MALDI-TOF MS for the identification of bacteria from *Acropora cervicornis* samples with White Band Disease

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Abstract— White Band Disease (WBD) is one of the most harmful coral syndromes in the Caribbean, deteriorating the structure of many corals during the eighties and nineties. Since its etiology is not entirely known, the use of techniques aimed at identifying the associated agents could provide an approach to the composition of the populations involved in the deterioration of *A. cervicornis*. Therefore, this research's objective was to isolate and characterize bacteria associated with WBD in *A. cervicornis* from hanging nurseries of Rosario and San Bernardo Corals National Natural Park, Colombia, using MALDI-TOF MS and PCR. The results showed the presence of *Bacillus cereus* bacteria in individuals with bleaching signs from nurseries and transplant areas on Isla Tesoro and Isla Ceiner. One of these strains sequenced and phylogenetically analyzed was closely related to *Bacillus thuringiensis*, with similarities of up to 99%. The presence of *B. cereus* could indicate a special relationship between this microorganism and WBD. However, it is imperative to carry out more studies on identifying the bacteria associated with this disease to support these findings.

Keywords— Coastal ecosystems, coral disease, coral nursery.

I. INTRODUCTION

Coral reefs are considered one of the most intricate and varied ecosystems on the planet, of great ecological importance given their role as home to almost a third of the marine species discovered [1]. Coral diseases, being listed as one of the main sources of mortality of these specimens, are defined as changes, either temporary or persistent, in the physical characteristics of the affected individual. However, the etiology of many diseases is difficult to determine, given the great diversity of microorganisms that their holobiont naturally harbors [2]

In the Caribbean, during the 1980s and 1990s, white band disease (BBD) caused notable losses in the genus Acropora, contributing to the deterioration of the structural complexity of reefs in this area [3].

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The cause is not fully identified, nor has it been proven to be the same in Acropora species. However, there are multiple candidates associated with this syndrome [4]. On the other hand, lesions with a pattern similar to this condition appear in hanging nurseries of *Acropora cervicornis* in the Parque Nacional Natural Corales del Rosario y San Bernardo (PNNCRSB), Colombia [5]. Then, the need arises to develop a system that allows the recognition of its causative agents from changes in the composition of the holobiont.

Additionally, Matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF MS) is an analytical technique based on the detection of specific ribosomal proteins at the level of genus, species, or bacterial subgroup [6], [7]. Hence, MALDI-TOF MS has been considered a promising technology in the identification of isolates of marine origin, obtaining results hours after culture, given its ability to analyze whole bacterial cells, and requiring little sample preparation time, which increases its cost-effectiveness [8], [9].

In this context, the definition of the associated microorganisms of this syndrome would allow taking measures that can guarantee their survival in the natural environment, which would contribute significantly to the restoration of ecological integrity, and preservation of the ecosystem services they provide. Thus, through the implementation of MALDI-TOF MS and amplification of species-specific genes, this project will seek to provide information related to the microbial communities present in *A. cervicornis* corals with visible signs of WBD in hanging nurseries.

II. METHODOLOGY

A. Collection of biological material

ANLA (National Environmental Licensing Authority) permitted collecting a total of forty-nine *A. cervicornis* fragments. The samples, collected on November 3, 2018, were taken at five PNNCRSB points: the Isla Tesoro (T) and Isla Ceiner (O) coral nurseries; the coral transplant area of each nursery (TT and OT), and an area with specimens in the wild

near Isla Tesoro (TC) (Figure 1). Coral fragments of approximately 3 cm in length were obtained, by the intentional selection, at a depth between 6 and 10 m after visual recognition of WBD symptoms, cutting at the interface of living tissue and dead skeleton [10]. In addition, we also collected healthy samples of asymptomatic coral colonies and sediment and water samples from the nurseries.

We placed all the fragments in collecting flasks for biological samples, refrigerate between 2 - 4 °C, and transport them to the Biology Laboratory of the Faculty of Basic Sciences of the Universidad Tecnológica de Bolívar. There, they were stored at -20°C until further processing. We previously sterilized all instruments, storage bags, and containers for transport and disinfected them to avoid cross-contamination.

