulic drilling system (Shinn et al., 1982). The cores from Looe Key were collected in 1981 and the core from Sombrero Reef was collected in 2015 (Shinn et al., 1982; Toth et al., 2018). The ages of the corals were determined by radiocarbon dating (Shinn et al., 1982; Toth et al., 2018). This information is summarized in Figure 1. Samples for aDNA analysis were cut from the internal skeletons of the corals using a tile saw dedicated to that purpose. Those samples were then individually sonicated in warm reverse osmosis water to remove any macroscopic surface contamination, dried overnight and sealed in sterile sample bags prior to analysis. Further information on all samples is listed in Table S1.

Samples were sent to Harvard Medical School, where sample processing, DNA extraction and library preparation were performed in dedicated aDNA clean rooms which had never processed coral samples previously. Samples were subjected to ultraviolet light (254 nm twice for 5 min) for surface decontamination and subsequently milled in a Retsch Mixer Mill.

2.2 | DNA extraction

DNA was extracted for high-throughput sequencing according to Rohland et al. (2018). All extractions were performed in a clean room, and no other coral processing had been done there previously. This protocol is optimized for the retrieval of short DNA fragments using a silica-based extraction method. Our four samples were processed in this fashion using DNA binding buffer D (5 M guanidine hydrochloride, 40% [v/v] 2-propanol, 0.12 M sodium acetate and 0.05% [v/v] Tween 20) and silica beads.

2.3 | Library preparation and sequencing

Libraries were prepared for sequencing as in Rohland et al. (2015). We applied partial uracil-DNA-glycosylase treatment to libraries to utilize the advantages of retaining some characteristic aDNA patterns for downstream authentication while still repairing most damage in ancient molecules to reduce biases during analysis. Samples were sequenced in 2019 using an Illumina HiSeq X 10 instrument to obtain 2 × 101-bp paired end reads and 2 × 7-bp for the index reads.

2.4 | Read processing

Paired-end reads were merged to single-end reads before alignment to filter out long fragments and trim adapters. A minimum of 15 bp of overlap was required, allowing for up to one mismatch at positions with base quality of at least 20, and up to three mismatches at positions of lower base quality. At mismatch positions, the base call of higher quality was retained, and the corresponding base quality was the difference of the two base qualities. For matching positions, the base quality was the maximum of the two base qualities. Initial read

