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Actinobacteria diversity associated with marine sediments and a wetland system, Agulhas-South Africa

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Abstract

Background South Africa is known for its great biodiversity. The Agulhas Plain represents one such unique environment where low-gradient topography has resulted in extensive wetland formation. It is fed by two major river systems, bringing in brackish, alkaline water. It has been exposed to major marine transgression and regression events, and harbours great Fynbos diversity as well as a Mediterranean-type climate, thereby creating unique ecosystems. It is therefore surprising that little is known about the bacterial diversity associated with the Agulhas Plain and associated marine ecosystems.

Methods In this study, we focused on the actinobacterial diversity (Phylum *Actinomycetota*) associated with an emerging peatland on the Agulhas Plain (SF; Areas 1–3) and a marine site (ANP; Ocean, Rocky, Dry) located 10 km away from SF. A combined metataxanomics and isolation approach was taken to evaluate the actinobacterial diversity of the sampling sites and to determine the effect of environmental physicochemical parameters on these populations. Various genome analyses were performed on an Sva0096 marine bin to gain insight into its ecological role.

Results Metataxanomics showed that the two sites shared defined major taxa, including *Blastococcus*, *Geodermatophilus*, *Microbacterium*, *Mycobacterium*, *Nocardioides*, *Streptomyces*, and the Sva0996 marine group. Analysis of the biosynthetic potential of an Sva0996 marine bin134 (obtained from GenBank) provided insights into the potential ecological role of this group of bacteria in both the marine and terrestrial environments. Higher actinobacterial diversity (Shannon index > 5) was observed for Areas 2 and 3 (SF), as well as the ANP Dry samples. The actinobacterial population composition was found to be driven by salinity, pH, Mn, and Ca, with certain areas of SF exhibiting similar (and even higher) salinity (SF: 70–100 Ω vs. ANP: 100–160 Ω) and lower pH levels (SF: 6.3-8.0 vs. ANP: 8.6–8.9) to that of the marine environment.

Conclusion This snapshot study has provided some insights into the actinobacterial diversity of the two sites studied. Analysis of an Sva0096 marine bin134 provided further insights into the potential ability of the Sva0096 marine group to survive in a unique terrestrial environment that is periodically exposed to environmental pressures that mimic the marine environment.

Keywords Actinobacteria, Agulhas, Biodiversity, Marine sediments, Peatlands, Sva0096 marine group

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Introduction

The Agulhas Plain in South Africa has a unique low-gradient topography that has resulted in extensive wetland formation in this area (Gordon 2012), which includes permanent lacustrine systems (e.g., Soetendalsvlei) and several endorheic pans (Russell and Impson 2006). Two major river systems (Nuwejaars River and Heuningsnes River) feed into the Agulhas plain, bringing in brackish (< 2 g/kg salts), alkaline (pH 6–8) water (Russell and Impson 2006; Gordon et al. 2012). Many of the endorheic pans are not connected to these systems and occasionally dry out completely, resulting in strongly saline saltpans (e.g., Soutpan has a salinity of 16–86 g/kg salt). The salinity of the Agulhas Plain is also linked to the major marine transgression and regression events it was exposed to over the past 8000 years, with many of the wetland systems containing deposits of marine-derived organic matter such as marine bivalve shells (Gordon et al. 2012; Kirsten et al. 2018). In addition, the area is known for its great Fynbos diversity and Mediterranean-type climate, creating unique ecosystems (Gordon et al. 2012). The preservation of ecosystems and their biodiversity, as well as their sustainable development, are guided by soil conservation strategies (Dos Santos et al. 2013). In the Agulhas region, the Working for Wetlands group, as well as the South African National Parks (SANParks), have assigned various areas as key conservation foci (Russell and Impson 2006). It is surprising that these environments have not been thoroughly studied for microbial diversity, particularly since changes in microbial diversity patterns could serve as indicators of environmental health (Weels et al. 2022). Such studies play a crucial role in conservation planning and monitoring the impact of climate change.

In both marine and terrestrial ecosystems, bacteria play a crucial role in nutrient cycling, including carbon and minerals, organic matter decomposition, and bioremediation. This significantly impacts the overall functioning of the ecosystems they inhabit (Lew et al. 2018; Heinrichs et al. 2020; Liu et al. 2020; Miller et al. 2020). The phylum *Actinomycetota* Goodfellow 2021 (Oren and Garrity 2021) represents one of the largest bacterial phyla within the domain Bacteria and is one of the most abundant groups of bacteria found in soil (22 ± 4%; Araujo et al. 2020). These organisms are of great interest to various researchers and industries, primarily due to the search for novel secondary metabolites such as pigments and antibiotics. They are also studied for their industrially relevant enzymes and their role in natural and engineered processes, e.g., floc formation in wastewater treatment systems (Bérdy 2012; Le Roes-Hill and Prins 2016). Furthermore, actinobacteria are known to be involved in the degradation of lignocellulosic materials (and ultimate carbon and nitrogen cycling; Rich et al. 2003; Hill et al. 2011), the stabilization of clay particles, the increase of soil moisture uptake, plant productivity enhancement, and the stabilization of organic matter, thereby playing an essential role in maintaining the health of various types of soil-based environments (Araujo et al. 2020). Despite extensive research on this group, little is known about their diversity in unique wetland ecosystems, such as those found on the Agulhas plain or the nearby marine environment.

Various studies focused on microbial diversity have reported the impact of the physicochemical properties of these ecosystems on diversity patterns. In the evaluation of the 'actinobiome' of > 900 soil samples from Australia and Northern Antarctica, Araujo et al. (2020) showed that environmental conditions drive the endemicity of certain actinobacterial taxa. In studies focused on peatlands, the major factors influencing microbial diversity range from water table level and vegetation composition (Kotiaho et al. 2013), pH (Jenkins et al. 2009; Zhang et al. 2017; Lew et al. 2018; Too et al. 2018), total carbon and total nitrogen (Zhang et al. 2017), Ca, P and Fe availability (Sun et al. 2016), ammonia and pH (Lin et al. 2012), changes in general nutrient supply (Parvina et al. 2018), to vegetation and pH (Sun et al. 2014). Similarly, studies focused on marine bacterial communities have found that various factors play a role in community composition. These factors include salinity, total N and P levels (Nimnoi and Pongslip 2020), as well as salinity, organic carbon, pH, and CO_2 partial pressure (Héry et al. 2014). Chen et al. (2016), on the other hand, identified pH, calcium, total organic carbon, total P, and total N as important factors (Chen et al. 2016).

The Agulhas region is home to several key conservation areas that have not been subjected to microbial diversity studies. To address this, we selected a wetland system (identified as an emerging peatland) on the Agulhas Plain and a marine site within the Agulhas National Park for analysis of actinobacterial diversity (culturable and non-culturable), and their occurrence was evaluated in the context of the environmental physicochemical properties.

Materials and methods

Chemicals and reagents

Unless otherwise stated, all chemicals and reagents used in this study were obtained from Merck-Millipore (South Africa).

Sampling

Samples were collected from Springfield Farm (SF; 34°44'15.3"S, 19°54'38.6"E) located at the base of the Soetanys Mountain, Agulhas Plain (August 2019) (Fig. 1a

Fig. 1 **A** Satellite image of the Agulhas plain and location of the Springfield Farm (SF) and Agulhas National Park (ANP) sampling sites as obtained from Google Maps; **B** location of the SF sampling site (yellow pin) in relation to key wetland ecosystems, Soetendalsvlei (blue dot) and the Soutpan (green dot); **C** location of the marine sampling site ANP; **D** photograph of SF sampling area 1; **E** photograph of SF sampling area 2; **F** photograph of SF sampling area 3; and **G** photograph of the marine sampling site, ANP

and b). Three sites were selected for sampling (Fig. 1d, e, and f). For each site, three subsites (1 m apart) were selected, and soil was collected from each subsite at three levels: top (first 5 cm), middle (10–15 cm), and deep (20–25 cm) (total of nine samples per site; 27 samples in total). In addition, samples were collected from Suiderstrand, Agulhas National Park (ANP; 34°48'51.3"S 19°56'25.4"E; Fig. 1a and c), with permission from the South African National Parks (SANParks, Permit number: CRC/2019–2020/013–2019/V1) (September 2019). Samples were collected from three sites (Fig. 1g), termed 'Ocean' (sediment below the low tide level and therefore always inundated with seawater), 'Dry' (sediment above the high tide level and therefore nearly always dry), and 'Rocky' (sediment from around the boulders on the beach area). Six sediment samples were collected for each site, each spaced 3 m apart, giving a final number of 18 samples. For both sites, samples were collected using a hand trowel (cleaned and sterilised between sampling events), with depth measurements taken using a Powerlock metric tape measure. Samples collected from SF and ANP were stored in sterile bags and transported at 4 °C. Samples were immediately processed for metagenomic DNA (mgDNA) extraction and composite samples were prepared and sent to Pathcare Bemlab (Strand, South Africa) for physicochemical analyses. The samples were classified as clay, loam, or sand according to the Soil Classification Working Group (1991). The pH of each sample

was determined electrometrically by the KCl method (McClean 1982). Total organic carbon content (C%) of each sample was determined by the Walkey-Black method (Nelson and Sommers 1982). Boron (B) was extracted from each sample with the hot water extraction method (Fertilizer Society of South Africa 1974), while calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na) were extracted from each sample using ammonium acetate extraction (Doll and Lucas 1973). The Bray II and Olsen extraction method (Thomas and Peaslee 1973) was used to extract phosphorus (P) from each sample, while the calcium-phosphate extraction method (The Non-affiliated Soil Analysis Working Committee 1990) was used to extract sulphur (S) from each sample. Quantification of these elements (B, Ca, Mg, K, Na, P, and S) was then performed with an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES; Varian Vista MPX, Australia). The copper (Cu), iron (Fe), manganese (Mn), and Zinc (Zn) content of each sample was determined by the EDTA extraction method (Beyers and Coetzer 1971). In addition, the electrical resistance of each sample was determined in a saturated paste using a standard cup method (The Non-affiliated Soil Analysis Working Committee 1990).

