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NOTE

Investigating seed bank potential of crustose coralline algae using DNA metabarcoding

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Abstract

To examine the potential for the autogenic ecosystem engineers, crustose coralline algae (CCA), to serve as seed banks or refugia for life stages of other species, it is critical to develop sampling protocols that reflect the diversity of life present. In this pilot study on two shallow water species of CCA collected from Raoul Island (Kermadec Islands; Rangitāhua) New Zealand, we investigated two preservation methods (ethanol vs. silica gel), sampled inner and outer regions of the crusts, and used DNA metabarcoding and seven genes/gene regions (16S rRNA, 18S rRNA, 23S rRNA, *cox*1, *rbc*L, and *tuf*A genes and the ITS rRNA region) to develop a protocol for taxa identification. The results revealed immense diversity, with typically more taxa identified within the inner layers than the outer layers. As highlighted in other metabarcoding studies and in earlier work on rhodoliths (nodose coralline algae), reference databases are incomplete, and to some extent, the use of multiple markers mitigates this issue. Specifically, the 23S rRNA and *rbc*L genes are currently more suitable for identifying algae, while the *cox*1 gene fares better at capturing the diversity present inclusive of algae. Further investigation of these autogenic ecosystem engineers that likely act as marine seed banks is needed.

KEYWORDS
algae, crustose coralline algae, diversity, DNA metabarcoding, seed bank

Terrestrial seed banks are recognized in ecological and evolutionary theory as "biodiversity reservoirs" (Plue et al., 2021; Yang et al., 2021). After extreme events (e.g., drought, fire, eruptions), population persistence and recovery of primary production are supported by the below-ground storage of seeds (Yang et al., 2021). This seed bank maintains species and genetic diversity for post-disturbance recovery and hedges against the risk of environmental change (Yang et al., 2021). Is there a functionally equivalent biodiversity reservoir, or "seed bank," in marine ecosystems, conferring resilience and protection from biotic and abiotic stressors (Hoffmann & Santelices, 1991; Schoenrock et al., 2021)?

Most primary production in the ocean is the result of photosynthesis by phytoplankton (microalgae) and macroalgae. Both micro- and macroalgae have complex lifecycles, often including alternative life history stages. Many phytoplankton taxa (e.g., dinoflagellates, diatoms) have cysts or resting stages, often present in marine sediments. Macroalgae with heteromorphic life histories also have microscopic stages that enable survival in periods of unfavorable conditions. Although identified as forming a marine "seed bank," these microscopic life forms remain poorly studied (Edwards, 2000; Ellegaard & Ribeiro, 2018; Fredericq et al., 2014, 2019; Hoffmann & Santelices, 1991;

Abbreviations: ASV, amplicon sequence variant; CCA, crustose coralline algae; OTU, operational taxonomic unit; sscinames, subject scientific names; *tuf*A, elongation factor Tu.

Krayesky-Self et al., 2017; Saunders & Brooks, 2023; Sauvage et al., 2016). Recently, resting stages of phytoplankton and macroalgae have been found within rhodoliths, a nodose form of crustose coralline algae (CCA; Fredericq et al., 2019; Krayesky-Self et al., 2020). Cryptic endolithic diversity in mesophotic rhodoliths has revealed both heterotrophic and autotrophic operational taxonomic units (OTUs), the latter including representatives of seven classes of algae, with the most numerous from the Florideophyceae, Chlorophyceae, Cyanophyceae, and Bacillariophyceae (Fredericq et al., 2019). Furthermore, Fredericq et al. (2019) noted that "Further exploration worldwide is also essential to … ascertain whether the rhodolith interior functions as seed banks for algal stages, as temporary reservoirs for life history stages of algal bloom-forming species, or as refugia for ecosystem resilience following environmental stress" (Conclusion section).

Crustose coralline algae are slow-growing, long-lived, rock-like red algae with calcium carbonate cell walls that are ubiquitous in shallow waters of the world's oceans (Nelson, 2009; Riosmena-Rodríguez et al., 2017). Important as ecosystem engineers, CCA cover an average of 52.5% of the surface of coastal reefs (van der Heijden & Kamenos, 2015). Given that the rhodolith form of CCA contains resting stages (Fredericq et al., 2019), we hypothesized that the more ecologically dominant encrusting CCA might be one of the most important components of the marine seed bank, particularly focusing on thick crustose growth forms.