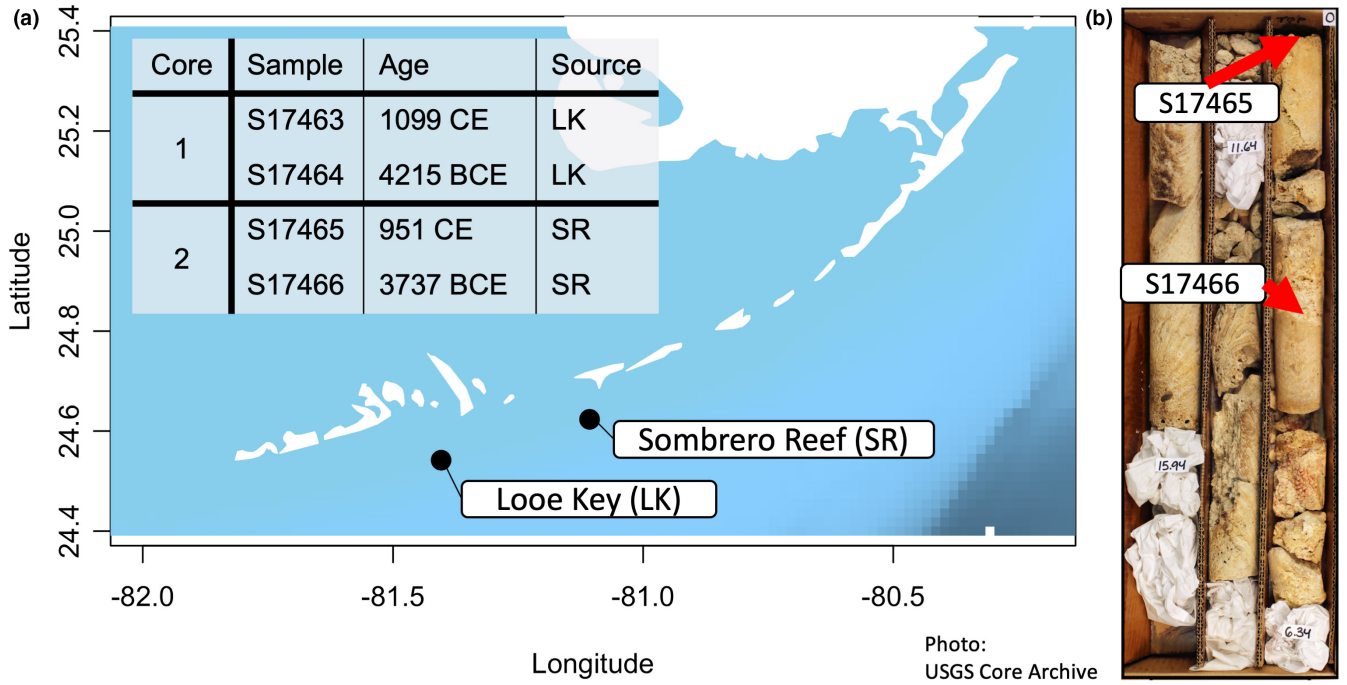

FIGURE 1 Samples originated from two geographically distinct cores dated between 4215 BCE and 951 CE. (a) Geographical source of the two cores used in this study. Inset gives the sample names associated with each core and the median radiometrically determined age of each. (b) Photograph of Core 2 from USGS data release (Toth et al., 2018). Red arrows indicate locations from within the core from which our samples were taken.

processing was conducted using software ADNA-TOOLS (DReichLab/ADNA-Tools, 2018/2021).

Modern *Acropora* spp. genomic samples ($N = 60$) were retrieved for comparison from NCBI BioProject PRJNA473816 (Kitchen et al., 2019). These samples span five locations: Belize, the Bahamas, Curacao, the US Virgin Islands and the Florida Keys. Modern-day microbial samples ($N = 81$) from two Florida Keys reefs (Looe Key and Molasses Reef) were retrieved from NCBI BioProject PRJNA299413 (Westrich et al., 2016). An additional 96 microbial samples from Florida Keys acroporids were retrieved from NCBI BioProject PRJNA546259 (Rosales et al., 2019). Modern comparison samples, their NCBI BiopProject accessions and source publications are summarized in Table S4. All modern samples were aligned to the master reference, described below, using "local" BOWTIE2 mapping parameters for analysis and "end-to-end" parameters for damage characterization.

The sequences from all samples were mapped to a concatenated reference genome of *Acropora millepora* (Fuller et al., 2020), *Symbiodinium* sp. (Shoguchi et al., 2018), *Breviolum minutum* (Shoguchi et al., 2013), *Cladocopium* sp. (Shoguchi et al., 2018), *Durusdinium* sp. (Dougan et al., 2022) and 103 assorted bacterial genomes (Cárdenas & Voolstra, 2021a, 2021b). Mapping of ancient sequences was first carried out using the default end-to-end option in BOWTIE2 version 2.3.4 (Langmead & Salzberg, 2012), to retain C→T/G→A misincorporations at terminal bases for damage pattern analysis. Sequences were then mapped to the concatenated reference genome a second time, after trimming 1 bp from each read end to remove putative ancient damage. All analyses except the "mapDamage" analysis were done using these trimmed reads. Only reads of mapping quality >30 were retained for further analysis.

2.5 | Characterization of aDNA damage

We determined patterns of characteristic aDNA damage of reads mapping to each reference using MAPDAMAGE2.2.0 (Jónsson et al., 2013). For coral and Symbiodiniaceae, misincorporation plots were visually inspected for terminal base misincorporations. For microbial analysis, groups of reads mapping to each metagenome-assembled genome were algorithmically sorted into modern and potentially ancient categories, using the following criteria: (i) the greatest C→T/G→A misincorporation rate at the terminal base of the read group, (ii) 5′ C→T and 3′ G→A misincorporation rates higher at the terminal position than the average misincorporation rates $\pm 2SD$ at this position, and (iii) the C→T/G→A misincorporation rates must be less than the average misincorporation rates $\pm 2SD$ at the next 10 base positions. Read groups mapping to a given microbial species are scored by the number of criteria they meet. Misincorporation rate and read length distribution plots were also visually assessed for any potentially ancient read groups, but no discordance was found.

2.6 | Comparison of modern and ancient coral data

We compared ancient *Acropora palmata* data to modern data with two different methodologies. The first method follows the procedure in Kirch et al. (2021), considering ancient samples in principal component analysis (PCA) construction. In brief, we used ANGSD (Korneliussen et al., 2014) to create genotype likelihood files. The output files were then subset for positions covered in at least one of our four ancient samples. We then used PCANGSD (Meisner & Albrechtsen, 2018) to create PCAs of all samples at ancient-covered positions. Genotype likelihoods from ANGSD were input to NGSADMIX (Skotte et al., 2013) to analyse admixture in our samples for $K = 2$ to $K = 7$. In parallel, we applied a method similar to Shinde et al. (2019). Here, ancient samples are projected onto modern-day variation. To accomplish this, we determined biallelic single nucleotide variants (SNVs) in modern-day samples using "bcftools mpileup" (Li et al., 2009). Pseudohaplotype calls were then made using a combination of "samtools mpileup" and SEQUENCETOOLS "pileupCaller" (Schiffels, 2015). Finally, using EIGENSOFT "smartpca" (Galinsky et al., 2016), PCAs were first constructed using data from modern samples, then ancient samples were projected into this space in order to reduce shrinkage effects on the ancient samples. To determine uncertainty about location of modern and ancient samples, we jackknifed our data about the 14 chromosome-level scaffolds plus two "pseudo-chromosomes" constructed from unplaced scaffolds in our *A. millepora* reference. We then projected the resulting "leave-one-out" samples into PCA space. All visualizations were completed in R with GGPLOT2 (R Core Team, 2021; Wickham, 2009).

