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Photosynthetic Microorganisms from Three Mexican Archaeological Sites

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ABSTRACT

Historical monuments and buildings represent an important part of the cultural legacy of past civilizations, in addition to being considered sites of endemic diversity, knowledge of this is essential, especially in photosynthetic organisms, since they are the pioneers in the establishment of those communities. Even though the morphological identification of photosynthetic microorganisms represents a challenge due to the phenotypic plasticity they present, so the use of Molecular Biology techniques is essential. The objective of this work was to identify species of microalgae and cyanobacteria present in three archaeological zones: Malinalco and Teotihuacan of the State of Mexico and Yohualichan, of the State of Puebla, through the partial amplification of the genes that code for the 16S rRNA and 18S rRNA subunits by the PCR technique using 2 pairs of primers and the sequencing of the amplified ones. In this way, 3 of the 10 strains isolated at the species level were identified: *Chlorella sorokiniana*, *Chlorococcum vacuolatum* and *Klebsormidium crenulatum*, and the other just at genus level because some initiators did not provide enough information. In this way, the use of morphological and molecular characters contributes to a more complete and reliable taxonomic identification.

Introduction

Biodeterioration is the physical, chemical, or aesthetic damage caused by different types of organisms on objects, monuments, or buildings that belong to cultural heritage (Gaylarde, 2020). The microbial populations present on the construction substrate are generally the result of successive colonization by different microorganisms over several years, this process is based on the ability of the substrate to provide a protective niche in which microorganisms can develop (Negi and Sarethy, 2019). The microbiota that grows on the stone material represents a complex ecosystem that develops in different ways, this will depend on the environmental conditions and the physicochemical properties of the material in question, this microbiota is known as a biofilm and is defined as an organized ecosystem, composed of one or more microorganisms associated with different types of

surfaces with functional characteristics and complex structures, which is embedded in a matrix of extracelular polymeric substances (EPS) that increase the resistance of the community and allow organisms to survive in hostile environments (Liu *et al.,* 2020; Soffritti *et al.,* 2019).

Microbial colonization most often begins with phototrophic organisms that accumulate in a visible film of organic-enriched biomass on the surface. When microbial biofilms establish themselves on the walls of buildings, they not only cause aesthetic deterioration, but also cause degradation of their structure through the production of acid or alkaline conditions, the retention of humidity and/or the differential absorption of heat by color deposits on the surface. If the buildings have historical importance, the losses caused compromise the cultural heritage of a town (Negi and Sarethy, 2019).

At present, studies focused on the conservation of monuments and historical sites are increasing, because these sites represent an important part of the cultural legacy. Knowledge about the microorganisms that grow and develop on the surface of historical monuments is important for the development of strategies to counteract the presence of these organisms. The use of morphological and molecular tools in the identification of photosynthetic microorganisms is widely recommended by authors such as Pineda-Mendoza *et al.,* (2011), as well as Komárek and Anagnostidis (2005) since they agree that approximately 50% of the strains of these organisms in the collections are misidentified since the identification is subject to researcher identification skills, which is based in morphological characters.

Traditionally, the identification of photosynthetic microorganisms, such as microalgae and cyanobacteria, has been based on their morphological characters, however, different authors (Soffritti *et al.,* 2019; Pineda-Mendoza *et al.,* 2011) have observed that morphological characteristics, such as the photosynthetic pigments and lipid composition change depending on environmental characteristics. During the biomass production of the strains, changes in the composition of photosynthetic pigments were observed as a result of a possible depletion of mineral nutrients in the medium, which can lead to an erroneous morphological identification. Studies of 16S or 18S rRNA are currently used because they are conserved sequences throughout evolution and allow the design of primers to measure genetic diversity (Valenzuela-González *et al.,* 2015; Rodicio and Mendoza, 2004). In this way, those molecules are used as molecular markers in the identification and classification of bacteria, by means of partial or total sequencing of the same; this technique is based on the similarity of sequences between different individuals and is demonstrative of the variation of their genomes, because for practical purposes if two organisms have an identity greater than 97% they can be considered closely related at the genomic level and therefore belong to the same species (Felis and Dellaglio, 2007; Větrovský *et al.,* 2013; Caro *et al.,* 2015).

The objective of this work was to identify the strains of photosynthetic microorganisms isolated from three archaeological sites in Mexico.

Materials and Methods

Nine photosynthetic microorganisms, isolated from three archaeological zones: Malinalco and Teotihuacan from the State of Mexico and Yohualichan from State of Puebla, were used; two cyanobacteria and seven microalgae. The cyanobacteria *Arthrospira maxima* and the microalgae *Chlamydomonas reinhardtii* were used as positive controls for molecular identification.

Cultivation of strains of photosynthetic microorganisms

Depending on the characteristics of each strain, they were seeded in BG11 mineral medium (Castenholz, 1988) in liquid form or solidified with 1.3% bacteriological agar and maintained with a 16-h photoperiod and constant aeration. After 4 to 12

weeks of growth, the biomass was harvested and concentrated and used for genomic DNA extraction. Total DNA extraction was carried out using the method described by Allers and Lichten (2000), modified by Rodríguez-Tovar (2004).