Here, we investigated two preservation methods (ethanol and silica gel) and seven genes/gene regions to develop a DNA metabarcoding protocol for broad taxa identification (inclusive of animals, bacteria, fungi, and plants) within inner and outer encrusting CCA layers. We collected samples from the subtropical waters of Raoul Island (Kermadec Islands; Rangitāhua), where CCA species can form thick crusts (Appendix S1: Figure S1 in the Supporting Information). Sample collection took place in November 2021 under the authority of Ngati Kuri as mana moana (Appendix S1: Table S1). The two CCA samples collected, CCA43 (tentatively identified as an undescribed genus in the Hapalidiales) and CCA94 (an undescribed genus in the Corallinales), have had voucher material lodged in the Herbarium of Auckland Museum (AK386376 and AK386377, respectively). The samples were 4–5 cm thick and allowed for separation of the pigmented surface portions and inner regions (ca. 2–4 cm within the crusts) and subsampling within each layer as inner/outer replicates. CCA43 was preserved in ethanol (100%) and five subsamples were taken for each of the inner (ethanol: 43EIA-43EIE) and outer (43EOA-43EOE) layers. CCA94 was split in two at the time of collection, and one half was preserved in ethanol, the other in silica. Three subsamples were taken for each of the inner and outer layers (ethanol: 94EIA-94EIC and 94EOA-94EOC; silica: 94SIA-94SIC and 94SOA-94SOC).

The largest subsamples from the ethanol-preserved CCA94 outer and inner layers were selected for preliminary DNA extraction kit testing. Each subsample was frozen in liquid nitrogen and crushed into a powder using a pestle and mortar. After crushing, the subsample was split equally into six to allow duplicates per DNA extraction kit, per layer. Three different DNA extraction kits were tested, DNeasy Powersoil DNA Isolation Kit (QIAGEN), DNeasy Plant Mini Kit (QIAGEN), and Nucleospin Tissue DNA Kit (MACHEREY-NAGEL) as per each manufacturer's instructions. For the DNeasy kits, the bead beat steps were omitted as the subsamples were already a powder. The Nucleospin Tissue Kit extracted a higher quantity and better quality of DNA (0.8% agarose gel and Nanodrop assessment), and thus, this kit was used for the remaining extractions.

Seven different gene or gene regions were targeted for PCRs; 16S V3–V4 rRNA region (Klindworth et al., 2013), 18S V4 rRNA gene (Bradley et al., 2016; Fiore-Donno et al., 2018), 23S V5 rRNA gene (Sherwood & Presting, 2007), *cox*1 gene (Geller et al., 2013; Leray et al., 2013), ITS rRNA region (White et al., 1990), *rbc*L gene (Hadi et al., 2016), and *tuf*A gene (elongation factor tu; Famà et al., 2002; Sauvage et al., 2016). Polymerase chain reaction (PCR) profiles were either followed from the literature or modified to improve amplification (Appendix S1: Table S2). The PCRs were performed in triplicate using MyTaq Red Mix (Bioline; Meridian Bioscience) master mix: 6.25 μL MyTaq Red Mix, 0.25 μL of each primer (10 μM), 5.75 μL DNase/RNase-free water (4.75 μL if BSA added), 0.5 μL DNA, and 1 μL BSA (1%; added when necessary). Negative controls were included in the DNA extractions (DNA extraction blank—no tissue added) and subsequently used in the PCRs to check for possible contamination. Further potential contamination was also monitored by including a PCR blank (no DNA added) in every PCR run. The PCR triplicates were pooled together by subsample per gene/gene region before proceeding with DNA clean-up using Agencourt AMPure XP (Beckman Coulter) and following the Illumina protocol for PCR clean-up for all gene regions (Illumina, 2013). The concentration of the purified PCR products was determined using a Qubit dsDNA HS Assay Kit (Invitrogen; ThermoFisher Scientific) following the manufacturer's instructions. All PCR products were brought to 2 ng $\cdot$ μL^{-1} where possible. The PCR products were pooled further to reduce indexing. Sequencing was completed through Auckland Genomics, where indexing (Nextera DNA Library Prep Kit) and the second round of PCR cleanup occurred before sequencing on an Illumina MiSeq System (2 × 300 pair-end; single lane).