To calculate f_4 statistics, we used ADMIXTOOLS (Patterson et al., 2012). Fifteen *A. millepora* whole genome sequencing samples were used as the outgroup population (from NCBI BioProject PRJNA593014; Fuller et al., 2020). We tested f_4(*A. millepora* population, ancient population; C population, D population) and f_4(*A. millepora* population, B population; C population, D population) where B, C and D are all possible permutations of *A. palmata*, *A. cervicornis* and *A. prolifera*. Significance was determined at $|Z| \geq 3$.

2.7 | Microbial data analysis

Relative abundance of modern microbes was compared to ancient microbes by first restricting analysis solely to bacterial genomes present in at least one ancient sample. For each modern sample, counts of high-quality ($Q > 30$) reads mapping to each ancient MAG were calculated with SAMTOOLS, then visualized in R with GGPLOT2. Additional analysis looked at overlapping presence of MAGs between modern and ancient samples and relative abundances of ancient–present MAGs in all samples.

To further confirm the specificity of mapped bacterial reads, we determined the taxonomic affiliation of all reads that mapped to bacterial genomes using KAIJU (Menzel et al., 2016) against the NCBI nr_euk database that includes all proteins belonging to viruses, archaea, bacteria, dinoflagellates and other microbial eukaryotes (June 25, 2019). The taxonomic level that best captured the genome taxonomy was reported (Figure S5).

3 | RESULTS AND ANALYSIS

3.1 | Recovery and characterization of coral and prokaryote aDNA

We generated 58–70 million Illumina read pairs for each sample (Table S2). To minimize issues associated with potential reference bias, we mapped to a concatenated reference consisting of *Acropora millepora*—a species equally diverged from all coral species found in the Florida Keys and the Caribbean. We also carried out all analysis mapping to *Acropora palmata* and observed similar results (Günther & Nettelblad, 2019). In addition, we mapped sequences to obligate endosymbionts *Symbiodinium*, *Breviolum*, *Cladocopium* and *Durisdinium* (formerly clades A–D; LaJeunesse et al., 2018) and 103 extant coral MAGs and cultured bacterial genomes (Cárdenas & Voolstra, 2021a, 2021b). DNA recovered from ancient or historical material contains signatures of DNA damage over time that can be used to authenticate the origins of the genomic data collected. Characteristic aDNA molecules have short read-lengths and a high 5′ C→T or 3′ G→A misincorporation rate at the terminal ends of reads (Briggs et al., 2007).

To examine these properties, after filtering for mapping quality ($Q > 30$), we characterized aDNA damage patterns for reads mapping to each reference (Figure 2). We identified between 30,615 and 293,984 putatively ancient reads from each fossil sample, mapping to the coral reference and 10 of the MAGs (Table S2). Of those, between 741 and 6566 mapped to our coral reference, with strong variation in data recovery depending on sample age. This translated to 1.47×10^{-6} to 1.38×10^{-5} genomic coverage of the *A. millepora* reference. The coral-mapped proportion of each sample showed expected misincorporation patterns from UDG-half prepared libraries, as well as shortened length distributions (Figure 2). This is in contrast to modern sequences which showed neither signature of aDNA damage (Figure S2). These patterns are in line with aDNA recovered from similarly dated carbonate shells of marine molluscs—a comparable preservation matrix (Der Sarkissian et al., 2020).

3.2 | Failure to recover Symbiodiniaceae reads

Notably, we recovered few (an average of 243 per sample) Symbiodiniaceae reads prior to ancient filtering, and no samples showed an elevated C→T/G→A misincorporation rate at the terminal base pair (Table S2; Figure S1). Given Symbiodiniaceae are only found in the endoderm, it is probable that Symbiodiniaceae DNA was not preserved within the skeletal reef matrix. Alternatively, the endogenous content may have been too low to detect using shotgun sequencing efforts.