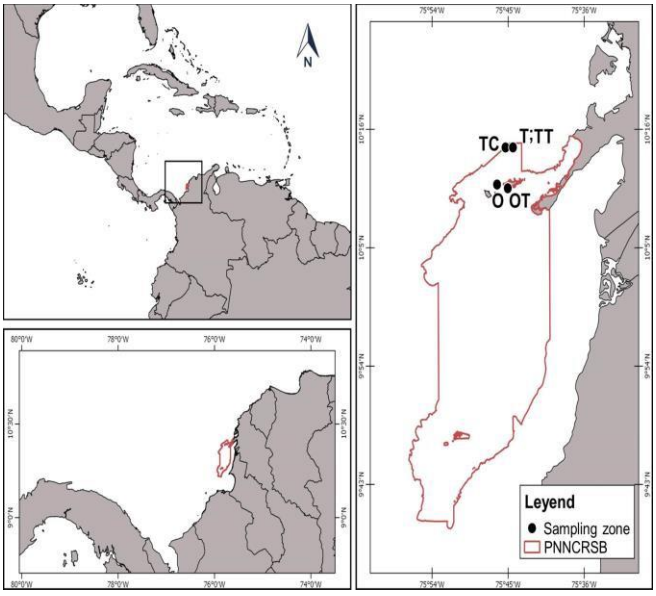

Figure 1. Sampled areas of the Parque Nacional Natural Corales del Rosario y San Bernardo (PNNCRSB): Isla Tesoro (T, TT, TC) and Isla Ceiner (O, OT).

B. Coral Sample Processing

Samples were thawed and incubated at room temperature. A fragment was cut from each coral using a sterile mortar and pestle [11]. Each sample was separately macerated and homogenized with sterile saline buffer to release microorganisms present in the tissues. Subsequently, we added 2 ml of each macerated coral sample to sterile tubes with sucrose-enriched seawater (SSW) and incubated at 37°C for 24 hours. These colonies were seeded by streak exhaustion in Marine Agar, incubating at 30°C for seven days. Finally, the colonies obtained were transferred into Eppendorf tubes with SSW broth, diluted in sterile 60% glycerol solution, and frozen at -70°C for preservation [12].

C. Bacterial identification using MALDI-TOF MS

We carried out the identification procedure through MALDI-TOF MS following the standardized protocols within the Human Proteomics and Mycoses Research Unit of the Pontificia Universidad Javeriana in Bogotá. Bacterial isolates obtained from marine agar were reactivated in a culture medium at 37ºC for one day [13], [14].

We identified using either the transfer methodology with direct extended plate extraction or ethanol and formic acid extraction. For direct extraction, we picked a single colony with a wooden stick, and a fragile layer of the microorganism was applied to the MALDI steel plate, allowing it to dry at room temperature. Next, we added 1µl of 100% formic acid and covered it with 1µl of HCAA matrix (α-cyano-4-hydroxycinnamic acid-HCCA), drying at room temperature [14]. Ethanol and formic acid extraction were performed only for isolates that presented a low score with the direct methodology, following the manufacturer's instructions (Bruker Daltonik GmbH, Bremen, Germany) [13].

To visualize the profiles, we use FlexControl software (version 3.0) and MALDI Biotyper RTC. For calibration and as a positive control, we used the standard bacterial test (BTS), which contains the protein profile of an *Escherichia coli* strain (DH5a) (Bruker Daltonik GmbH, Bremen, Germany). To read the results, we use the following identification criteria:

- A score ≥ 2.0, identification to species level
- A score between 1.7 and 1.9, identification at the gender level
- A score < 1.7, no identification.

Scores between 1.7 and 2.0 were considered valid for identification at the genus and species level.

D. Molecular detection and amplification of species-specific genes

We extracted Bacterial DNA from samples identified by MALDI-TOF MS from pellets obtained after seeding colonies in nutrient broth at 37°C for 24 hours, using the MO BIO® UltraClean™ Fecal DNA kit. The purity and concentration of the extracted DNA was determined quantitatively by Nanodrop using a NanoPhotometer® N60, and qualitatively by the agarose gel electrophoresis method, before storage at -80°C [15].

We amplified the total DNA using Polymerase Chain Reaction (PCR). Their final concentration conditions were 0.5X PCR Buffer, 10.0 µl of CorpoGen® PCR Mix, 0.8 µl of each primer, 5.4 µl of water, and 3.0 µl of template DNA in a final volume of 20 µl, following the following PCR program [16]. We performed all PCR reactions with a Bio-Rad T100™ Thermal Cycler, and the PCR products were visualized on 1% gel electrophoresis and revealed with ethidium bromide (EtBr). Then, to confirm the results obtained with MALDI-TOF MS, the resulting amplification products were sequenced at Gencore-Uniandes, using the Applied Biosystems 3700 (ABI-3700) system.