Raw sequencing data underwent primer removal (Cutadapt v3.5; Martin, 2011), and the DADA2 (Callahan et al., 2016) pipeline within Qiime2 (v2022.2; Bolyen

et al., 2019) was used to retain high-quality amplicon sequence variants (ASVs; Callahan et al., 2017). Only ASVs that had more than 10 reads per subsample were retained. The ASVs were then run through GenBank v2022-07 (Benson et al., 2013) using MegaBLAST from the BLASTn suite v2.13.0 (Morgulis et al., 2008), and taxonomy, where possible, was assigned using World Register of Marine Species (accessed November 2022; Horton et al., 2022). To note, GenBank DNA sequence (nucleotide) database entries are not always provided with the full taxonomic classification to species-level, and thus, BLASTn assignments (subject scientific names—sscinames), despite having an exact/high confidence match (≥90%) to the respective DNA sequences deposited in GenBank, can result in an ambiguous sscinames, e.g., "prasinophyte sp. MBIC10622" or "uncultured Nitrospiraceae bacterium." Therefore, sscinames were placed into four categories; "yes," "uncultured assigned," "uncultured unassigned," or "no." If "yes" was assigned, then the sscinames did not start with "uncultured." If the sscinames started with "uncultured," then the results were further divided into "assigned" (at a minimum the phylum was determined) or "unassigned" (the phylum was not determined). Lastly, assigning "no" meant no sscinames assignment was possible when applying the filtering parameters (qcovhsp ≥90; pident ≥85). The data subsequently underwent quality filtering in R Studio v1.4.1106 (R v4.1.0; R Core Team, 2021). These final datasets were then used to create phylogenetic trees by aligning sequences using MAFFT (Katoh et al., 2002) and building the trees using FastTree (Price et al., 2009) within Qiime2. R was used to produce the figures, mainly relying on ggplot2 v3.3.3 (Wickham, 2016) and ggtree v3.1.4.992 (Yu et al., 2017).

The Nucleospin Tissue Kit extracted higher quantities of DNA than the other kits, based on agarose gel visualizations. In general, more DNA was visible from the inner subsamples (2×94EIA per extraction kit) than the outer (2×94EOA per extraction kit), irrespective of the extraction kit used. Only the Nucleospin extractions for 94EIA did not generate Nanodrop warnings and had approximately extracted 5× more DNA compared with extractions done by the other kits (Appendix S1: Table S3). The remaining extractions were completed using the Nucleospin kit, and the low DNA concentrations for the outer subsamples were only seen for sample CCA94, for both ethanol (94EOA-94EOC) and silica (94SOA-94SOC) preservations. These outer subsamples had variable visible amplification but were consistent among PCRs, which aligns with the DNA gel results. Although ethanol and silica preservations were effective for CCA94 samples, a greater quality and quantity of DNA could be extracted from the ethanol-preserved sample.

Seven genes/gene regions were tested with variable amplification success. For some genes, multiple primer sets were tested, and those that did not work were noted (Appendix S1: Table S2). Strong amplification was produced by the *cox*1, 16S rRNA, 18S rRNA, and 23S rRNA genes. Amplification was weaker for the *rbc*L gene, the *tuf*A gene, and the ITS rRNA region. DADA2 filtering removed ~70% of the *tuf*A gene total raw reads, while the *rbc*L gene had ~50% removed; the ITS rRNA region and 16S rRNA gene, 40%; 18S rRNA gene, ~30%; and 23S rRNA and *cox*1 genes, ~25% (Appendix S1: Table S4). Post R filtering (e.g., >10 reads/ASV/subsample) ~75% of ASVs were retained (~1% reads removed). No reads were retained for 94EOC (18S rRNA gene) and 94SOB (18S rRNA and *rbc*L genes), each of which had a low number of raw reads. In general, more subsamples had higher numbers of ASVs identified from the inner CCA substrate than the outer, except for the ITS rRNA region (CCA94 and CCA43) and 16S rRNA gene (CCA94; Appendix S1: Table S5). This trend remained when investigating algae taxa.