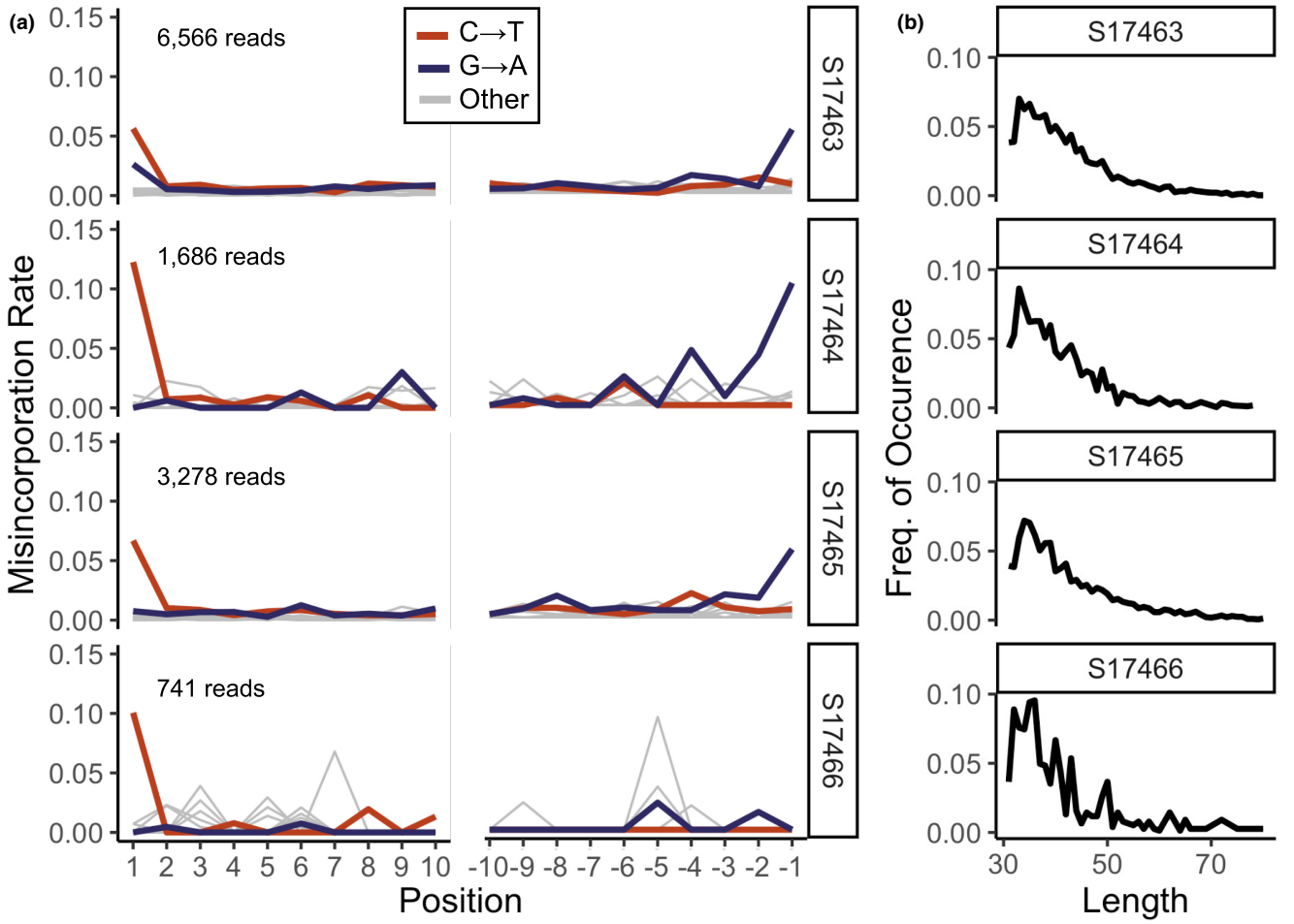

FIGURE 2 Terminal base misincorporation patterns and length distributions verify aDNA from coral host. (a) Misincorporation rate of C→T and G→a substitutions across the first and last 10 bp of all mapped reads, given by sample. The expected pattern is seen across all samples, except at the 3′ end of S17466. This is probably an artefact of the low number of reads recovered from this sample. Inset numbers give mapped read pairs retrieved. (b) Read length distribution (base pairs) given by sample. All putatively ancient samples show similar distributions.

3.3 | Comparisons with extant acroporids

To place ancient samples in the context of modern-day variation, we retrieved modern-day whole genome sequencing data of *A. palmata*, *A. prolifera* and *A. cervicornis* ($N = 60$) as well as microbial DNA samples associated with *A. palmata* ($N = 177$) from three recent studies (Kitchen et al., 2019; Rosales et al., 2019; Westrich et al., 2016). These samples were collected from across the western Atlantic and overlapped with our study region in the Florida Keys (Table S4).