E. Bacterial nucleotide diversity and taxonomic composition

For the phylogenetic analyses, we compared one of the obtained sequences with partial sequences from different

geographical origins, housed in the NCBI Genebank (https://www.ncbi.nlm.nih.gov/), using the BLASTN subroutine (nucleotide-nucleotide) v. 2.2.27 [17]. We reconstructed a phylogenetic tree from sequences corresponding to the detected bacterial group. The sequences were analyzed with the Geneious program (Biomatters) and aligned with the ClustalW program [18].

We calculated the nucleotide substitution model that best fit the data with the Mega 7.0 program. Using Bayesian analysis, we also performed the phylogenetic reconstruction and implemented the General Time Reversible Substitution Model (GTR), with 30,000,000 million generations. We conducted the analyses using the MrBayes program [19], and the levels of nucleotide diversity levels were evaluated with the program DnaSP v.5.1 [20] through descriptive sequence comparison. This program was also used to evaluate the deviations from neutrality using the D and F statistics of Fu & Li [21], and D of Tajima [22].

III. RESULTS

A. *Collection and characterization of biological material*

Samples were labeled to indicate their place of extraction Isla Tesoro nursery (T), Isla Ceiner nursery (O), coral transplant zone of Isla Tesoro (TT), coral transplant zone of Isla Ceiner (OT), and control zone (TC). We collected 26 asymptomatic fragments and 23 fragments with WBD signs at the five selected sampling points. Of these fragments, six corresponded to fully bleached branches of *A. cervicornis*, three to a possible case of predation by *Hermodice carunculata* fireworm, and one sample to possible infection by fungus (Figure 2).

B. *Coral Sample Processing*

From the 49 coral fragments, we obtained bacterial colony growth only on 21 marine agar samples. Of these, nine corresponded to asymptomatic individuals (TC3, TC8, O10, OT7, OT1, TT1, TT14, TT18, TT20-1), four to the fragments with more significant whitening (T28, TC4, O8, OT1), and eight to samples with the transition from healthy to bleached tissue (T29, TC2, O4, O9, OT5, OT8, TT4, TT16). In addition, we found five different morphologies of white colonies from these isolated colonies, most of them with creamy surface appearance, flat elevation, irregular shape, and slightly wavy edges, and to a lesser extent, rhizoid-shaped colonies with ruffled margins, crateriform elevation, and rough surface.

C. *Bacterial identification using MALDI-TOF MS*

We performed the identification procedure through MALDI-TOF MS from the bacterial isolates obtained in marine agar, reactivating in duplicates at 37ºC for 24 hours. As a result, we detected *Bacillus cereus* strains with scores higher than 2.1 in 5 samples (T28, TC4, O8, OT1, TT16), two strains of *Bacillus licheniformis* (OT5 and OT10), and one of *Staphylococcus warneri* (TT14), with maximum values between 1.7 and 2.0 (Table 1, Figure 3, and Figure 4).

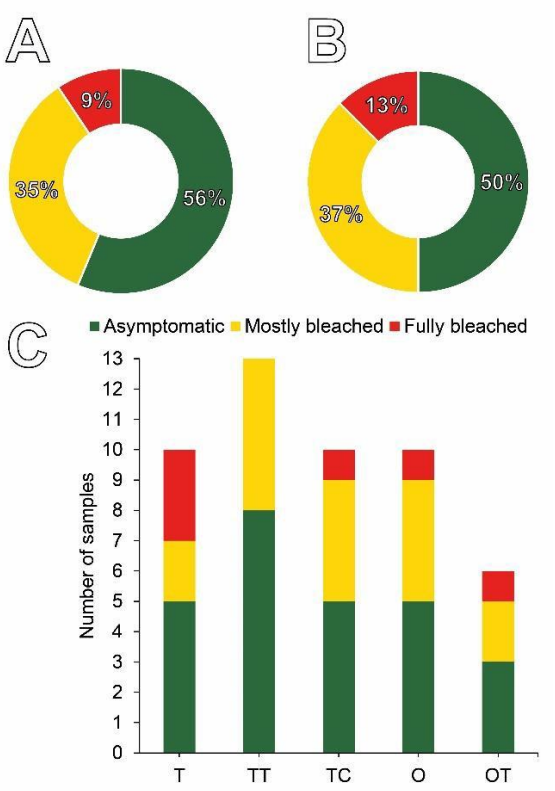

Figure 2. Characterization of *Acropora cervicornis* fragments sampled in Isla Tesoro (A), Isla Ceiner (B), and frequency in each sampled area according to bleaching level (C).