Microbial community analyses

mgDNA was isolated from 0.1 g of each sample using the DNeasy PowerSoil DNA Isolation Kit (Qiagen), according to the manufacturer's instructions. The amplification of the 16S rRNA gene was performed using the method described by Schäfer et al. (2010). The actinobacterial-specific 16S rRNA gene primer pair, Com2xf: 5'-AAA CTCAAAGGAATTGACGG-3'; Ac1186r: 5'-CTTCCT CCGAGTTGACCC-3', was used in the following PCR reaction mix: 1x KAPA Taq Readymix (1.5 mM $MgCl_2$ and 0.2 mM of each dNTP), 0.2 µM of each primer, 1 µL of template (10 ng of mgDNA) and PCR-grade water to a final volume of 25 µL. The amplification program was started with an initial denaturation step at 95 °C for 3 min, followed by 25 cycles of the following steps: denaturation at 94 °C for 30 s, an annealing gradient with temperatures between 51.6 and 60.2 °C and extension at 72 °C for 30 s. The amplification program was ended with a final extension step of 72 °C for 15 min. Genomic DNA from *Streptomyces polyantibioticus* SPR^T was used as a positive control. Amplicons were analysed by electrophoresis on a 0.8% (w/v) agarose gel (containing 10 µg/mL ethidium bromide) and visualised under UV_{254nm} light. Amplicons were excised from the agarose gel, purified using the Machery-Nagel gel purification kit (purchased from Separations), and submitted to the DNA sequencing facility at the Central Analytical Facility (CAF), Stellenbosch University. Sequencing was performed using an Ion Torrent S5, and raw data was received in FASTQ format for further metabarcoding analysis. Metadata is available in the NCBI BioSample database (http://www.ncbi.nlm.nih.gov/biosample/) under accession numbers SAMN35158336-35158364. The data have been deposited with links to BioProject accession number PRJNA974022 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

The open-source package, mothur (v1.44.0, Schloss et al. 2009), was used to perform the metabarcoding analyses. Batch scripts were used to conduct the analyses remotely on the Centre for High Performance Computing (CHPC), hosted by the Council for Scientific and Industrial Research (CSIR) situated at their Rosebank Campus (Cape Town, South Africa). Scripts were queued in batches to the CHPC PBSPro scheduler via the ssh protocol, and the outputs were intermittently checked to ensure no errors occurred during processing. The raw sequencing reads were filtered to remove bases below a minimum Phred score of 20, followed by removing ambiguous bases and merging the reads into a single file. VSEARCH was used to remove chimeric sequences. Taxonomic classification was performed using Bayesian classification, with the SILVA 16S rRNA gene database (v138, Quast et al. 2013) as reference. Lineages classified as non-prokaryotic were removed. The remainder were aligned to the reference database, ensuring the region of interest overlapped across all samples, after which redundancies and overhangs were removed. Subsequently, the data was normalised by subsampling to the minimum library size, and the sequences clustered into Operational Taxonomic Units (OTUs) at a distance-matrix cut-off of 0.03. The resultant OTU table and taxonomy file were used for downstream analyses.

Statistical analyses

The alpha and beta diversity were analysed using RStudio containing R Core version 4.2.0 (R Core Team 2022). The data was filtered to focus on the phylum *Actinobacteriota* (reclassified as *Actinomycetota*; Oren and Garrity 2021). Principal Coordinate Analysis (PCoA) and non-metric multidimensional scaling (NMDS) were performed based on the Bray-Curtis dissimilarity matrix. Data visualization was performed using the R packages "phyloseq", "dplyr" and "ggplot2" (McMurdie and Holmes 2013; Wickham 2016; Wickham et al. 2022). Additional analyses were performed using Primer 7® software (Quest Research Limited, Auckland, New Zealand) according to the developers' instructions (Clarke and Gorley 2015; Clarke et al. 2016). Analysis of similarity (ANOSIM) was based on Spearman rank similarity.

Furthermore, multivariate analyses on the square root transformed microbial community data and the

fourth root transformed physicochemical data were performed. For the physicochemical data, Principal Component Analysis (PCA) plots were constructed from similarity matrices based on Euclidean distances. To determine the effect of physicochemical parameters on the microbial communities, the most important drivers were determined through constrained cluster analyses (LINKTREE), which were performed using Bray-Curtis similarity matrices of the microbial community and physicochemical data sets (determined using BEST analyses). To determine whether any biomarker genera could be identified for the different classes (sampling sites), Linear Discriminant Analysis Effect Size (LefSe) analysis was performed via the MicrobiomeAnalyst 2.0 platform, using the default parameters (Lu et al. 2023). Core microbiome analysis was also performed, with parameters set to a sample prevalence of 20% and a relative abundance of 0.01%. The outputs were visualised as heatmaps.

Sva0996 marine group bin134 analyses

The Sva0996 marine group bin134 genome sequence (Slaby et al. 2017) was downloaded from GenBank (GenBank assembly accession: GCA_002239105.1). The Galaxy Platform (The Galaxy Community 2022) was used to perform the following analyses: QUAST (Galaxy Version 5.2.0 + galaxy1; Gurevich et al. 2013), Busco (Galaxy Version 5.4.6 + galaxy0; Simão et al. 2015), and Prokka (Prokaryotic genome annotation; Galaxy Version 1.14.6 + galaxy1; Seemann 2014). The genome was visualised using Proksee (Grant et al. 2023), and specific features identified in the genome using the Alien Hunter tool (version 1.1.0) (Vernikos and Parkhill 2006), CRISPR/Cas Finder tool (version 1.1.0) (Couvin et al. 2018), and the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) tool (version 1.1.1) (Alcock et al. 2020). The biosynthetic potential of the Sva0996 marine group bin134 was evaluated using dbCAN3 (Zheng et al. 2023), eggNOG-mapper 2.1.9 (Cantalapiedra et al. 2021), and antiSMASH version 7.0.0 (Blin et al. 2023).

Isolation of actinobacteria from SF samples

Actinobacterial strains were isolated using three different selective isolation techniques: (1) Soil samples (1 g of each sample) were removed from the sampling bags using a sterilised spatula, spread out onto a sterile Petri dish (90 × 15 mm), and were incubated at room temperature (22 ± 3 °C) and with variable (day/night) artificial fluorescent lighting exposure for 7 days before performing isolations; (2) 1 g of soil was added to 9 ml Ringer's solution (g/L: 7.2 NaCl, 0.17 $CaCl_2$, 0.37 KCl, pH 7.3–7.4; filter-sterilised and autoclaved) containing 25 µg/mL rifampicin and 100 µg/mL cycloheximide and mixed for 15 min at 37 °C on a rotary platform at 15 rpm; and (3) 1 g of soil was added to 9 mL Ringer's solution containing thirteen glass beads (4 mm; Merck-Millipore) and mixed at room temperature (22±3 °C) on a rotary platform at 15 rpm for 15 min. Ten-fold serial dilutions of the different pre-treated samples were prepared in Ringer's solution (up to 10^{-3}), and 100 µL of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated in duplicate onto the following media: International *Streptomyces* Project medium 2 (ISP2; Shirling and Gottlieb 1966), ISP2 supplemented with calcium carbonate (ISP2-C; also called GYM *Streptomyces* medium or DSMZ medium 65), ISP2-C diluted 1:10 with a Red Sea Salt (RSS; Coral Pro Salt, Red Sea; 38.2 g/L) solution (ISP2-C 1:10), and M1 (Hames-Kocabas and Uzel 2012) (Table S1). All media were adjusted to pH 7.0 using 1 M KOH before autoclaving and supplemented with either 5, 15, 30, 40, or 50 µg/mL rifampicin and 100 µg/mL cycloheximide or 50 µg/mL nalidixic acid and 100 µg/mL cycloheximide after autoclaving.

In addition, an enrichment process was employed: 1 g of soil was added to 9 mL enrichment media (10%, v/v, M1, g/L: 1 soluble starch, 0.4 yeast extract, 0.2 peptone, 19.1 RSS) supplemented with 25 µg/mL rifampicin and 100 µg/mL cycloheximide and was incubated at room temperature (22 ± 3 °C), mixing at 15 rpm for 3 months. Samples were removed after 2 weeks, 1 month, 1½ months, 2 months, and 3 months. Ten-fold serial dilutions were prepared (up to 10^{-9}) in an RSS solution (38.2 g/L), and 100 µL of the 10^{-1} to 10^{-5} dilutions were plated for the 2 weeks and 1-month samples, while the 10^{-5} to 10^{-9} dilutions were plated for the 1½, 2- and 3-months samples. All dilutions were plated in duplicate onto M1 supplemented with RSS (M1-RSS; pH 8.0) and 50% M1/50% RSS agar (pH 8.0) (Table S1). Isolation media were supplemented with either 5, 15, 30, 40, or 50 µg/mL rifampicin and 100 µg/mL cycloheximide or MAST® Selectavial (STAPH/STREP; prepared and used as per the manufacturer's instructions) and 100 µg/mL cycloheximide.

Isolation plates were incubated at 30 °C and duplicate sets at room temperature (22 ± 3 °C) with a variable (day/night) artificial fluorescent light exposure and evaluated for bacterial growth on a weekly basis. Actinobacterial colonies were selected based on morphology and re-streaked onto agar media lacking antibiotics. Pure cultures were maintained as stock cultures in 20% (v/v) sterile glycerol at -20 °C and − 80 °C.

Isolation of actinobacteria from ANP samples

Actinobacterial strains were isolated from untreated sediment samples, as well as mechanical and physical pre-treated samples. For the mechanical pre-treatment, 1 g of sediment was added to 9 mL Ringer's

solution containing eleven glass beads (4 mm) and mixed on a rotary platform at 15 rpm for 15 min at room temperature (22±3 °C). For the physical pre-treatment, 5 g of sediment was incubated in sterile Petri dishes (90×15 mm) at 37 °C for 24 h (variable day/night artificial fluorescent light exposure). Ten-fold serial dilutions (up to 10^{-3}) were prepared of untreated and pre-treated sediment samples in Ringer's solution, and 100 µL of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated in duplicate onto the following media: ISP2-C, ISP2-C 1:10, M1-RSS and International *Streptomyces* Project medium 5 (ISP5; Shirling and Gottlieb 1966) (Table S1). Before autoclaving, the pH of ISP2-C, ISP2-C 1:10, and M1-RSS were adjusted to 7.2 using 1 M KOH, while the pH of ISP5 was adjusted to 7.0 with 1 M HCl. Media were supplemented with 15 µg/mL rifampicin and 100 µg/mL cycloheximide (15R/C), 15 µg/mL rifampicin and 50 µg/mL potassium dichromate (R15/K), 25 µg/mL rifampicin and 100 µg/mL cycloheximide (25R/C), 25 µg/mL rifampicin and 50 µg/mL potassium dichromate (R25/K), 100 µg/mL penicillin and 100 µg/mL cycloheximide (P/C) or 100 µg/mL penicillin and 50 µg/mL potassium dichromate (P/K). In addition to serial dilution and plating, the stamping method (Hames-Kocabas and Uzel 2012) was used for untreated and physical pre-treated samples. To do this, the samples were lightly ground with a sterilised pestle and mortar, and sterile cotton wool was used to stamp the ground sediment onto duplicate agar plates following a clockwise spiral pattern.

Furthermore, during a second round of isolations, serial dilutions were prepared of untreated and mechanical pre-treated samples, and 100 µL of the 10^{-1} dilutions were plated in duplicate onto the following media: ISP2-C, ISP2-C 1:10, chitin, and artificial seawater (Hames-Kocabas and Uzel 2012) (Table S1). Artificial seawater and chitin agar were adjusted to pH 7.2 with 1 M HCl prior to the addition of the agar and autoclaving. The ISP2-C and ISP2-C 1:10 agar was supplemented with 15 µg/mL rifampicin and 100 µg/mL cycloheximide (15R/C) or 25 µg/mL rifampicin and 100 µg/mL cycloheximide (25R/C). In contrast, the seawater and chitin agar were supplemented with 25 µg/mL nalidixic acid and 100 µg/mL cycloheximide (N/C).

Isolation plates were incubated at 30 °C and duplicate sets at room temperature (22 ± 3 °C; exposure to variable day/night artificial fluorescent light) and evaluated for bacterial growth every week. Actinobacterial colonies were selected based on morphology and re-streaked onto agar media lacking antibiotics. Pure cultures were maintained as stock cultures in 20% (v/v) sterile glycerol at -20 °C and − 80 °C.