We employed two different methods to compare the ancient and modern acroporid data. These approaches account for uncertainty in ancient genotypes in different ways. The first analysis followed the method of Kirch et al. (2021), where PCANGSD was used to determine genotype placement—restricted to genomic sites where SNVs were covered by at least one ancient sample. Of the initial unfiltered set of 110,508,546 sites with genotype likelihoods from ANGSD, 90,931 were covered by at least one ancient sample. PCANGSD is designed for placing low-depth next-generation sequencing data using genotype likelihoods, which accounts for uncertainty in genotypes (Meisner & Albrechtsen, 2018). Notably, this method uses both ancient and modern samples to calculate principal component loadings.

In parallel, we carried out an analysis in the spirit of Shinde et al. (2019), which first used modern coral sequences to establish axes of variation upon which to project ancient samples. This approach potentially reduces bias which may make ancient samples artificially appear most related to each other (i.e., poorer data quality, damage patterns, etc.). A total of 11,527,322 SNVs were identified from the modern *A. palmata*, *A. cervicornis* and *A. prolifera* data. Of these SNVs, 3476 were covered by S17463, 641 were covered by S17464, 2262 were covered by S17465 and 209 were covered by S17466. As the number of reads we had available from the ancient coral sequences was low, we examined uncertainty in the PCA positions of these samples using a chromosome-level block jackknife.

Both approaches yielded qualitatively similar results: the aDNA samples clustered closest with modern-day *A. palmata* in PCA space

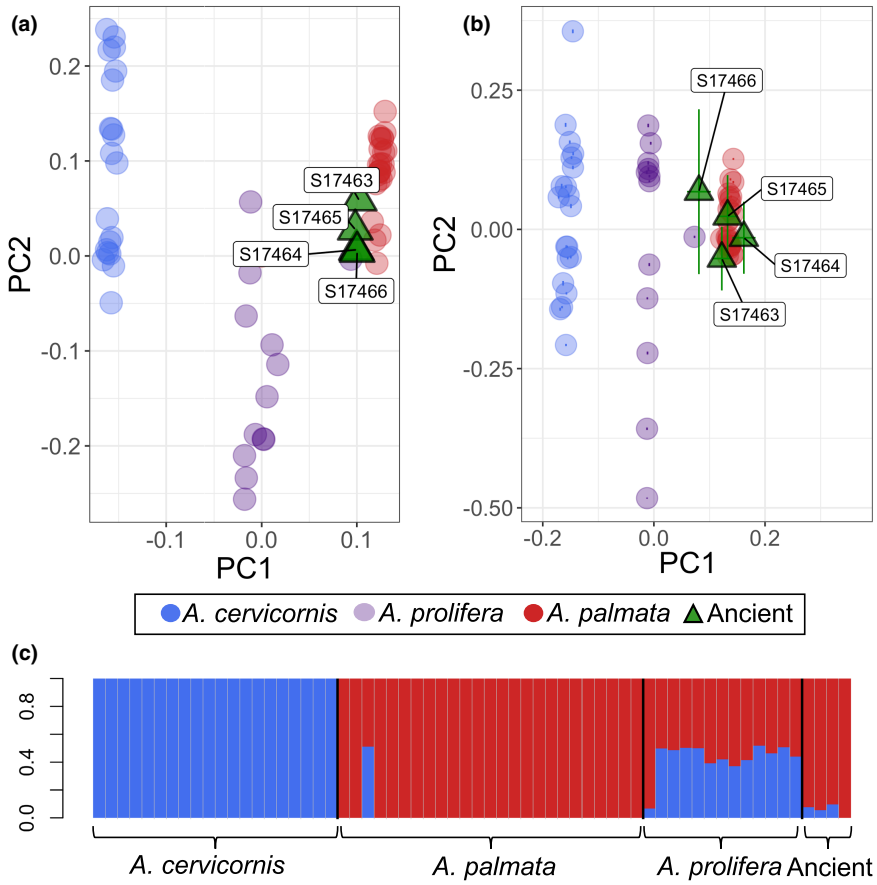

FIGURE 3 Comparison of coral aDNA with extant acroporids shows the predominance of *Acropora palmata* ancestry in ancient samples. (a) PCANGSD-constructed PCA showing positions of ancient samples with respect to modern species clusters. Ancient samples (green triangles) group closest with *A. palmata*. (b) Projection-based PCA construction yielded qualitatively similar results. Bars about points represent PC-coordinate uncertainty calculated from chromosome jackknifing. (c) NGSADMIX plot showing ancestry of different groups with $K = 2$ (for $K = 3$, see Figure S4). Each horizontal bar represents one individual from the labelled population. The *x*-axis labels show the population each sample was assigned to morphologically prior to admixture analysis. Coloured bars represent the proportion of ancestry from each group.

Population				Statistic	
Outgroup	**Ancient**	**C**	**D**	f_4	**Z**
Acropora millepora	Ancient	*A. cervicornis*	*A. palmata*	0.019	5.72
		A. cervicornis	*A. prolifera*	0.012	4.08
		A. prolifera	*A. palmata*	0.008	3.56

TABLE 1 f_4 statistics validate assignment of ancient samples to *Acropora palmata*.