Table 1. Characterization of strains analyzed by MALDI-TOF MS

Code	Symptom	Protein extraction	Score	Detected species
T28	Fully bleached	YES	2.209	*Bacillus cereus*
T29	Mostly bleached	NO	1.559	N/D
TC2	Mostly bleached	N/A	N/A	N/D
TC3	Asymptomatic	N/A	N/A	N/D
TC4	Fully bleached	YES	2.256	*Bacillus cereus*
TC8	Asymptomatic	N/A	N/A	N/D
O4	Mostly bleached	N/A	N/A	N/D
O8	Fully bleached	YES	2.302	*Bacillus cereus*
O9	Mostly bleached	N/A	N/A	N/D
O10	Asymptomatic	NO	1.640	N/D
OT1	Fully bleached	NO	2.135	*Bacillus cereus*
OT5	Mostly bleached	NO	2.011	*Bacillus licheniformis*
OT7	Asymptomatic	N/A	N/A	N/D
OT8	Mostly bleached	N/A	N/A	N/D
OT10	Asymptomatic	NO	1.784	*Bacillus licheniformis*
TT1	Asymptomatic	N/A	N/A	N/D
TT4	Mostly bleached	NO	<0	N/D
TT14	Asymptomatic	NO	1.786	*Staphylococcus warneri*
TT16	Mostly bleached	YES	2.319	*Bacillus cereus*
TT18	Asymptomatic	N/A	N/A	N/D
TT20-1	Asymptomatic	N/A	N/A	N/D

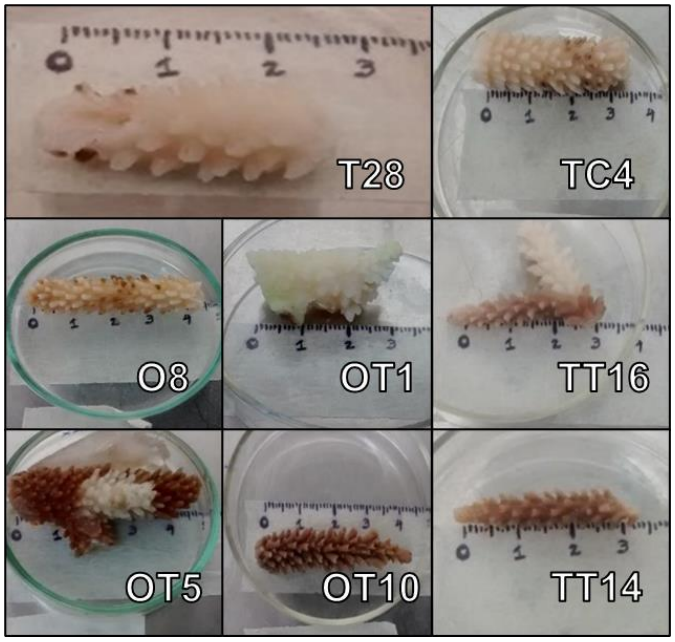

Figure 3. *A. cervicornis* coral samples from which bacterial isolates were identified with MALDI-TOF MS.

The mass spectra obtained for *B. cereus* presented their maximum peak near 4500 m/z. At a general level, the Bacillus species exhibited peaks similar to each other, except for sample OT10, which also presented the lowest score of all identified samples (Figure 4). In addition, the profile obtained for the *S. warneri* strain showed a certain similarity with those obtained for *B. cereus*, which, added to the level of distance between groups obtained in the dendrogram (Figure 5), would indicate a correlation between the identified species.