Isolation of genomic DNA and 16S rRNA gene sequence analysis

Genomic DNA was isolated from SF actinobacterial isolates using a modified bead-beating method (Miller et al. 1999) as described by Maibeche et al. (2022). A colony PCR protocol was used for the ANP isolates. An actinobacterial colony was transferred to 100 µL sterile distilled water and vortexed for 5 s. The samples were heated at 95 °C for 15 min and then cooled at 4 °C for 10 min. A total of 1 µL of the final sample was used to amplify the 16S rRNA gene by PCR. If the colony PCR was unsuccessful, genomic DNA was extracted from ANP isolates with the Quick-DNA™ Fecal/Soil Microbe MiniPrep Kit (Zymo Research; supplied by Inqaba Biotech) according to the manufacturer's instructions.

The 16S rRNA gene sequence was amplified by PCR to determine the taxonomic position of the isolates. The universal bacterial primers F1 and R5 and the PCR amplification program described by Cook and Meyers (2003) were used. The amplified DNA was purified using an MSB® Spin PCRapace kit (Stratec Molecular) and sequenced by the DNA sequencing facility at CAF (Stellenbosch University, South Africa). The sequences were analysed and edited using SnapGene Viewer (5.0.7). For identification at the genus level, partial 16S rRNA gene sequences were submitted to the 16S-based ID tool on the EzBioCloud platform (http://www.ezbiocloud.net; Yoon et al. 2017). The 16S rRNA gene sequences were also submitted to GenBank and have been assigned the following accession numbers: OQ993219 – OQ993328.

Results

Actinobacterial diversity – metataxanomics

The actinobacterial diversity of the sampling sites was determined using actinobacterial-specific 16S rRNA gene primers. Rarefaction analysis showed good sequencing coverage (> 99%; Fig. S1). The observed and the predicted species richness (Fig. 2a and b) were higher for SF Areas 2 and 3, while the ANP Ocean and SF Area 1 Deep (A1D) had the lowest species richness. Higher actinobacterial diversity (Shannon index > 5) was also observed for SF Areas 2 and 3, as well as the ANP Dry samples (Fig. 2c). The alpha diversity visualised at Order, Family, and Genus level can be seen in Figs. S2a, S2b and S2c.

Both sampling sites (SF and ANP) showed variation in actinobacterial population composition even within the three sub-sites of the main sampling areas (Fig. 3). The major taxa shared by both sites mostly include unclassified and/or uncultured taxa. Defined major shared taxa include *Blastococcus*, *Geodermatophilus*, *Marmoricola*, *Microbacterium*, *Mycobacterium*, *Nocardioides*, *Streptomyces* and the Sva0996 marine group. Taxa unique to the ANP site, include unclassified *Dermabacteraceae*

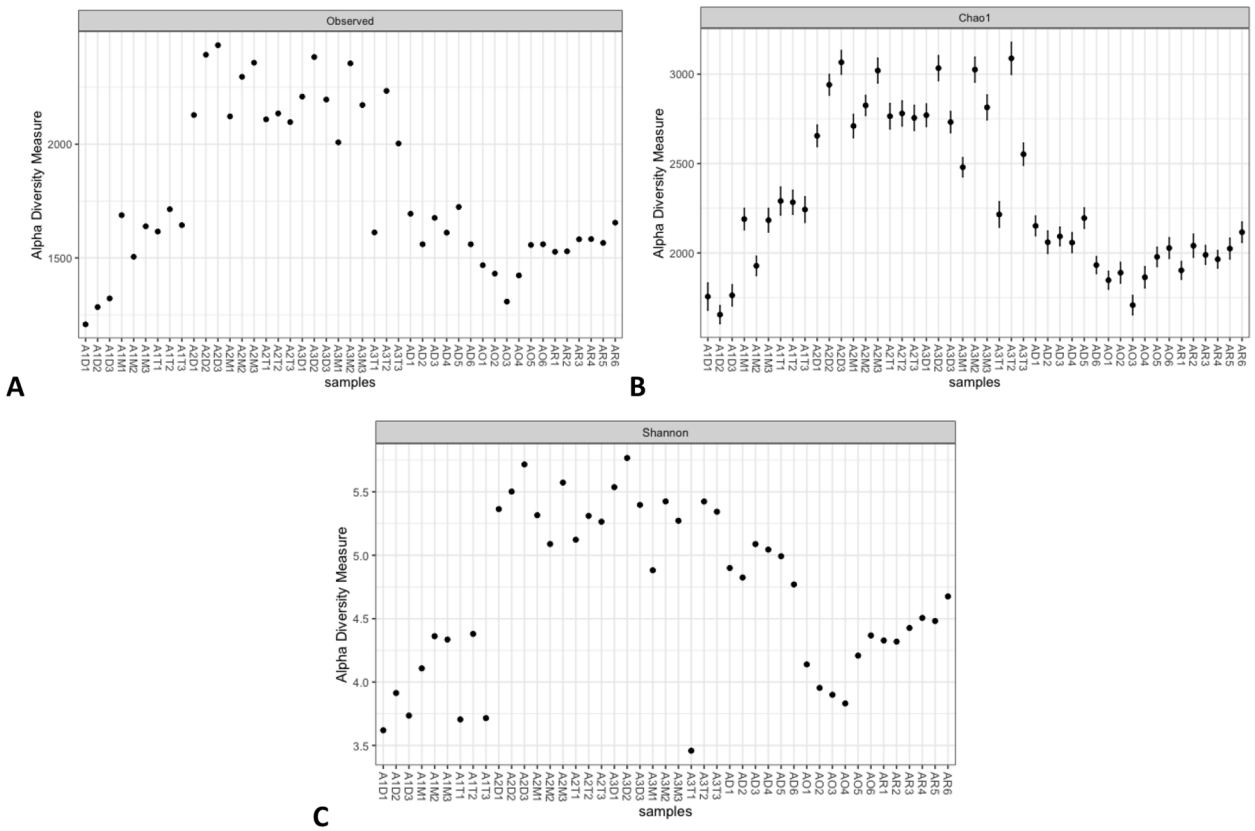

Fig. 2 **A** Actinobacteria species richness observed, and **B** predicted (Chao 1 index) for the Springfield Farm (SF) and Agulhas National Park (ANP) samples, as well as **C** the actinobacterial diversity (Shannon index). A1D: Area 1 Deep; A1M: Area 1 Middle; A1T: Area 1 Top; A2D: Area 2 Deep; A2M: Area 2 Middle; A2T: Area 2 Top; A3D: Area 3 Deep; A3M: Area 3 Middle; A3T: Area 3 Top; AD: ANP Dry; AO: ANP Ocean; AR: ANP Rocky

and *Promicromonosporaceae*, uncultured *Janibacter*, *Euzebya*, *Brevibacterium*, and candidatus *Planktoluna*. Taxa unique to the SF site is more extensive, and include *Actinocorallia*, *Actinomadura*, *Actinomyces*, *Amycolatopsis*, *Cryptosporangium*, *Curtobacterium*, *Frankia*, *Gryllotalpicola*, *Homoserinibacter*, *Jiangella*, *Kineospora*, *Kitasatospora*, *Kocuria*, *Kribbella*, *Nonomuraea*, *Tsukamurella*, unclassified *Cellulomonadaceae*, unclassified *Frankiaceae*, unclassified *Kineosporiaceae*, unclassified *Streptosporangiaceae*, and *Virgisporangium* (Fig. 3).

LefSe analysis showed the presence of 61 features (genera) with a log Linear Discriminant Analysis (LDA) score of more than 2.0 (Fig. 4). The dominant classified biomarker genus was identified as the Sva0996 marine group (LDA = 6.2; $p < 0.001$) for site ANP, as *Frankia* (LDA = 5.13; $p < 0.001$) for SF Area 1, as *Streptomyces* (LDA = 4.85; $p < 0.001$) for SF Area 2 and as *Mycobacterium* (LDA = 5.99; $p < 0.001$) for SF Area 3. Of these features, the Sva0996 marine group, *Streptomyces* and *Mycobacterium* occur in all four sites, while *Frankia* was only detected in the SF sampling sites (Table S2). Unclassified *Actinobacteria* (now *Actinomycetes*) had the highest log LDA score for SF Area 2 (LDA = 6.08; $p < 0.001$), while unclassified *Acidimicrobiia* had the highest log LDA score for SF Area 1 (LDA = 5.98; $p < 0.001$) (Fig. 4).

Core microbiome analysis showed that unclassified *Actinobacteria*, unclassified *Acidomicrobiia*, and *Mycobacterium* dominate the SF Area 1 microbiome (Fig. 5a). Unclassified and uncultured *Actinobacteria* (assigned as uncultured_ge), unclassified *Actinobacteriota*, unclassified *Acidomicrobiia*, unclassified *Microtrichales*, *Mycobacterium* and the Sva0996 marine group dominates the SF Area 2 (Fig. 5b) and 3 (Fig. 5c) microbiomes, with unclassified *Micrococcales*, *Nocardioides*, *Geodermatophilus*, *Micromonospora*, and unclassified *Nocardioideceae* also dominating the SF Area 3 microbiome. The ANP microbiome is dominated by the Sva0996 marine group, unclassified *Microtrichales*, unclassified *Actinobacteriota*, unclassified *Ilumatobacteraceae*, uncultured *Actinobacteria*, unclassified *Acidomicrobiia*, and *Nocardioides* (Fig. 5d). The predicted core microbiomes of the four sites were also reflected in the visualisation of the alpha diversity of the top ten genera with the highest actual abundance (Fig. 6).

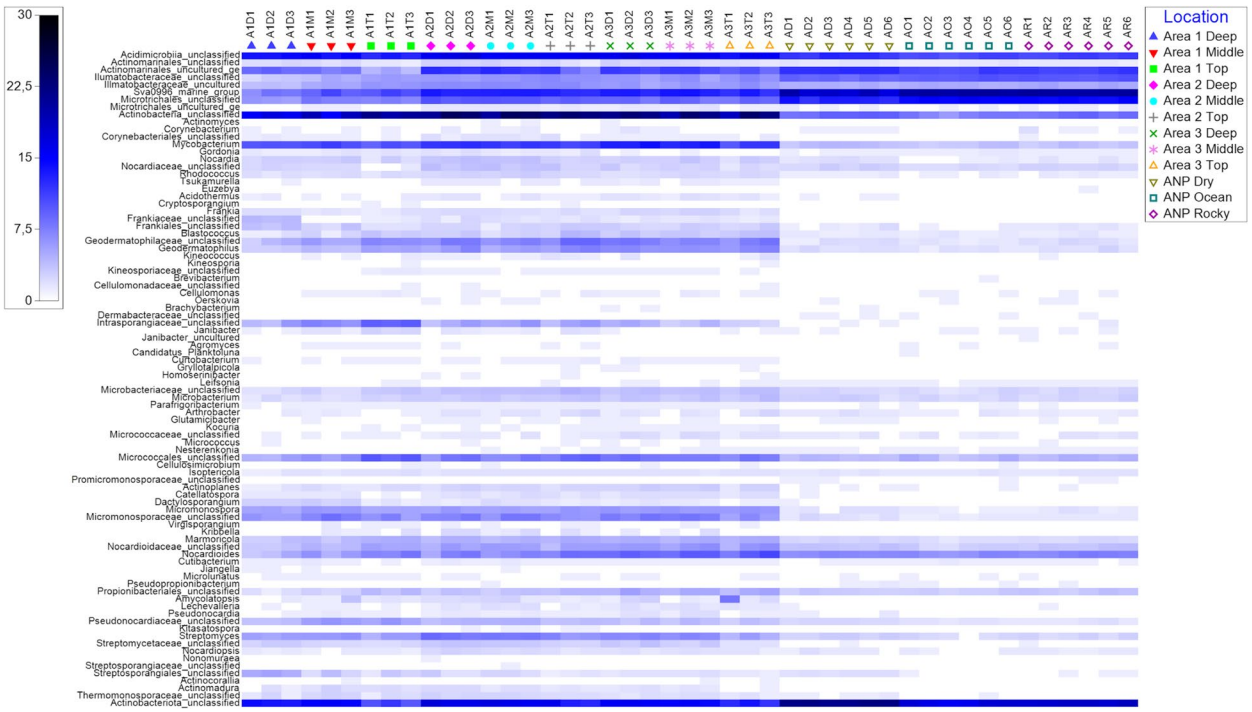

Fig. 3 Shade plot of the square root transformed relative abundance of the actinobacterial operational taxonomic units detected for the Springfield Farm (SF; Areas 1-3; A1-A3) and Agulhas National Park (ANP; AD1-6; AO1-6; AR1-6) samples