Note: f_4 statistics calculated for f_4(*Outgroup*, *Ancient Individuals*; *C*, *D*), where C and D are all possible combinations of *A. cervicornis*, *A. prolifera* and *A. palmata*. Significance is determined by $|Z| \geq 3$. Significant admixture is only found between ancient individuals and *A. palmata* or hybrid *A. prolifera*.

(Figure 3a,b). This confirms expectations, as ancient samples were morphologically identified as *A. palmata* (Table S1). Further, modern individuals clustered first by species (*A. palmata*, *A. cervicornis*, *A. prolifera)*, then by site (Figure S3). This recapitulates the results found by Kitchen et al. (2019), showing pseudo-haplotype data created in our projection-based approach accurately resolved known modern population clusters.

NGSADMIX analysis supports the results of the PCAs, confirming the assignment of our ancient samples to be mostly *A. palmata* (Figure 3c). Assuming two distinct genetic clusters ($K = 2$), there is clear species delimitation between *A. palmata* and *A. cervicornis*, with evidence of mixed ancestry in the known hybrid *A. prolifera*. The small proportion of ancient samples attributed to *A. cervicornis* is likely to be noise in the data, disappearing for S17464 when $K = 3$, and presumably does not reflect ancestry from this congener (Figure S4). Together, the separation of ancient samples from *A. palmata* in PCA-space and the small proportion of ancestry assigned to a second cluster suggest slight segregation between our ancient and modern samples (Figure 3a,b; Figure S4). This is potentially due to actual genetic differences accumulated over the last 1000–6000 years, or to artefacts arising from characteristics of aDNA (it also may reflect both phenomena together).

We next carried out formal analysis of ancestry and examined evidence (or lack thereof) of hybridization in our ancient samples through the application of f_4 statistics (Patterson et al., 2012). First, we established a phylogeny consisting of an outgroup (*A. millepora*) and three modern coral samples found in the Florida Keys (*A. palmata*, *A. cervicornis* and *A. prolifera*). *A. prolifera* is a well-known hybrid, and indeed f_4 statistics of the form f_4(*A*, *B*; *C*, *D*) where A, B, C and D are varied across all permutations of the modern corals, confirm this hypothesis (Table S3). Following this, we aimed to determine with which modern group our ancient samples shared the most

alleles by permuting f_4(*Outgroup*, *Ancient*; *C*, *D*), where *C* and *D* are all possible combinations of *A. palmata*, *A. prolifera* and *A. cervicornis*. In all cases, the ancient samples only showed significant ancestry from *A. palmata* or *A. prolifera* ($Z \geq |3|$; Table 1). Given allele sharing with *A. prolifera* is only positive when compared against *A. cervicornis*, but not when compared to *A. palmata*, we inferred our samples to be most closely related to *A. palmata*.

3.4 | Identification of ancient microbes

Next, we examined reads from our sequencing data that mapped to our bacterial reference set. Of the 103 bacterial genomes used as a "search space" in this study, a total of 10 had reads assigned from at least one ancient sample. These bacterial genomes corresponded mainly to bacterial populations associated with coral skeletons (Cárdenas et al., 2022). Mapping largely occurred in moderate to highly conserved gene regions shared across members of the same phylum (Figure S5) and indicating the presence of representatives of the phyla Bacteroidota, Desulfobacterota, Myxococcota, Proteobacteria and Spirochaetota in ancient samples. Only one bacterial representative from coral tissues was mapped by the ancient samples (R13_0) with enough taxonomic certainty to support the presence of members of the genus *Ruegeria* (Figure S5) in aDNA. Moreover, we found evidence for the presence of proteins involved in photosynthesis, nitrogen and carbon fixation, and sulphur oxidation among the ancient reads by means of mapping to the genes encoding the respective proteins.

3.5 | Comparison to modern microbiomes

Additionally, ancient metagenomic data shared considerable overlap with modern microbial data. To determine if our ancient microbes could be found in modern-day coral holobionts, we compared our data against two acroporid studies from the Florida Keys (Rosales et al., 2019; Westrich et al., 2016; Table S6). Of the 10 bacterial genomes to which the sequences from the ancient samples mapped, five were represented in both studies, and an additional two were present in data from Westrich et al. (2016) (Figure 4). Notably, microbe presence was variable within our ancient samples with the exception of Endolith_179, Endolith_99 and Endolith_149. More modern (S17463, S17465) aDNA samples showed higher microbial richness, though this may be due to preservation bias rather than reflecting a more diverse microbiome at that time point. Along the same vein, we are hesitant to draw conclusions about microbial absences (i.e., Endoltih_284, R13_0 and Endolith_131), as this may be attributable to sampling instead of function.