Taxonomic assignment success varied among the gene/gene regions (Appendix S1: Figure S2). On average, the rRNA genes 16S and 23S had the greatest ASV taxonomic assignment success as only 2.7% and 7.0% of ASVs had no assignments respectively. However, 82.3% of 16S rRNA gene ASVs were "uncultured" and either could be assigned to phylum-level or higher (52.2%; e.g., "uncultured *Acaryochloris* sp."), or could not be assigned to a phylum (30.1%; e.g., "uncultured bacterium"). The remaining 15.3% of 16S rRNA gene ASVs (the lowest among gene regions) were assigned (i.e., "yes" category). This contrasts with the 23S rRNA gene results, which had 75% of ASVs assigned as "yes" (Figure 1a). The genes/gene regions with the largest numbers of ASVs with no assignment ("no" category) were *tuf*A gene (82.3%), ITS rRNA region (67.2%), and *cox*1 gene (58.2%).

Overall, the greatest number of phyla were identified by the 16S rRNA gene (25), and the least by the *rbc*L gene (10; Figure 1b). Focusing on algal taxa from phyla "phyta" (e.g., Chlorophyta and Rhodophyta etc.), Cyanobacteria, and Myzozoa (class Dinophyceae only), the 23S rRNA gene had the greatest number (10), and the ITS rRNA region had the lowest (two; Appendix S1: Figure S3). Considering assigned algae ASVs, the two gene regions with the highest percentages were 38.6% for the 23S rRNA gene (Figure 1c) and 36.8% for the *rbc*L gene. The lowest number of algal ASVs was identified in 16S rRNA V4 region (1.8%). The 23S rRNA and *rbc*L genes were also identified as the two gene regions with the highest percentage of algal reads, 91.1% (*rbc*L) and 79.2% (23S; Figure 1c). The ITS rRNA region (0.6%) and the *tuf*A gene (3.1%) had the lowest percentages of algal reads.

For the 23S rRNA gene, Bacillariophyceae, Ulvophyceae, and Cyanophyceae reads were predominant in the inner layer, irrespective of sample