Sva0996 marine group bin134

In order to gain insight into the potential role of the Sva0996 marine group in the environments studied (SF and ANP), an Sva0996 marine group bin134 genome sequence was analysed. The Sva0996 marine group bin134 genome sequence consists of 77 contigs, 3 684 518 bp and a GC content of 64.29% (Fig. S3). Analysis of the Prokka-annotated genome using BUSCO, showed the presence of 91.9% complete Benchmarking Universal Single-Copy Orthologs (BUSCOs), 4.8% fragmented BUSCOs, and 3.3% missing BUSCOs (see Supplementary information: BUSCO). The CARD Resistance gene identifier tool in Proksee detected the gene, *rpsL*, which can infer aminoglycoside resistance (specifically, streptomycin). The CRISPRCasFinder tool, identified two Cas-3 Type I and one Cas-4 Type I-II Cas-type/subtypes, as well as nine CRISPR sites. In addition, the Alien Hunter tool in Proksee predicted the presence of 123 putative horizontal gene transfer events. Further analysis of the annotated genome showed that the genes typically associated with linear genomes, *tpg*, *tap*, and *ttr* (Kirby 2011), were absent. An evaluation of the Sva0996 marine group bin134 genome for the presence of carbohydrate-active enzymes (CAZymes) through the online dbCAN3 server showed the presence of 17 predicted CAZymes (prediction based on three separate tools, HMMER: dbCAN, HMMER: dbCAN_sub, and DIAMOND: CAZy), and 21 probable CAZymes (prediction based on two tools) (Table 1). No signal peptide sequence was detected for any of the predicted CAZymes, and the predicted CAZymes included members of the glycoside hydrolase (GH) family, the glycosyl transferase (GT) family, the carbohydrate esterase (CE) family, and enzymes with auxiliary activities (AA).

Analysis of the annotated Sva0996 marine group bin134 genome with eggNOG-mapper showed that 20 of the 26 COG categories are represented, with categories A (RNA processing and modification), Y (nuclear structure), Z (cytoskeleton), W (extracellular structures), X (mobilome: prophages, transposons), and R (general function prediction only) not represented. Of the 2682 proteins annotated (Table 2), 145 were assigned to COG category L (replication, recombination, and repair), 57 to COG category V (defense mechanisms), and 308 were assigned to COG category C (energy production and conversion), including 33 proteins annotated as luciferase-like monooxygenases, and six as nitroreductases. Proteins potentially involved in carbohydrate transport and metabolism (COG category G; 94 predicted) include the glycoside hydrolases and glucosyl transferases predicted by dbCAN3. In an analysis of the 110 proteins representing COG category Q (secondary metabolites biosynthesis, transport, and catabolism), nine are notably predicted to be involved in taurine catabolism

Fig. 4 Linear Discriminant Analysis Effect Size (LefSe) output for the four classes (sites) analysed, representing the 61 features (genera) identified with a log LDA score > 2.0. Individual scores of the different features are provided as supplementary material (Table S2)

(dioxygenases, TauD, TfdA family). A single biosynthetic gene cluster (BGC) of 26 073 bp was predicted by the online tool, antiSMASH, showing a 38% sequence similarity to the terpene BGC, hopene, encoded for on the genome of *Streptomyces coelicolor* A3(Alcock et al. 2020), and with a MiBiG similarity score of 0.33 to a carotenoid from *Rhodobacter sphaeroides* (Fig. S4).

Taxonomic position of actinobacterial isolates

A total of 86 isolates were obtained from sediment samples collected from ANP (Table S3). Most isolates (51 isolates) were obtained from samples collected at the dry sites, while the least number of isolates (12 isolates) were obtained from samples collected at the rocky sites. Only 15 of the ANP isolates could be identified to genus level,

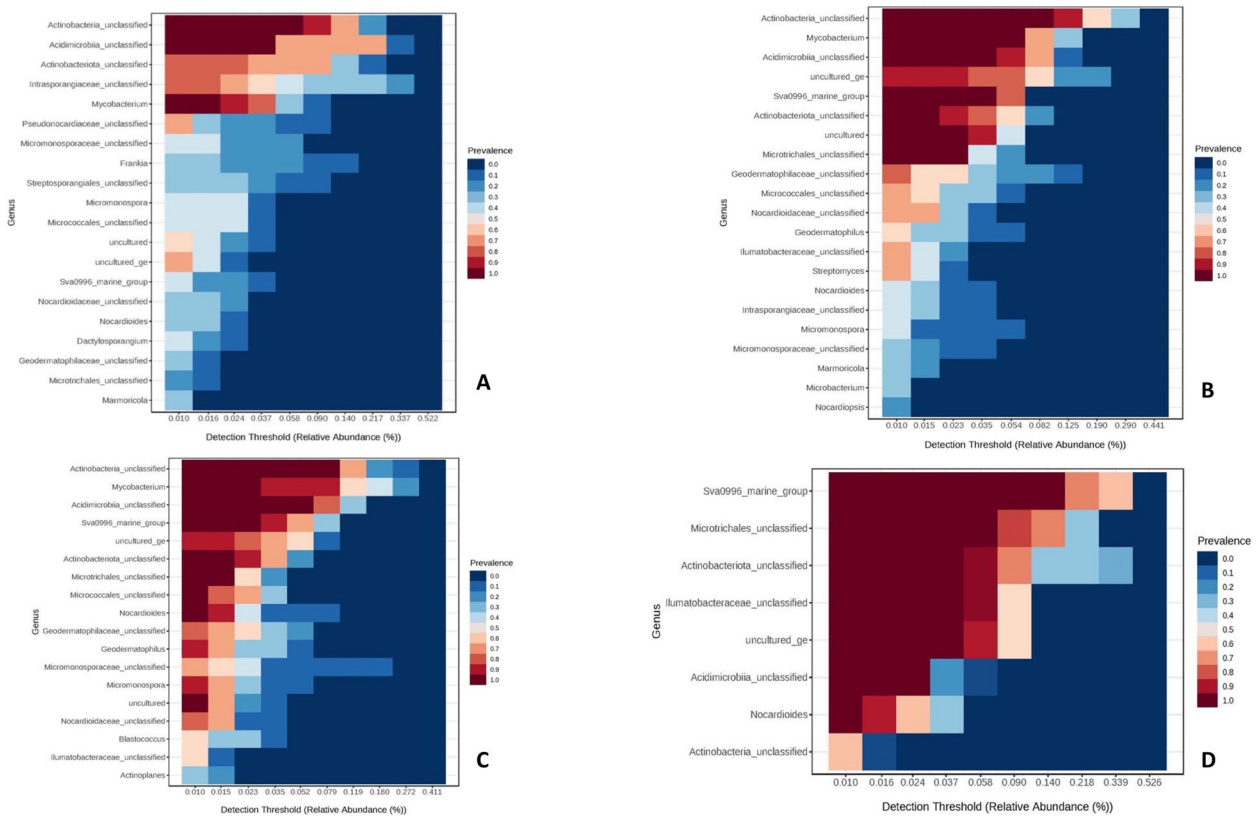

Fig. 5 Predicted core microbiomes of **A** Springfield Farm Area 1, **B** Springfield Farm Area 2, **C** Springfield Farm Area 3, and **D** Agulhas National Park

with all strains belonging to the genus *Streptomyces*. For the SF soil samples, 128 isolates were obtained (Table S4). Most of the isolates (Paix et al. 2019) were isolated from the Area 1 middle sample, followed by Area 1 deep (19 isolates) and Area 2 deep (9 isolates). Only one isolate was obtained from samples collected from Area 3. Sixteen actinobacterial genera representing twelve different families and eight different orders within the class *Actinomycetes* are represented among the isolates obtained.

Environmental factors driving actinobacterial diversity

Physicochemical properties were determined for bulk samples collected from SF at three depths for the three sampling sites and composite samples collected from ANP at three different sites (Table 3). The SF Area 1 top (A1T) sample consisted of loam (approximately 40% sand, 40% silt, and 20% clay; pH 6.3), while the Area 1 middle (A1M) and deep (A1D) samples consisted of clay (pH 6.8 and 7.0, respectively). All samples collected from SF Areas 2 and 3 consisted of sand, with a pH range of 6.9–7.4 for Area 2 and a pH of 8.0 for Area 3. Samples collected from ANP had a pH range of 8.6–8.9 and mainly consisted of granular particles (Fig. S5). Samples from SF Area 1 had a higher total organic carbon content than samples from the other two SF sites and samples from ANP (Table 3). Samples from SF Area 1 also had a much higher content of macronutrients and micronutrients than Areas 2 and 3, with an almost ten-fold higher concentration of Na, Ca, Mg, and soluble S, and a five-fold higher concentration of K and B. The ANP samples had a higher Ca concentration than the SF samples but had much lower amounts of Cu, Mn, and Fe. The Na levels were lower for the ANP samples than for SF Area 1 samples but higher than for SF Area 2 and 3 samples. Only the SF Area 2 middle (A2M) and Area 3 top (A3T) samples had resistance readings above 300Ω. The grouping of the samples based on the LINKTREE (Fig. S6) and principal component (Fig. S7) analyses showed that Ca, Mn, pH, and resistance (R) were the main determinants for the clustering of the different sampling sites.