4 | DISCUSSION

The presence of high-confidence assigned sequence reads and expected damage patterns show it is possible to obtain aDNA from fossil coral fragments up to 6000 years old. Ultimately, we only assigned ~0.01% of the original 50–70 million reads sequenced per sample to *Acropora millepora*. While this recovery rate is very low compared to typical data recovery rates in humans (e.g., Skoglund et al., 2012), these values are in line with studies from 6000–7000-year-old marine molluscs where only 0.02%–1.7% of shotgun sequenced reads were retained with comparable genomic coverage (Der Sarkissian et al., 2020).

The low coverage of the genetic data recovered poses a significant barrier to downstream analysis. While we are able to place our coral samples with fairly high confidence at the species level, further classifications become more difficult. This is represented by the error bars in Figure 3b. Through jackknifing at the chromosome level, we assess the sensitivity of each sample's placement

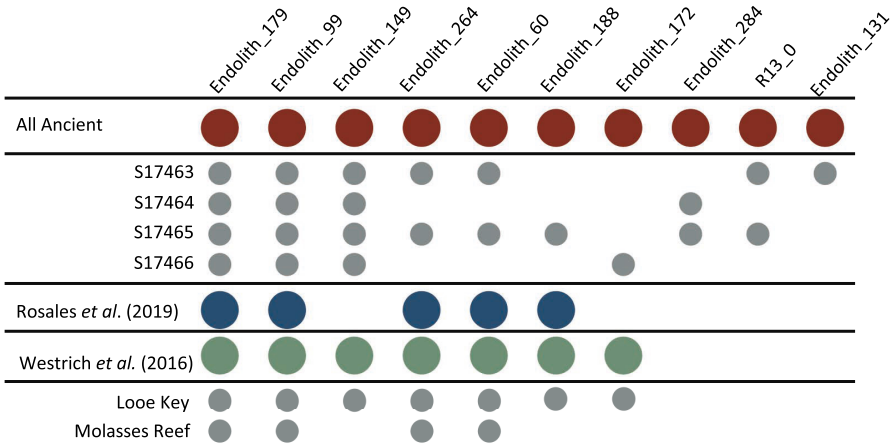

FIGURE 4 Ancient microbes overlap with modern-day Florida keys data sets. Plot of presence/absence of metagenome-assembled genomes (MAGs) present in ancient samples against two recent studies from the Florida keys. Grey dots represent subsets of samples from the study listed above them (i.e., Looe Key and Molasses Reef samples are both from Westrich et al.). Microbial richness was highest in the more modern samples (S17463 and S17465) and some microbes showed consistency across all groups (Endolith_179, Endolith_99, Endolith_149).

in multivariate space as groups of SNVs are removed from the set. This process determines if placement is robust to down-sampling. Position along PC1 remains stable, while sample position along PC2 varies greatly, giving confidence only to our species-level assignment along the first axis. However, when considering *A. palmata* samples in isolation, the slight segregation between modern and ancient *A. palmata* in Figure 3a,b becomes much more pronounced (Figure S6). While some of this segregation might be due to true genetic distinctiveness of ancient corals, low coverage (and low sample size) makes it impossible to ascertain this with our present data (e.g., Lou & Therkildsen, 2022).

In aDNA research applied to other organisms, target capture techniques have been used to increase target organism read retention or to restrict analysis to known polymorphisms (Armbrecht, Hallegraeff, et al., 2021; Fu et al., 2015; Haak et al., 2015) or enrich for damaged DNA molecules (Gansauge & Meyer, 2014). Specifically, Armbrecht, Paine, et al. (2021) successfully applied hybridization-capture methodology to marine eukaryotes to greatly increase eukaryote and archaea DNA capture with respect to bacteria, while retaining patterns of aDNA damage. In the future, applications of capture technology to coral aDNA may similarly increase both the feasibility and the efficiency of this method.

The recovery of ancient microbial sequences in addition to coral data allowed us to make preliminary comparisons between historical and modern microbiomes. It is well established that the coral holobiont can vary considerably over time and space, although certain coral species exhibit a remarkable extent of microbiome conservation (Guppy & Bythell, 2006; Klaus et al., 2005; Morrow et al., 2012; Neave et al., 2017). Disparities in the presence of microbes between ancient and modern samples may represent unique characteristics of the coral microbiome 1000–6000 years in the past which have not persisted to the modern day. Notably, our ancient samples had the highest similarity in presence and absence of ancient microbes with modern samples taken from the same reef (Looe Key). The fewest ancient MAGs were found in the data from Molasses reef—a reef geographically the furthest from the others. This result was not statistically significant (Fisher's exact test; $p = .3698$), but it raises interesting questions about microbiome stability in both time and space. While not the primary goal of this study, future directions in coral aDNA research could centre on characterizing marine microbiomes across these dimensions.