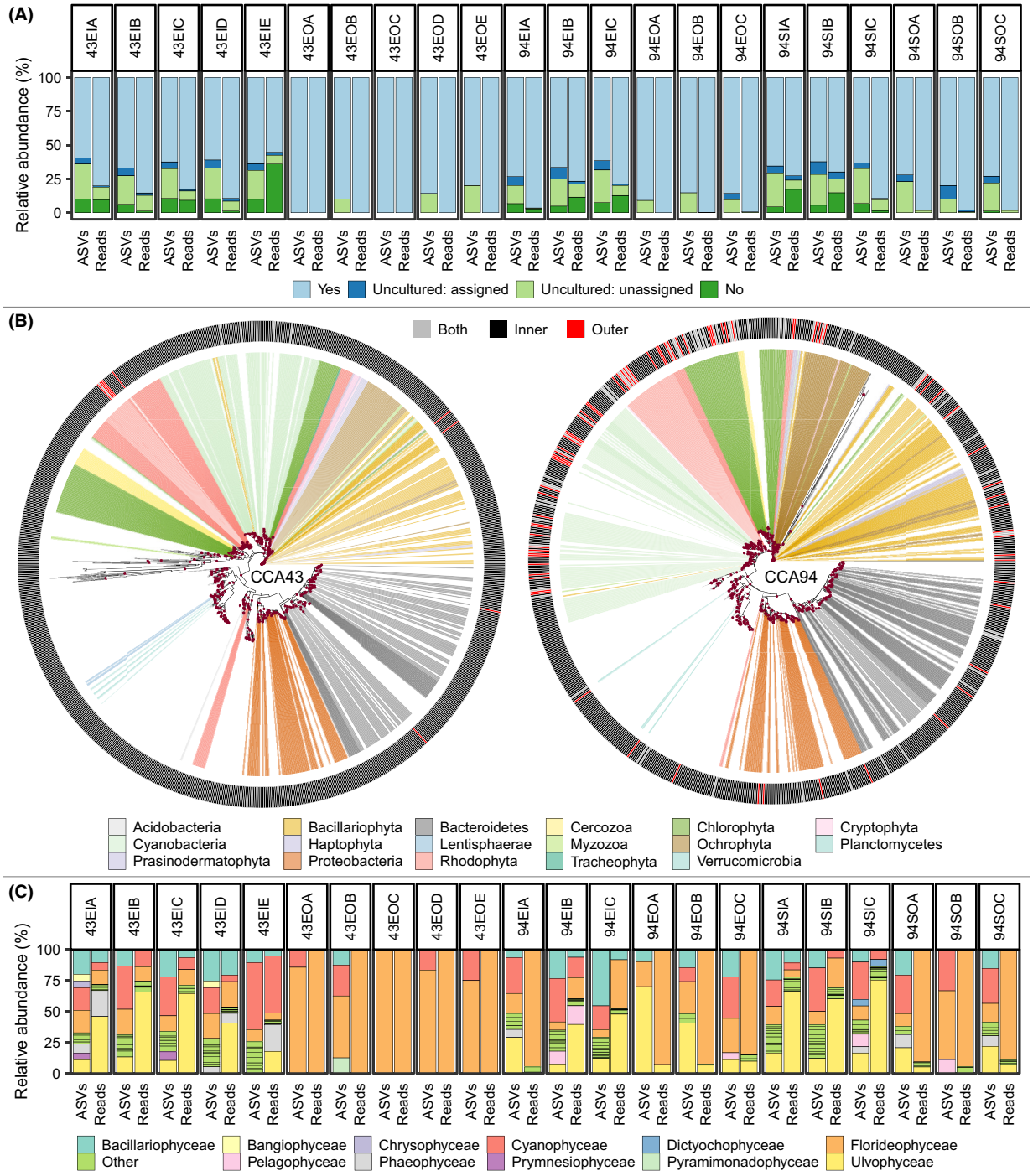

FIGURE 1 Information captured for crustose coralline algae samples, CCA43 and CCA94, using the 23S V5 gene region. Subsamples for each were taken for both the inner and outer layers; each subsample is denoted by the sample number (43/94), by whether ethanol or silica preserved (E/S), by whether from the inner or outer layer (I/O), and by which subsample (e.g., A–E). (A) Amplicon sequence variants (ASVs) were assigned using GenBank and categorized based on subject scientific names (sscinames). Four different categories were used: (1) "Yes"—taxonomy assignment for the ASV was successful and the sscinames did not start with "uncultured," (2) "Uncultured: assigned"—taxonomy assignment for the ASV was successful and the sscinames did start with "uncultured" but was still somewhat informative (at a minimum the phylum was determined), (3) "Uncultured: unassigned"—taxonomy assignment for the ASV was successful and the sscinames did start with "uncultured" but was not informative (phylum not determined) and, (4) "No"—taxonomy assignment for the ASV was unsuccessful using the given parameters. (B) Phylogenetic trees created using the ASVs (left CCA43 and right CCA94); branch lengths have been incorporated to show evolutionary relationships, and bootstrap values >0.9 are indicated as red dots. The outer ring indicates which CCA layer the ASV was identified from (both, inner, or outer) and the colors branching from the tree indicate the phylum of the ASV, if it could be assigned. (C) The algae composition ("phyta" phyla, e.g., Chlorophyta and Rhodophyta etc., Cyanobacteria, and Myzozoa—class Dinophyceae only) of each subsample at a class-level. If the relative abundance was ≤5, the class was assigned as "other" for the purpose of visualization. The number of "other" classes can be identified by black borders within "other."

(Figure 1c). The only algal class that had more reads in the outer layer was Florideophyceae (Figure 1c). This was similarly seen for the *rbc*L gene; however, Cyanophyceae reads were predominant in the outer layer for CCA43, and Florideophyceae had a greater number of reads in the inner layer of CCA94. Pinguiophyceae (<20 reads identified in each sample) was the only other class identified in the outer layer of CCA94 and not in the inner layer, yet the reverse was seen for CCA43.