According to the BEST analysis (Fig. 7), Ca, pH, and Mn are the key environmental drivers determining the actinobacterial communities associated with the two sampling sites (SF and ANP). Non-metric multidimensional scaling (nMDS) analysis showed a clear difference in the actinobacterial communities of the ANP Dry samples compared to the ANP Ocean and Rocky samples (Fig. 8a; key drivers: P, K, Zn, Mn, Na, and resistance),

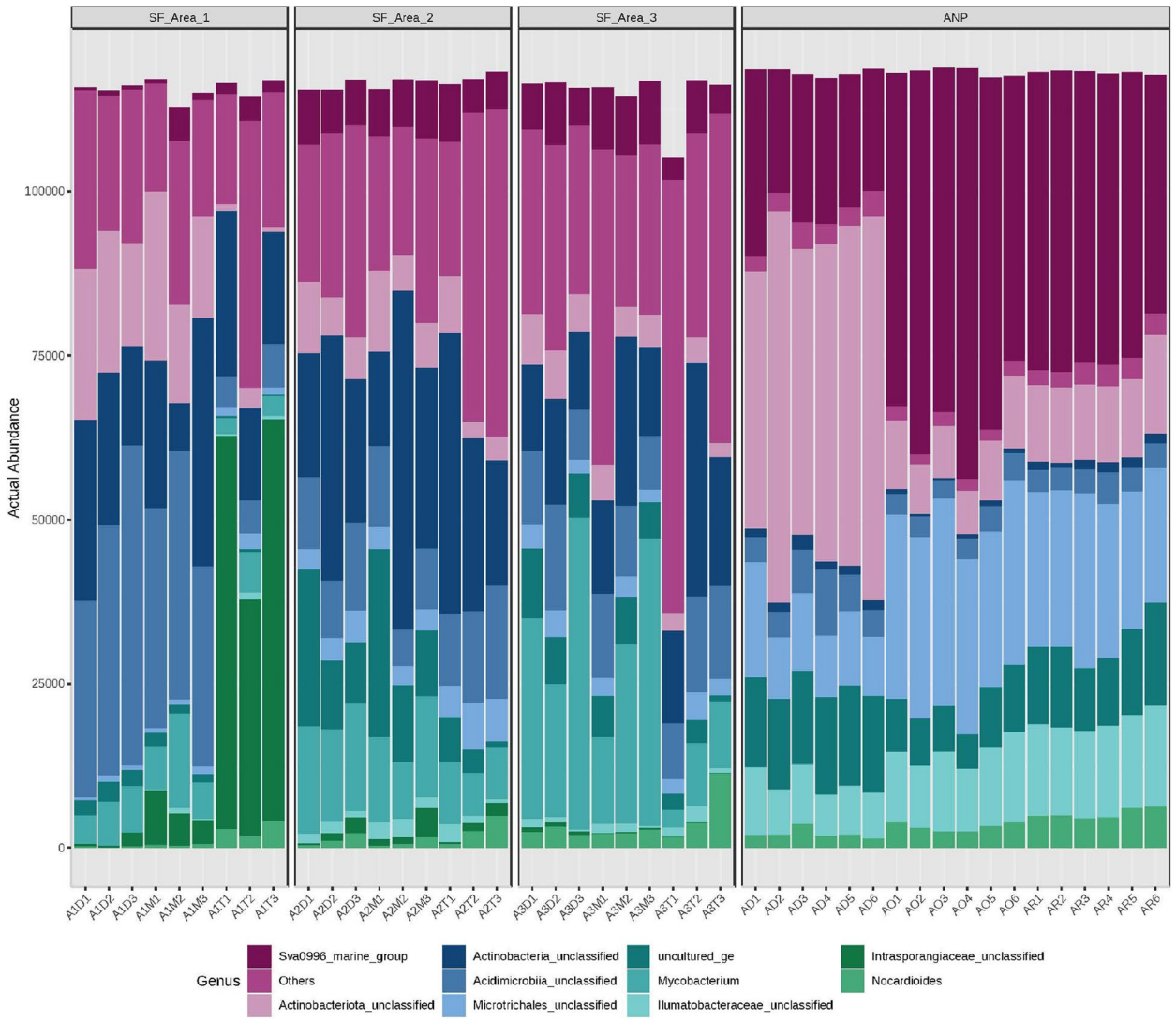

Fig. 6 Alpha diversity of the top ten genera with the highest count for Springfield Farm (SF) and Agulhas National Park (ANP). A1D: SF Area 1 Deep; A1M: SF Area 1 Middle; A1T: SF Area 1 Top; A2D: SF Area 2 Deep; A2M: SF Area 2 Middle; A2T: SF Area 2 Top; A3D: SF Area 3 Deep; A3M: SF Area 3 Middle; A3T: SF Area 3 Top; AD: ANP Dry; AO: ANP Ocean; AR: ANP Rocky

while Area 1 (SF) differed from the other two SF areas sampled (Fig. 8b; key drivers: all physicochemical parameters, except resistance).

Discussion

The Agulhas Plain and surrounding areas represent a unique ecosystem. It comprises a coastal marine region and inland wetland systems, and is encircled by the Cape Fold Mountain ranges. The Heuningsnes estuary and De Hoop Vlei, which form part of the Agulhas Plain are identified as Ramsar Wetlands of International Importance (Grenfell et al. 2019). In addition, the Agulhas Plain also forms part of the Cape Floristic Region, a recognised biodiversity hotspot. The coastal marine area is greatly impacted by the Agulhas current, where the warm waters of the Indian Ocean mix with the colder Atlantic Ocean, greatly influencing the climatic conditions of the south to south-east coast of southern Africa (Tim et al. 2023). This study provided a snapshot of the actinobacterial diversity of two important sites on the Agulhas Plain, providing the basis for future studies.

Although the two environments harboured different actinobacterial communities, they shared selected defined major taxa: *Blastococcus*, *Geodermatophilus*, *Marmoricola*, *Microbacterium*, *Mycobacterium*, *Nocardioides*, *Streptomyces*, and the Sva0996 marine group.

Table 1 Results output from the dbCAN3 server for the analysis of the Sva0996 marine group bin134 genome sequence. Only results confirmed by >=2 tools are presented. GH=glycoside hydrolase; GT=glycosyl transferase; CE=carbohydrate esterase; AA=Auxiliary activity

Gene ID	EC#	HMMER	dbCAN_sub	DIAMOND	# of Tools
MPNQ01000001.1_211	3.2.1.21|3.2.1.126|3.2.1.37	GH3(32–240)	GH3_e175	GH3	3
MPNQ01000001.1_54	-	GH2(9-592)	GH2_e67	GH2	3
MPNQ01000001.1_95	-	GH15(489–848)	GH15_e19	GH15	3
MPNQ01000001.1_96	2.4.1.15	GT20(43–500)	GT20_e1	GT20	3
MPNQ01000002.1_14	-	GH3(89–315)	GH3_e96	GH3	3
MPNQ01000003.1_113	-	GH3(91–317)	GH3_e96	GH3	3
MPNQ01000004.1_122	-	GT4(234–384)	GT4_e2125	GT4	3
MPNQ01000004.1_15	-	GH3(63–270)	GH3_e99	GH3	3
MPNQ01000005.1_25	3.2.1.20|3.2.1.10	GH13_23(31–375)	GH13_e182	GH13	3
MPNQ01000009.1_27	-	GT4(215–369)	GT4_e3552	GT4	3
MPNQ01000018.1_42	3.2.1.21|3.2.1.126|3.2.1.37	GH3(33–245)	GH3_e175	GH3	3
MPNQ01000023.1_13	-	GH20(1-229)	GH20_e36	GH20	3
MPNQ01000025.1_3	-	GT4(179–324)	GT4_e2372	GT4	3
MPNQ01000027.1_4	3.2.1.-	GH2(3-579)	GH2_e66	GH2	3
MPNQ01000029.1_6	-	GT51(76–247)	GT51_e163	GT51	3
MPNQ01000038.1_27	-	GT4(188–346)	GT4_e3061	GT4	3
MPNQ01000041.1_21	-	AA3_2(2-526)	AA3_e57	AA3_2	3
MPNQ01000001.1_206	3.2.1.8	GH10(6-318)	GH10_e103	N	2
MPNQ01000001.1_56	-	GH1(11–400)	GH1_e108	N	2
MPNQ01000002.1_206	-	GT28(211–374)	GT28_e100	N	2
MPNQ01000003.1_115	-	GH26(121–242)	GH26_e26	N	2
MPNQ01000005.1_106	-	CE14(4-132)	CE14_e6	N	2
MPNQ01000006.1_88	3.5.1.115	CE14(8-142)	CE14_e1	N	2
MPNQ01000007.1_52	2.4.1.266	GT81(6-290)	GT81_e0	N	2
MPNQ01000021.1_16	-	AA3_2(3-504)	AA3_e4	N	2
MPNQ01000023.1_30	-	AA3(5-505)	AA3_e57	N	2
MPNQ01000030.1_9	-	CE4 (2-114)	CE4_e209	N	2
MPNQ01000035.1_12	-	GT4(201–349)	GT4_e2284	N	2
MPNQ01000035.1_13	-	GT4(189–344)	GT4_e1595	N	2
MPNQ01000035.1_23	-	GT4(158–309)	GT4_e2034	N	2
MPNQ01000040.1_22	-	GT2(2-157)	GT2	N	2
MPNQ01000047.1_19	-	CE9(5-357)	CE9_e30	N	2
MPNQ01000050.1_16	-	CE14(13–129)	CE14_e46	N	2
MPNQ01000035.1_11	-	N	GT4_e939	GT4	2
MPNQ01000035.1_27	-	N	GT2+GT4_e1547	GT2+GT4	2
MPNQ01000035.1_3	-	N	GT2	GT2	2
MPNQ01000001.1_169	-	N	GT94_e7	GT0	2
MPNQ01000002.1_51	-	N	AA2_e1	AA0	2

An evaluation of reports on the occurrence of these taxa provided some insights into their occurrence in the ANP and SF sites. Members of the family *Geodermatophilaceae*, such as *Blastococcus* and *Geodermatophilus*, are known to produce a range of extracellular enzymes, allowing them to survive in diverse environments (Montero-Calasanz et al. 2022). As an example of their metropolitan nature, members of the genus *Blastococcus* have been isolated from various environments: sediment from the Baltic Sea (Ahrens and Moll 1970); extreme hyper-arid Atacama Desert soil (Castro et al. 2018); an ancient Roman pool in Tunisia (Hezbri et al. 2016); ruins of a Roman amphitheatre in Tunisia (Hezbri et al. 2017); Gurbantunggut Desert in China (Yang et al. 2019); beach sediment in Korea (Lee 2006); sea-tidal flat sediment (Lee et al. 2018); an archaeological site in Greece (Urzì

Table 2 Sva0996 marine group bin134 genome analysed using eggNOG to predict COG categories represented based on the proteins annotated

COG Category	Number of proteins annotated
J – translation, ribosomal structure and biogenesis	143
K – transcription	104
L – replication, recombination and repair	145
B – chromatin structure and dynamics	0
D – cell cycle control, cell division, chromosome partitioning	36
V – defense mechanisms	57
T – signal transduction mechanisms	33
M – cell wall/membrane/envelope biogenesis	71
N – cell motility	2
U – intracellular trafficking, secretion, and vesicular transport	29
O – posttranslational modification, protein turnover, chaperones	66
C – energy production and conversion	308
G – carbohydrate transport and metabolism	94
E – amino acid transport and metabolism	185
F – nucleotide transport and metabolism	90
H – coenzyme transport and metabolism	100
I – lipid transport and metabolism	180
P – inorganic ion transport and metabolism	105
Q – secondary metabolites biosynthesis, transport and catabolism	110
S – function unknown	466
Combinations:	
BDLTU, BQ	2
CH, CJ, CO, CP	17
DHM, DJ	4
EGP, EH, EQ, EHP, ET, EFGP, EG, EP	66
FG, FJ, FP	4
GK, GM	14
HP, HQ	4
IM, IQ	124
JK, JM	4
KL, KLT, KT	8
LU	2
MU	1
NOU, NU	5
OV, OU, OT	3
Not assigned to a COG category	95

et al. 2004); and from a marble sample collected from the Bulla Regia monument in Tunisia (Louati et al. 2022).