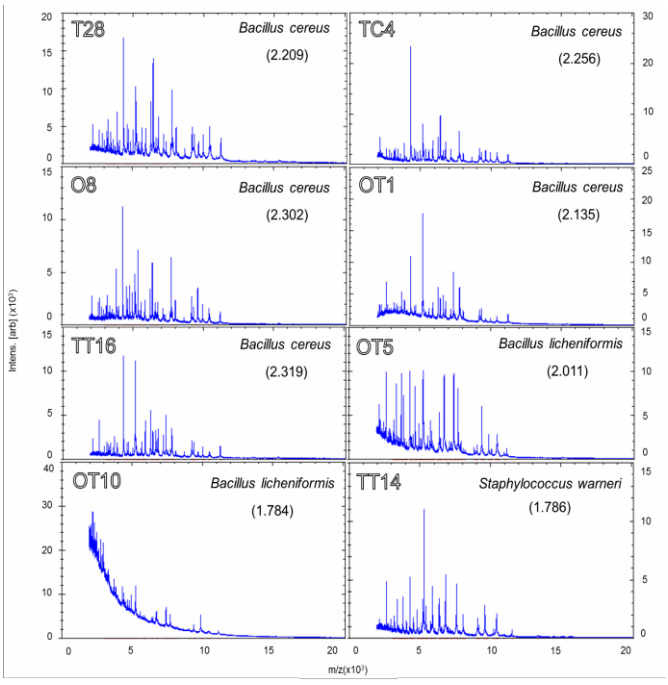

Figure 4. MALDI-TOF MS profiles for *B. cereus*, *B. licheniformis* and *S. warneri* identified with MALDI-TOF MS in marine agar.

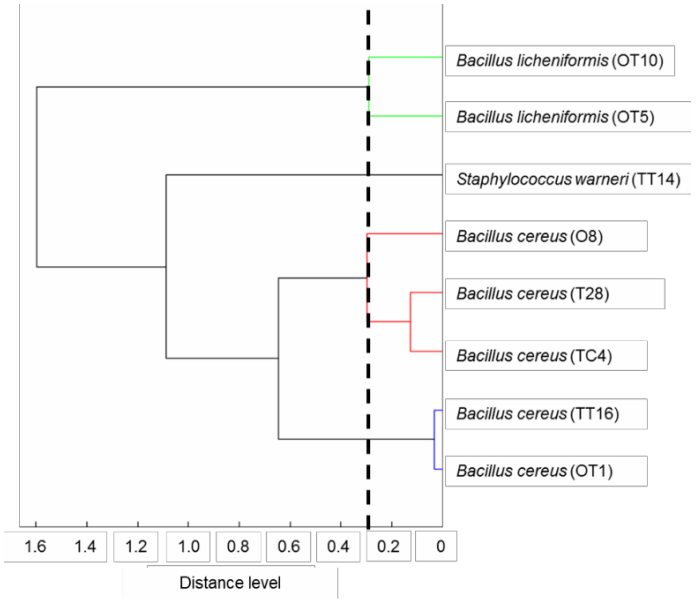

Figure 5. MALDI-TOF MS dendrogram of bacteria isolated from marine agar.

D. Molecular detection and amplification of species-specific genes

We obtained bands with an approximate length of 575 bp in the samples referred to as *B. cereus*, which coincides with the molecular weight of the DNA of this species [23]. Therefore, it validates the results obtained with MALDI-TOF MS. In the *S. warnerni* sample, we obtained a band of approximately 800 bp [16], which confirms the presence of bacterial DNA in the sample (Figure 6).

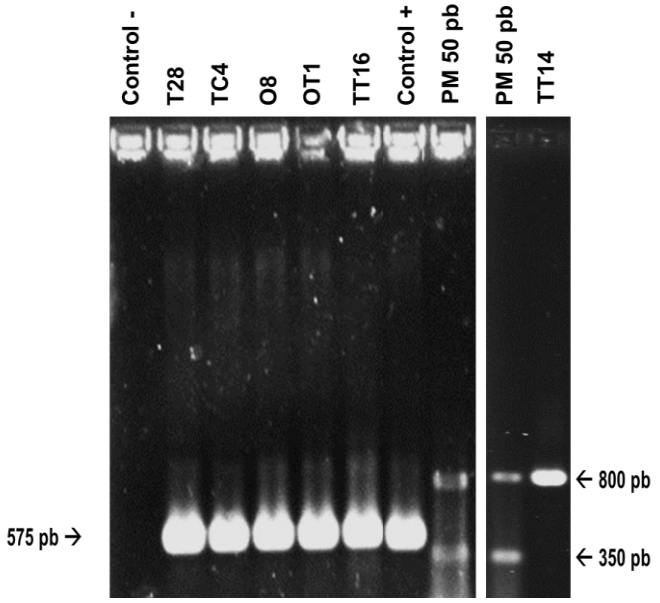

Figure 6. Agarose gel electrophoresis and positive DNA control using species-specific primers for *B. cereus* (BCFomp1 and BCRomp1) and 16S rRNA (QUGPRn1 and QUGPRn6) for *S. warneri*. PM: O'RangeRuler DNA marker 50 bp.