The results reveal variable diversity within different areas of a CCA sample and show that relying on a single subsample from each layer does not adequately represent the diversity held within. At an ASV level, minimal overlap was seen within a layer among subsamples, but a greater overlap could be achieved if considering taxonomic assignment levels (e.g., family level). Based on the results presented here we recommend multiple extractions from each layer, per sample, be undertaken to reflect the diversity present. Even then, the results should be interpreted cautiously. As seen in this study, the ASV overlap between subsamples is likely to be minimal irrespective of the layer or gene/gene region used. Sample preservation proved more successful when using ethanol for DNA extractions, and this method is recommended for future studies undertaking DNA work on CCA.

As has been highlighted in many DNA metabarcoding studies, reference databases are incomplete, and this can only be somewhat mitigated by using multiple gene regions to increase taxonomic breadth (Alberdi et al., 2018; van der Reis et al., 2020, 2023; Zhang et al., 2018). Evident from our results was the number of ASVs that could not be assigned at a known genus level (i.e., either the sscinname was ambiguous and did not contain the taxonomic information required for genus-level assignment or the pident was <95%), and this highlights the volume of missing marine algae references for all gene/gene regions tested (as also noted in other algae studies; Fredericq et al., 2019; Yoon et al., 2016). Nevertheless, using multiple genes/gene regions was paramount for targeting a broad range of taxa and identifying the scope of each gene or gene region. At present, 23S rRNA and *rbc*L genes are more suitable to identify algae, but the opportunity to identify other taxa and assess diversity beyond algae is limited. To note and based on ASVs, the *rbc*L gene favored Chlorophyta taxa due to its primer design, but did still amplify other algae phyla (Appendix S1: Figure S3). The *cox*1 gene fared better: it was more universal while still capturing algae and may be an option as databases increase their references over time. GenBank (non-curated database) is currently the only "one-fits-all" reference database, but we do note that if a more in-depth study were to be done using GenBank, then ASV assignments would need to be inspected in more detail. For example, a noticeable incorrect assignment for an Annelida species was identified in the *rbc*L gene data, but the GenBank entry was referring to an unidentified endosymbiont and not the annelid. However, not all assignments that appear incorrect necessarily are. An interesting assignment was for a chloroplast gene region in a Tracheophyta for 23S rRNA gene, which indicates high similarity in chloroplast genes linking terrestrial to marine plants, requiring further investigation (chloroplast genome review in Dobrogojski et al., 2020).

This pilot study shows the immense diversity held within CCA, with more diversity typically identified within the inner layers than the outer. These results also confirm studies undertaken on rhodoliths, which have been identified as supporting a high diversity and abundance of marine species at both a micro- and macroscopic levels (e.g., Foster et al., 2013; Fredericq et al., 2019; Nelson et al., 2012). Specifically, this work inspected the presence of algae in shallow-water encrusting CCA and calls for further investigation into these autogenic ecosystem engineers that likely act as marine seed banks with the ability to harbor algal taxa during periods of stress.

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AUTHOR CONTRIBUTIONS

Aimee L. van der Reis: Conceptualization (supporting); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (supporting); software (lead); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Mary A. Sewell:** Conceptualization (lead); data curation (supporting); funding acquisition (lead); investigation (supporting); methodology (supporting); project administration (lead); resources (equal); supervision (equal); validation (supporting); visualization (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Wendy A. Nelson:** Conceptualization (lead); data curation (supporting); funding acquisition (supporting); investigation (supporting); methodology (supporting); project administration (lead); resources (equal); supervision (equal); validation (supporting); visualization (supporting); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

All codes used are available on https://github.com/AvdReis/CCA_seed_bank/. The sequencing data will be made available upon reasonable request in consultation with Ngāti Kuri as mana whenua and mana moana of Rangitāhua.

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SUPPORTING INFORMATION

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

Appendix S1. Appendix S1 contains the Tables S1–S5 and Figures S1–S3.

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