Recently, Wang et al. (2023) proposed the reclassification of *Marmoricola* as *Nocardioides*. Members of this genus have also been isolated from diverse environments, including marine sediments, estuaries, forest and agricultural soils, volcanic ash, marble, a hot spring, and marine sponges, and it is therefore not surprising that this genus was detected in both the SF and ANP sites (Wang et al. 2023). Members of the genus *Microbacterium* play an important role in the health of soil (e.g., the release of essential nutrients, plant growth promotion, and removal of toxic metals) (Gómez-Ramírez et al. 2015; Liu et al. 2022). They are also highly adaptive and have been found in diverse environments, including the high-altitude Atacama Desert (Mandakovic et al. 2020) and various marine environments (Kageyama et al. 2007; Williams et al. 2007). Notably, *Microbacterium oxydans*, which was

Table 3 Physicochemical properties of the bulk samples collected from Springfield Farm at three depths for the three different sampling areas (A1T = Area 1, top; A1M = Area 1, middle; A1D = Area 1, deep; A2T = Area 2, top; A2M = Area 2, middle; A2D = Area 2, deep; A3T = Area 3, top; A3M = Area 3, middle; A3D = Area 3, deep), as well as composite samples collected from Suiderstrand, Agulhas National Park (ANP) at three different sites (ANP Ocean; ANP Rocky; ANP Dry)

Sample	Soil type	pH (KCl)	*P* (mg/kg) Olsen	*P* (mg/kg) Bray II	Na (cmol/kg)	K (cmol/kg)	Ca (cmol/kg)	Mg (cmol/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	B (mg/kg)	Fe (mg/kg)	C %	S (mg/kg)	Resistance (Ohm)
A1T	Loam	6.3	-	16	14.45	1.64	14.24	16.92	1.0	9.0	28.1	6.35	331	5.06	309	100
A1M	Clay	6.8	-	8	10.33	1.18	8.29	10.95	0.2	0.7	21.0	6.36	74	2.49	243	90
A1D	Clay	7.0	6	9	12.48	1.19	7.38	10.36	0.1	0.3	16.1	4.99	31	1.60	295	70
A2T	Sand	7.0	4	4	1.16	0.21	3.86	2.36	0.2	0.4	31.4	1.98	74	1.63	38	290
A2M	Sand	6.9	-	2	0.97	0.09	1.46	1.36	0.1	0.2	9.0	0.98	22	0.49	22	310
A2D	Sand	7.4	5	2	1.83	0.12	1.19	1.60	0.1	0.2	11.3	0.79	17	0.54	52	160
A3T	Sand	8.0	2	3	0.35	0.13	4.63	1.15	0.2	0.3	21.7	1.45	55	0.67	47	580
A3M	Sand	8.0	1	2	1.13	0.09	1.84	1.05	0.1	0.2	7.5	1.20	37	0.42	40	240
A3D	Sand	8.0	1	2	2.51	0.25	9.42	1.62	0.1	0.1	5.9	1.47	19	0.37	62	140
ANP Ocean	Sand	8.8	2.82	3.76	4.70	0.12	26.08	1.39	0.02	0.19	0.41	1.31	2.29	0.16	127	160
ANP Rocky	Sand	8.6	1.92	4.16	4.61	0.11	27.98	1.19	0.01	0.14	0.25	0.98	1.69	0.20	113	100
ANP Dry	Sand	8.9	1.25	0.81	4.36	0.11	25.41	1.40	0.01	0.15	0.13	1.23	2.11	0.15	124	100

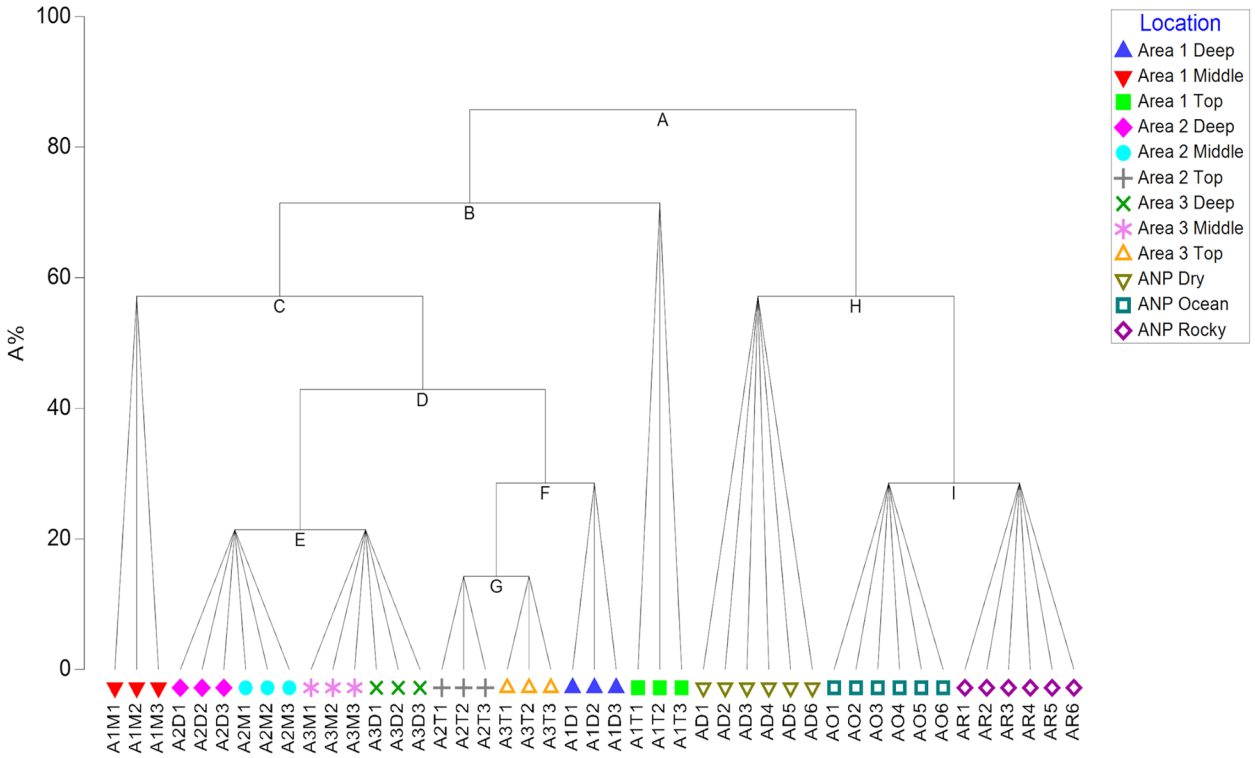

Fig. 7 LINKTREE analysis showing the most important environmental parameters identified using BEST analysis selecting the actinobacterial community structure in the Springfield Farm (Areas 1–3) and Agulhas National Park (ANP) samples. A: R = 1,00; B%=100; Ca < 0,0146(> 1,05) or pH < 0,152(> 0,837) or Mn>-0,406(<-0,92); B: R = 0,47; B%=52; pH>-1,22(<-1,79) or Ca<-0,433(> 0,0146); C: R = 0,40; B%=47; pH<-1,22(>-1,1); D: R = 0,41; B%=40; Mn < 0,0993(> 0,549); E: R = 0,75; B%=21; pH<-0,533(> 0,152) or Mn>-0,116(<-0,256) or Ca<-1,17(>-1,14); F: R = 1,00; B%=63; Mn > 1,07(< 0,549) or Ca<-0,878(>-0,623); G: R = 0,19; B%=29; pH<-0,989(> 0,152) or Mn > 1,98(< 1,07) or Ca<-0,95(>-0,878); H: R = 0,81; B%=32; pH > 1,18(< 1,07) or Ca < 1,05(> 1,11) or Mn<-0,946(>-0,935); I: R = 0,32; B%=16; pH > 1,07(< 0,837) or Ca < 1,11(> 1,29) or Mn>-0,92(<-0,935)

isolated from a coastal marshland, showed the ability to produce alginate lyase and laminarinase, two enzymes required for the degradation of seaweed, and is therefore directly involved in carbon cycling in the marine environment (Kim et al. 2013). Similarly, environmental mycobacteria are widespread, occurring mostly in cool, wet, acidic soil (Walsh et al. 2019), but may also occur in other environments, such as coastal swamps and estuaries, depending on their environmental preferences (Falkinham III 2009). Very few environmental mycobacteria are pathogenic; while some undescribed mycobacterial lineages include pathogenic mycobacteria, they are hardly ever detected in soil environments (Walsh et al. 2019). Falkinham III (2009) also highlighted the importance of mycobacteria in the formation of biofilms and their potential to protect themselves, as well as other microorganisms, against toxic metals that may be present in the immediate environment.

The occurrence of the genus *Streptomyces* in both marine and peat environments is well documented. The members of this genus, due to their abundance, have been readily detected, including within eutrophic peatlands (Golovchenko et al. 2022), boreal *Sphagnum* peat bogs where several isolated strains exhibited cellulolytic activities (Pankratov and Dedysh 2009), low-moor peats with varying levels of water-content (Zenova et al. 2007), as well as from wetland-associated water samples where the isolated *Streptomyces* strain exhibited several antimicrobial activities (Benhadj et al. 2020). Moreover, due to their ubiquitous nature, members of the genus *Streptomyces* have been detected in numerous marine environments, often with a particular focus on strains producing natural products. These include several sites across the Philippine archipelago (Tenebro et al. 2021), marine sediments in the South China Sea where genomic analyses revealed common characteristics of marine adaptation (Tian et al. 2016), a tunicate-associated *Streptomyces* strain that produces griseorhodin A (Li and Piel 2002), coastal sediments in São Paulo, Brazil (Tangerina et al. 2020), and a seasonal study on marine sediments collected at the Stellwagen Bank Marine Sanctuary near Massachusetts, USA, with the relative abundance of *Streptomyces* ranging from