E. Nucleotide diversity of the motB gene

We selected 88 partial sequences of *B. cereus* and *Bacillus thuringiensis*, corresponding to the gene responsible for encoding the flagellar motor rotation protein (motB) during the phylogenetic analyses. As a result, we obtained the sequence of the OT1 strain of *B. cereus* in the present study, and we used an *Escherichia coli* sequence as external control. As a result, the nucleotide diversity value (π-Pi) of *B. cereus* was 0.22, corresponding to the 47 sequences from different geographical regions, including the OT1 sequence.

Meanwhile, the 40 *B. thuringiensis* sequences yielded a π-Pi value corresponding to 0.22 and an average of 0.44. Regarding the neutrality test of the *B. cereus* and *B. thuringiensis*, there is no significance between the statistics D and F of Fu & Li, and D of Tajima ($P < 0.02$).

Additionally, the total average of *B. cereus* and *B. thuringiensis* is not statistically significant, observing a probability of 5.09, with $P < 0.02$ and $P < 0.05$ (Table 2), showing no deviation from the neutral pattern for the evolution of the MotB gene.

Table 2. Nucleotide diversity for the motB gene.

Bacteria	n	π	D of Fu & Li	F of Fu & Li	D of Tajima
B. cereus	47	0.2220	2.0851	2.5985	2.3202
B.thuringiensis	40	0.2202	2.0230	2.4933	2.1974
Total	87	0.4422	4.1081	5.0918	4.5175
Average	43.5	0.2211	2.0541	2.5459	2.2588

n = Number of sequences analyzed; π (Pi) = nucleotide diversity; Test D = D of Fu & Li; Test F = F of Fu & Li; D for Tajima. Fu & Li's D: Statistical significance: **, $P < 0.02$; Tajima's D: Statistical significance: *, $P < 0.05$.

F. Phylogenetic reconstruction of the motB gene

We performed a phylogenetic reconstruction using Bayesian analysis for a partial sequence of the motB gene (Figure 7) from different sequences and bacterial species belonging to the genus Bacillus (*B. cereus* and *B. thuringiensis*), in order to identify the OT1 sequence obtained in this study. We evidenced two clades or groupings, which presented supports with bootstrap indices of 100% and 90% for clades 1 and 2, respectively. In the first clade, we grouped the sequences belonging to *B. cereus* and *B. thuringiensis*, showing a high similarity corroborated by the bootstrap index and sequence homology, grouping the OT1 sequence of *B. cereus* together with the rest of *B cereus* sequences from other geographic regions.

This phylogenetic analysis confirmed the identity of the sequenced Colombian strain, confirming that it corresponds to the species *B. cereus* (Figure 7). Likewise, there is an important evolutionary divergence since the branches have different lengths than the tree's root sequence used as an external group, corresponding to *E. coli*.

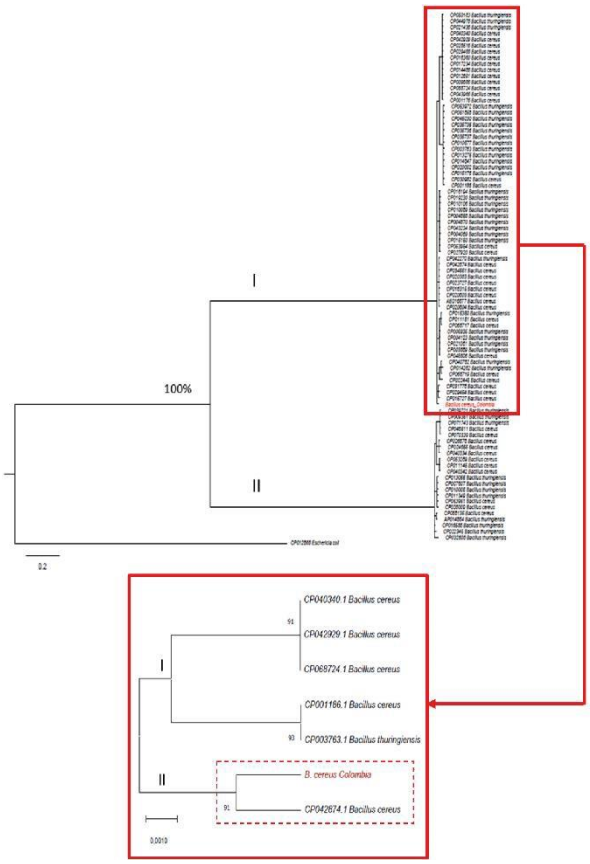

Figure 7. Phylogenetic reconstruction of the motB gene using Bayesian analysis to determine the taxonomic identity of the Colombian *B. cereus* strain.