Conversely, the presence of extant coral microbiome members in ancient samples reflects their (long-lasting) specificity and, by inference, their functional importance. Members of the phyla Myxococcota and Desulfobacterota are common associates of skeletal samples and presumed to play a role in sulphur cycling (Yang et al., 2019, 2020), or nitrogen fixation if residing in the mucus (Lema et al., 2012). Our results corroborated the presence of genes involved in sulphur cycling, and carbon and nitrogen fixation from these taxa in ancient samples, reaffirming their ecological niche in nutrient cycling, although their contribution to the coral holobiont remains unknown. Members of the Myxococcota have been linked to disease resistance in extant *A. palmata* (Rosales et al., 2019), and the presence of this bacterial group in ancient corals highlights the ancestry as well as consistency of beneficial microbial associations with putative insights into the evolution of coral holobiont health. Similarly, *Ruegeria* species are prevalent coral associates exhibiting a high taxonomic and functional diversity across coral compartments (Luo et al., 2021), with putative beneficial roles for the holobiont in pathogen control (Garren et al., 2014; Miura et al., 2019) and larval development (Apprill et al., 2009; Freire et al., 2019).

Long-term variation in marine microbes and eukaryotes using aDNA has previously yielded exciting results about marine community composition at distant timescales. For example, Armbrecht, Paine, et al. (2021) revealed the dynamics of harmful algal blooms using marine sediment cores up to 9000 years in the past. In a similar vein, del Cabrera et al. (2019) explored variability of coral-associated macroalgae in the sediment record over the past 750 years through paleoecological DNA, correlating it with the coral fossil record. Because coral microbiomes generate specific microbial fingerprints depending on the host physiological state or environmental condition, extending coral reef aDNA studies to include their associated microbial communities would provide a holistic perspective of the evolution and role of microbial associations in coral health.

In the western Atlantic, gaining insight into evolutionary trajectories of coral reefs is increasingly important as extant corals are declining at an unprecedented rate. Since the 1980s, coral cover has declined by 90% in the region and reef-building corals such as *A. palmata* have been hit particularly hard (Gardner et al., 2003; Kuffner & Toth, 2016). The ability to detect genetic diversity declines associated with population bottlenecks and instances of genetic turnover (i.e., replacement of earlier populations with new, genetically distinct, ones) of key coral taxa in response to past stressors will enable better prediction of extant corals' responses to continued environmental disturbances in the future. As the field of coral aDNA is further being developed, particularly with the adoption of hybridization capture technology (e.g., Armbrecht, Paine, et al., 2021), it will be possible to investigate these past changes directly. Through coral aDNA we can gain a "window into the past" to evaluate the evolutionary history of the coral holobiont.

Overall, this study serves as a promising proof-of-concept to root future applications of aDNA in coral reef biology. Due to low ancient read recovery rate, future coral aDNA studies will have to utilize target enrichment technologies such as hybridization capture to make broader scale aDNA surveys feasible. However, we demonstrate that it is possible to extract nuclear DNA from corals up to 6000 years old and place these data in the context of modern-day genetic variation, for both the coral host and associated microbes. Future studies can also work to clarify the separation observed between ancient and modern populations, how coral hosts and their associated microbes change over time, and the presence of evolutionary constraints that may limit their adaptive potential. Building on this work could allow for a holistic perspective on coral ecology over millennial timescales and beyond.

AUTHOR CONTRIBUTIONS

Conceptualization, M.V.M., L.T.T. and D.R.; Methodology, D.R., N.R., L.T.T., M.M., A.C. and C.R.V.; Software, V.M.N. and M.M.; Validation, C.B.S.; Formal Analysis, C.B.S.; Investigation, N.R. and L.T.T.; Resources, D.R., M.V.M., L.T.T., C.R.V. and A.C.; Data Curation, C.R.V. and C.B.S., Writing—Original Draft, C.B.S; Writing—Review & Editing, All; Visualization, C.B.S.; Supervision, M.V.M., D.R., C.R.V. and V.M.N.; Project Administration, M.V.M.; Funding Acquisition; M.V.M., C.R.V. and D.R.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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 in NCBI BioProject with accession numbers (PRJNA757238, PRJNA757245). Code used to replicate results and figures can be found at github.com/cb-scott/coral-aDNA.

DATA AVAILABILITY STATEMENT

All DNA sequences from fossil coral cores can be found at NCBI BioProject #PRJNA757238 (Scott et al., 2022a). Coral holobiont endolith bacterial genomes can be found at NCBI BioProject #PRJNA757245 (Scott et al., 2022b). Code used for data processing as well as protocols used for library preparations can be found at https://github.com/cb-scott/coral-aDNA.

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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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