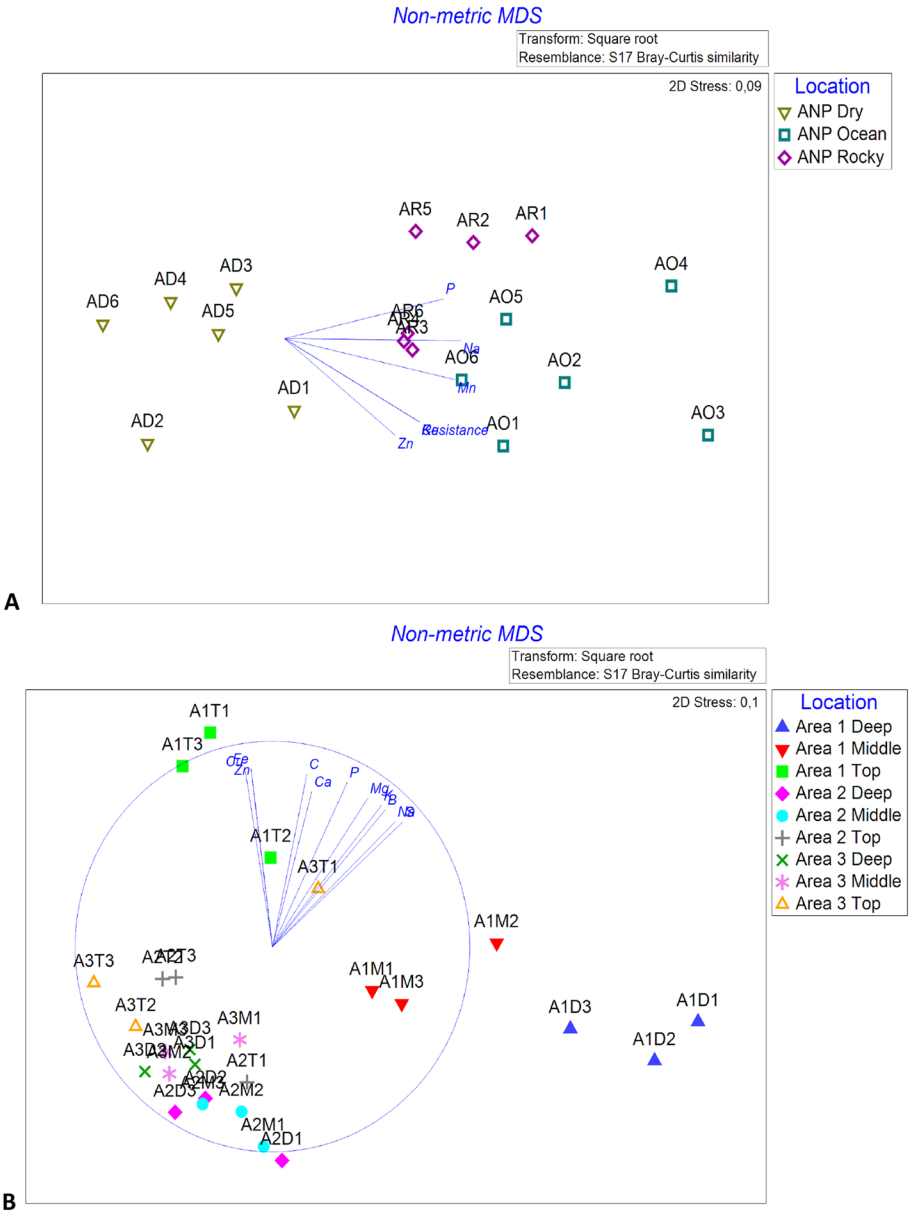

Fig. 8 Non-metric multidimensional scaling (nMDS) plots for the **A** Agulhas National Park (ANP) samples and the **B** Springfield Farm (Areas 1–3) samples, showing the actinobacterial community similarities and the key environmental drivers for these communities

1.57% prevalence in summer, compared to 0.96% in fall samples (Heinrichs et al. 2020), to name a few.

The Sva0996 marine group has been detected in various marine microbial community studies. The presence of this group has been reported in deep-sea sediments collected along the Southwest Indian Ridge (Chen et al. 2016), detected in the Laptev Sea water column above and outside a methane seep field (Samylina et al. 2021), in temperate and polar coastal sands (Miksch et al. 2021), found as a major component of a microbial community thriving on biofilm fouling release coating (Papadatou et al. 2021), in seawater samples from the South Sea of Korea (Seo et al. 2017), shown to be involved in community succession during kelp degradation (Brunet et al. 2021), present in the water columns of the South China Sea (Li et al. 2021), as a major bacterial clade of the South Pacific Gyre (Reintjes et al. 2019), and as an indicator species occurring in deep subsurface fluids of a borehole, as well as seawater (Jungbluth et al. 2016). In addition, the SVA0996 marine group has also been detected in the mucus, tissue, and skeleton of the reef coral *Porites lutea* (Kuang et al. 2015), the Mediterranean coral, *Astroides*

calycularis (Biagi et al. 2020), found in association with marine sponges (Slaby et al. 2017; Turon et al. 2019; Verhoeven 2019; Indraningrat et al. 2022), found in association with the brown alga, *Laminaria digitata* (Ihua et al. 2020), the macro-alga, *Taonia atomaria* (Paix et al. 2019), and ascidians (*Tunicata*) (Steinert et al. 2015).

Although the role of the Sva0996 marine group in sponges is unknown, Orsi et al. (2016) showed that this group is involved in dissolved protein cycling in the ocean, while Indraningrat et al. (2022) reported that this taxon may be present where high nitrate concentrations are found, as well as in high productivity areas. A study by Biagi et al. (2020) confirmed the potential of this marine group to utilise organic nitrogen, where it has been shown that stressed reef-building corals produce mucus with a higher protein content. Brunet et al. (2021) followed the changes in bacterial community structure over time during the degradation of kelp. During the first phase, epiphytic bacterial communities rapidly changed to algal polysaccharide degraders, followed by a second phase dominated by the Sva0996 marine group. Brunet et al. (2021) suggested that this group may act as opportunistic scavengers, utilising substrates exposed by the first degraders. At the same time, the authors noted a change in the bacterial communities in the underlying sediments, where anaerobic taxa involved in sulphur and nitrogen cycles dominated. Similarly, a study by Chun et al. (2021) proposed that the Sva0996 marine group (along with other clades) is involved in the creation of species-engineered microenvironments and are referred to as 'skeleton'-forming microorganisms or keystone species that are responsible for stabilising microbial modules. The authors concluded that the Sva0996 marine group is part of an essential core group of bacterial clades, providing resilience to microbial communities under biotic and abiotic changes in the coastal waters (Chun et al. 2021).

In a study conducted on water columns of the South China Sea, Li et al. (2021) showed that the Sva0996 marine group could adapt to either a particle-associated or free-living lifestyle at the seawater surface. In addition, the group was not limited to the upper photic ocean but was also found in meso- and bathypelagic seawaters, where they are mostly free-living. Furthermore, the occurrence of the Sva0996 marine group as a major bacterial clade of the South Pacific Gyre (referred to as a marine biological desert) highlights the versatility of this group and its important role in biogeochemical cycles (Reintjes et al. 2019). In this study, the Sva0996 marine group was detected in both the marine and the SF sampling sites, with the highest abundance in the marine environment and SF Area 3. The presence of this marine group in the SF terrestrial environment is most probably due to the continued pressures from the brackish (< 2 g/kg salts), alkaline (pH 6–8) water of the two main river systems in the area (Russell and Impson 2006; Gordon et al. 2012).

In a study by Slaby et al. (2017), which focused on the metagenome binning of a marine sponge microbiome, an Sva0996 marine group genome bin134 was obtained. This metagenome-derived genome was downloaded and analysed during this study to gain more insight into the biosynthetic potential and lifestyle of this representative of the uncultured Sva0996 marine group. The Sva0996 marine group is classified within the Order *Acidimicrobiales* (Class *Acidimicrobiia*, Phylum *Actinomycetota*), and the proposed unclassified Candidatus *Hopanoidivoraceae* family, which is related to the validly published *Acidimicrobiaceae, Iamiaceae*, and *Ilumatobacteriaceae* families. The Sva0996 marine group bin134 genome size (3.68 Mbp) and G + C% content (64.3%) falls within the reported size range (2.16 Mbp – 6.22 Mbp) and G + C% content (47.7–74.9%) when compared to other members of the Order *Acidimicrobiales* (Table 4). The fact that the typical genes associated with linear genomes were not detected does not necessarily mean that the genome is circular. Annotation of the genome is difficult due to the taxonomic lineage, the sequence may be incomplete, the genes may be found on a plasmid, or the bacterium may house homologues to *tpg* and *tap*.

Further analysis of the genome sequence highlighted the potential ability of this bacterium to adapt to various environments. For example, an adaptive immune system (CRISPR-Cas; Xu and Li 2020), an antibiotic-resistance gene, various CAZymes, nitroreductases, taurine (and other) degrading enzymes, and a biosynthetic gene cluster encoding for the production of a carotenoid pigment, are all present. In addition to the CRSIPR-Cas system and the antibiotic-resistance gene, the bacterium's potential to produce a carotenoid pigment is of great importance in terms of self-protection/defence. Carotenoids can protect from UV radiation and negate oxidative stress/damage due to their antioxidant properties. Carotenoids have also been reported to be involved in membrane fluidity, allowing producers to adapt cell membrane fluidity and structure under low-temperature conditions (Vila et al. 2019), supporting the potential of this Sva0996 marine group to survive at different ocean depths. The potential role of the Sva0996 marine group as secondary colonisers can be seen in the biosynthetic potential of the strain represented by bin134 to produce degradation enzymes. The presence of CAZymes (as predicted by dbCAN3 and eggNOG mapper), shows the potential of the strain to utilise polysaccharide polymers as potential carbon sources (e.g., GH3, GH2, GH10, GH13), but lacks the enzymes required for the release of these polymers from

Table 4 The genome size and G + C% content of representative type species of validly published genera assigned to validly published families within the Order *Acidimicrobiales*

Family	Genus	Type species	Genome size (Mbp)	G + C% content
Acidimicrobiaceae	*Acidiferrimicrobium*	*Acidiferrimicrobium austral* DSM 106,828^T	4.07	73.2
	Acidimicrobium	*Acidimicrobium ferrooxidans* DSM 10,331^T	2.16	68.3
	Acidithrix	*Acidithrix ferrooxidans* Py-F3^T	4.02	47.7
	Ferrimicrobium	*Ferrimicrobium acidiphilum* T23^T	3.09	55.3%
	Ferrithrix	*Ferrithrix thermotolerans* DSM 19,514^T	2.49	51.1%
Iamiaceae	*Actinomarinicola*	*Actinomarinicola tropica* SCSIO 58,843^T	3.72	72.6
	Aquihabitans	*Aquihabitans daechungensis*	No genome sequence available	N/A
	Iamia	*Iamia majanohamensis* DSM 19,957^T	4.58	74.9
	Rhabdothermincola	*Rhabdothermincola sediminis* KCTC 49,500^T	3.29	70.5
Ilumatobacteraceae	*Desertimonas*	*Desertimonas flava* SYSU D60003^T	6.22	70.2
	Ilumatobacter	*Ilumatobacter fluminis* DSM 18,936^T	4.78	68.6

larger structures such as lignocellulose and algal biomass (no polysaccharide lyases or lignin-degrading enzymes predicted). Nitroreductases are responsible for reducing nitroaromatics, compounds typically introduced into the environment due to human activities, e.g., they are used in the production of pesticides, dyes, polymers, and explosives. By including nitroreductases in biodegradation pathways, bacteria have shown the ability to utilise these toxic compounds as sources of carbon, nitrogen, and energy (Ju and Parales 2010; Pitsawong et al. 2014). In addition, the presence of taurine degradation pathways further highlights the importance of this group of bacteria in the marine environment and supports their proposed role as keystone species (Brunet et al. 2021; Chun et al. 2021). Taurine is an amino acid-like compound released into the marine environment during the decomposition of marine organisms, including algae, as dissolved organic matter. The degraded taurine could potentially serve as a source of carbon, nitrogen, sulphur, and energy, as well as other important sulphur and nitrogen species (e.g., ammonium, sulphate, etc.) for the Sva0996 marine group and microorganisms found in their immediate environment (Clifford et al. 2019). The potential of the SVa0996 marine group to adapt to diverse environments may also explain its potential ability to survive in the SF terrestrial environment where, as in the marine environment, it can be envisaged playing a key role in stabilising/providing resilience to nearby microbial communities.