IV. DISCUSSION

Bacillus cereus and *Bacillus licheniformis* are Gram-positive bacteria, facultative anaerobes, elongated and sporulated [24], with metabolisms that facilitate their adaptation to adverse conditions, by producing a complexity of biofilms and generate highly resistant and adhesive spores, which increases their chances of dissemination and survival in this type of environment [25]. *B. cereus* and *B. licheniformis* are common inhabitants of seabeds and seacoasts [26], [27], and have been previously detected in corals, and in the case of *B. cereus*, both in healthy tissues of the coral *Antillogorgia elisabethae* [28]. and in diseased samples of the coral *P. lutea* [29]. In contrast, the polyphosphate kinase gene, expressed by this bacterium, has been detected in a sample of *Acropora cervicornis* infected with WBD [30].

In contrast, *Staphylococcus warneri* is not as widespread in the environment. This spherical Gram-positive bacterium is found mainly on human skin and mucous membranes, and is considered of clinical importance as a zoonotic pathogen associated with abortions in cattle, and the etiologic agent of some infections and sepsis in human tissues [31], although it has also been isolated from Acropora corals [32]. However, its role in the incidence of coral diseases has not been fully explored. The few investigations that relate *B. cereus*, *B.*

licheniformis and *S. warneri* with coral syndromes report them in both asymptomatic and diseased corals.

In this study, the isolation of five strains of *B. cereus* from bleached specimens of the *A. cervicornis* coral, distributed in the five sampled areas of the PNNCRSB, could indicate a probable correlation of this bacterium with the incidence of WBD in these points. Since *B. cereus* has not previously been reported as an agent associated with WBD, both in Colombian reefs and in other Caribbean areas, it is still necessary to corroborate this finding through metagenomic studies, also evaluating whether there are other species or associated consortia. to *B. cereus*, through the use of defined genetic sequences. In contrast, the detection of *B. licheniformis* in only one healthy and one diseased individual and the isolation of *S. warneri* from a healthy specimen in the Treasure Island transplant area do not provide sufficient evidence to associate these bacteria with the appearance of WBD in these areas.

Despite this, the isolation of these species is a first step for recognizing the microbial variety present in corals with signs of WBD in PNNCRSB nurseries. In this sense, the comparison between the OT1 sequence of *B. cereus* with partial sequences housed in the NCBI showed a 98-99% similarity with *B. cereus* and *Bacillus thuringiensis*, both belonging to the *Bacillus cereus* sensu lato group [33]. Similarly, the values obtained for the coefficients D and F of Fu & Li, and D of Tajima show an absence of changes in the neutral pattern of evolution of the motB gene, thus confirming its suitability as a specific marker for the molecular identification of *B. cereus*. Finally, in this first approach, it was possible to acquire new knowledge about the microbial diversity associated with the *A. cervicornis* coral, bearing in mind that the genus Acropora is one of the main reef-forming taxa in the entire Caribbean. In addition, it highlights the importance of researching this group of corals and the relevance of strengthening conservation programs.

V. CONCLUSIONS

The characterization of the state of these fragments allowed us to examine the state of health of the reef and the coral nurseries, being fundamental for the feedback and the maintenance of the conservation programs, based on their assisted reproduction, implemented in the PNNCRSB.

From the *A. cervicornis* samples collected, five strains of *Bacillus cereus*, two strains of *Bacillus licheniformis*, and one of *Staphylococcus warneri* were detected, showing that the MALDI-TOF MS test can be used as a rapid diagnostic tool. In conjunction with traditional DNA-based molecular identification methods, such as specific PCR.

We evidenced through a phylogenetic reconstruction using a Bayesian approach the genetic homology between *B. cereus* and *Bacillus thuringiensis*. Likewise, we determined the taxonomic identity for one of the *B. cereus* sequences isolated from an individual with WBD disease in the PNNCRSB.

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