The selective isolation of 'rare' actinobacteria from various types of environments has mostly been driven by bioprospecting studies focused on antibiotic discovery (Hayakawa 2008; Goodfellow and Fiedler 2010). Actinobacteria, notably members of the genus *Streptomyces* and *Micromonospora*, are well-known producers of antibiotics (Subramani and Aalbersberg 2013). Standard isolation techniques typically result in the re-isolation of known antibiotic-producing strains, notably streptomycetes, which drove the need for alternative isolation approaches. Since antibiotic-producing actinobacteria were mostly isolated from terrestrial soil, selective isolation techniques were previously mostly aimed at application to terrestrial samples (Hayakawa 2008). However, work performed by Mincer et al. (2002) and Maldonado et al. (2005, 2009) expanded the use of selective isolation techniques to the marine environment. The present study used various isolation techniques to isolate diverse actinobacterial genera, specifically to isolate more 'rare' type actinobacteria. A combination and/or variation of the protocols described by Mincer et al. (2002), Maldonado et al. (2005, 2009), and Jose and Jha (2017) were successfully applied. Even though Jose and Jha (2017) isolated a range of well-known actinobacterial genera (*Actinomadura*, *Glycomyces*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharomonospora*, and *Streptomyces*), when Huang et al. (2021) applied similar isolation techniques, they succeeded in isolating strains representing 24 genera, including 'rare' actinobacteria genera. *Micromonospora* and *Streptomyces* strains were also isolated in the present study. However, 'rare' type isolates included members of the genus *Actinophytocola*, *Actinopolymorpha*, *Blastococcus*, *Isoptericola*, *Kocuria*, *Myceligenerans*, *Ornithinimicrobium*, and *Promicromonospora*. There is a continued need for isolation studies since it allows for the generation of large culture collections that serve as a rich source of genomic data that can further provide insights into the

physiology and ecological role of the bacteria within the targeted environment (Huang et al. 2021). Understanding the target environment (e.g., physicochemical properties) can further provide insights into the type of selective isolation approaches to take in the search for novel 'rare' actinobacterial genera (Goodfellow and Fiedler 2010).

Analysis of the physicochemical properties showed that the majority of the samples were characterised as sand (0.37–0.67%), while samples from SF Area 1 were characterised as loam (5.06%) and clay (1.6–2.49%). Soil texture determines the organic carbon content (Bemlab, 2020), with loam or clay soils typically containing > 1.2% organic carbon. Therefore, it is not surprising that samples from SF Area 1 had a higher organic carbon content than the other samples. The cation exchange capacity (CEC) also tends to be higher in soils containing clay and organic matter, but will vary depending on the type of clay and the soil pH (Botta 2013; Eurofins Apal 2020). The CEC refers to the ability of soil to hold/adsorb and release exchangeable cations such as Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}, which in turn influences the soil structure stability, nutrient availability, and soil pH (e.g., the release of H^{+} ion will result in a decrease in soil pH). The fact that samples collected from SF Area 1 mainly consisted of loam and clay supports the occurrence of 10-fold or 5-fold higher levels of the cations. The higher concentration of sulphur in the samples collected from SF Area 1 is also not surprising, since sulphate ions leach out in sandy soils (Botta 2013).

The soil texture will also influence the levels of trace elements where sandy soil is found to be inherently low on trace elements (Eurofins Apal 2020). Furthermore, wetland environments that are typically sandy, saline, calcareous or compacted, and rich in organic matter typically have a Zn deficiency. Therefore, it is not surprising that samples collected in this study were greatly deficient in Zn, ranging from 0.1 to 9.0 mg/kg. In addition to Zn, Cu also typically occurs at low levels (10-40.5 mg/kg) (Dos Santos et al. 2013). Samples collected in this study were greatly deficient in Cu, ranging from 0.01 to 1 mg/kg. Furthermore, Cu, Mn, and Zn are less available and typically in insoluble form in high pH soils, as observed for the marine samples (Fig. 9). Similarly, Fe deficiency has been found to occur at pH_{KCl} > 6.0 (Bemlab 2020). This was observed for the marine samples during this study.

An analysis of the effect of the physicochemical properties on the actinobacterial communities of the two main sites targeted in this study showed that the overall community composition is mostly driven by pH, calcium, and manganese (Fig. 9). It is clear from the various studies on peat and marine samples that soil or sediment pH is

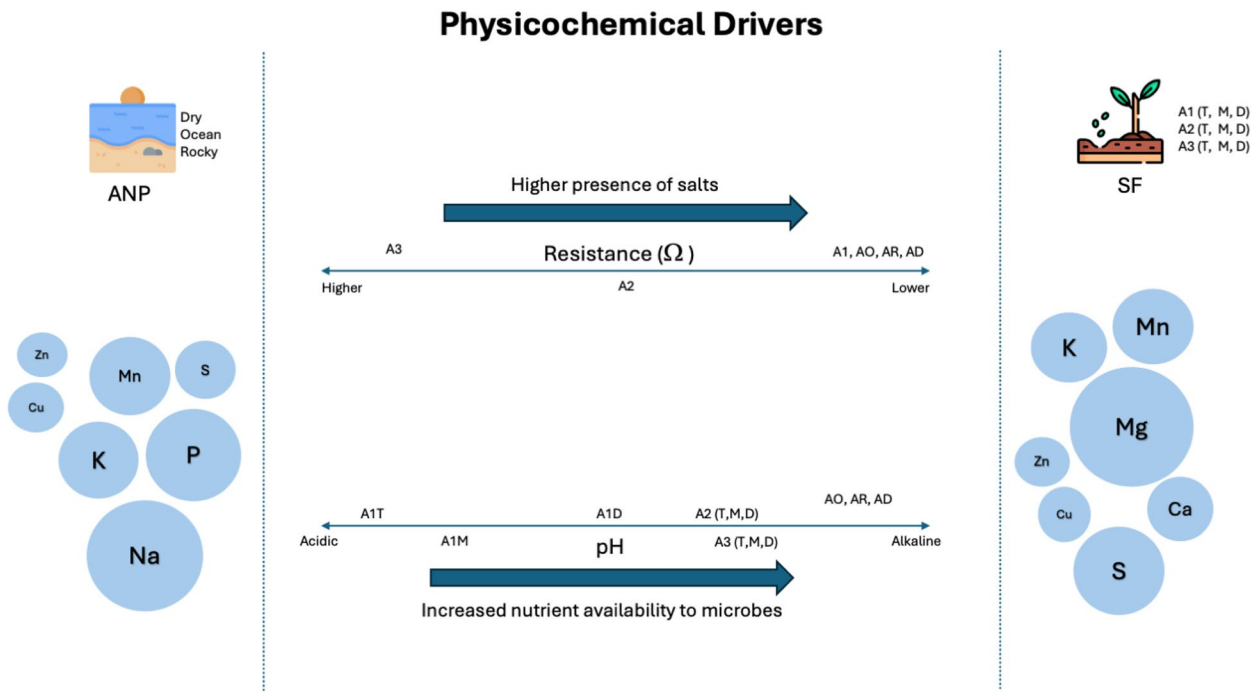

Fig. 9 Main physicochemical drivers influencing actinobacterial communities for the Agulhas National Park (ANP) and the Springfield Farm (SF) samples (A1D: Area 1 Deep; A1M: Area 1 Middle; A1T: Area 1 Top; A2D: Area 2 Deep; A2M: Area 2 Middle; A2T: Area 2 Top; A3D: Area 3 Deep; A3M: Area 3 Middle; A3T: Area 3 Top; AD: ANP Dry; AO: ANP Ocean; AR: ANP Rocky). Elemental factors are scaled – larger spheres indicated more pronounced effect on community structure

considered one of the key aspects that influences the soil biological, physical and chemical properties. For example, it has a considerable influence on the soil microbial population and the availability of soil nutrients (Motsara and Roy 2008; Botta 2013; Neina 2019). At low pH, trace elements become more soluble and available to plants and microorganisms. In contrast, mineralizable carbon and nitrogen increase with an increase in soil pH. It is therefore not surprising that microbial growth typically occurs at pH 5.5–8.8, where nutrient and trace element availability is at maximum (including calcium and manganese), and increased enzyme activities and biodegradation occur (Neina 2019). When separating the communities according to sampling site, it was found that the actinobacterial communities at the marine site (ANP) were driven by P, K, Zn, Mn, Na, and resistance. Low resistance is an indication of a high concentration of soluble salt, where soil with a resistance of < 300Ω is regarded as saline (Botta 2013; Bemlab 2020). Samples collected from all three areas showed a different degree of salinity, with SF Area 1 and the marine sediment samples (ANP) being the most saline (70–160Ω), followed by SF Area 2 (160–310Ω) and SF Area 3 (140–580Ω) (Fig. 9). A study by Lozupone and Knight (2007) showed that salinity is most probably the most important driver for the global environmental distribution of bacterial diversity, thereby influencing the bacterial community composition.

Conclusion

Bacteria and other microorganisms play an essential role in the health of an environment and are typically used as biomarkers/indicators of ecological health, yet we know very little about their diversity in many of our natural protected areas. This study served as a reminder of the interconnectedness of environments found in close proximity. We focused on a marine environment that is currently part of a national park, while the second site, which is approximately 10 km inland, forms part of the vast Agulhas Plain, which, up until 8000 years ago, was still inundated by the ocean. Remnants of marine actinobacteria are still visible in the terrestrial sampling site, as was evident in the detection of the Sva0996 marine group in the SF samples, most notably in Area 3. It is clear that we only have a snapshot of the bacterial diversity associated with both sampling sites. Additional research will be required to determine the diversity associated with the larger ecological niches in the area, including the saltpan, the mountainous region with a natural underground spring, and the extensive dune systems located near the SF and ANP sampling sites, and whether the Sva0996 marine group could potentially play a key role in these environments. The additional information on bacterial diversity will then provide us with a foundation from which we can build a better understanding of how ecological changes, such as those brought about by climate change and invasive species, may affect the marine environment, related ecosystems, and historically inundated marine sites. This knowledge can be gained through the continuous monitoring of microbial populations on a seasonal and annual basis and will be the focus of our ongoing studies.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
Conceptualization, M.L.R., L.A.M.; Methodology, A.P., S.S.D., J.V., L.A.M., M.L.R.; Validation, A.P., M.L.R.; Formal analysis, A.P., S.S.D., M.L.R.; Investigation, A.P., S.S.D., J.V., L.A.M., M.L.R.; resources, M.L.R.; Data curation, A.P., J.V., M.L.R.; Writing—original draft preparation, A.P., S.S.D., J.V., L.A.M., M.L.R.; Writing—review and editing, A.P., S.S.D, J.V., L.A.M., M.L.R. All authors read and approved the final manuscript.

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Availability of data and materials
The data presented in this manuscript is available from the sequence databases indicated in the manuscript. All other data sets are provided either in the manuscript or as supplementary material.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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