PCR

For the amplification of sequences of interest, two primers were used, one for cyanobacteria and the other for microalgae (Pineda-Mendoza *et al.,* 2011; Ferris *et al.,* 2005; Berdoulay and Salvado, 2009). In the case of cyanobacteria, a pair of primers specific for cyanobacteria was used, CYAF (5'-AGCAGTGGGGAATTTTCCG-3') and CYACR (5'-TCACYGCCGTATGCTGACC-3'); that amplifies sequences of the gene encoding the 16S rRNA subunit.

For microalgae, EUF (5' GTCAGAGGTGAAATT CTTGGATTTA 3') and EUR (5' AGGGCAGGGA CGTAATCAACG 3') were used, which amplifies a region of the gene that codes for the 18S rRNA subunit and is used for algae in general. The amplification reactions were carried out in a StratagenRobocycler Gradient 40 thermocycler, under the following reaction conditions: an initial denaturation cycle at 94°C for 5 min, followed by 30 cycles, denaturation at 94°C for 1 min, alignment at 50°C (depending on the species) for 1 min and extension at 72°C for 3 min and a final extension cycle at 72°C for 10 min.

Purified amplicons were sequenced with the CYAF and CYACR primer for cyanobacteria and with the EUF and EUR for microalgae, at Macrogen Inc., Korea on a 3730xl DNA Analyzer (Aplied Biosystems). The obtained sequences were manually analyzed and edited with the Chromas Lite v. 2.13., checking in detail that each peak corresponded to the registered nucleotide base. To obtain the consensus sequence by alignment, the Unipro UGENE version 1.28 program was used, the obtained sequences were aligned and compared with those deposited in the gene bank of the National Center for Biotechnology Information (NCBI) using the BLASTn program, the same which was used to obtain the distance tree.

Results and Discussion

The CYAF and CYACR primers allowed amplification in all 4 species of cyanobacteria. With the primers EUF and EUR, amplification was achieved in all species of microalgae. The molecular identification corresponding to each strain is shown in Table 1, it is observed that for each strain there is more than one species with the same percentage of identity, or with very high percentages; Also included are the access keys to the NCBI Gene Bank.

The use of the 16S rRNA molecular marker allowed the relationship between the morphological and molecular identification of the control strain *Arthrospira maxima* with a percentage of identity of 98% and as can be seen in Figure 1, the amplified sequence is phylogenetically related to three *Arthrospira* species, so there is a relationship between species of the genus, this through the use of the CYAF and CYACR primers which flank 5 of the 9 variable regions of the rrs gene that codes for the 16S subunit of rRNA (Figure 3), variable regions are those that provide the most useful information for phylogenetic and taxonomic studies and are of great help in designing universal primers that allow the amplification of the various hypervariable regions of the vast majority of rRNAs in microorganisms, even if they occur in a community (Valenzuela-González *et al.,* 2015).

Regarding the *Oculatella* strain, when performing the analysis using the distance tree (Figure 2), it is observed that the amplified sequence is closely related to three species of the genus: *O. atacamensis*, *O. coburnii* or *O. mojaviensis*, in addition that it is also aligned with *Leptolyngbya* sp., this species belongs to the Leptolyngbyaceae family, so the genetic closeness is possible, however, there is a greater correspondence with species of the *Oculatella* genus. The species of the genus *Oculatella* (except *O. subterranea*) were reported by

Osorio-Santos *et al.,* (2014) as new species, through the isolation of desert soil samples from the California Desert in the United States, until then the taxon *Oculatella subterranea* (the only species of the genus until 2014) had only been recovered from Mediterranean countries, so the results obtained in the present study could be the first report based on molecular identification in the country, this is reaffirmed with the alignment with the gene databases, since the sequence obtained in this study is aligned with the sequences obtained by Osorio-Santos *et al.,* (2014). In addition, this study affirms that some species of the genus are morphologically and/or ecologically very similar and that the cryptic species are identifiable mainly by sequencing since they are not diagnosable by morphology. Despite not obtaining a definitive species, the identification for the taxon corresponds to *Oculatella* sp. based on molecular data and it is suggested to use the 16S rRNA +16S-23S ITS subunit reported by Osorio-Santos *et al.,* (2014) as a molecular marker for identification to the species level. Regarding the presence of this species in archaeological zones, there are no studies that report it.

In the case of the *Mastigocladus* sp. strain, it was not possible to identify it at the species level by molecular biology techniques, since by using the CYAF and CYACR primers a PCR product was obtained whose sequence is aligned in GenBank with three genera different (Table 1), the molecular marker used did not provide enough information to specie level. Authors such as Woese (1987) report that this gene, being so conserved, may present as a disadvantage that there are no differences in the sequence between species, which does not allow identification to be carried out at said taxonomic level. Some authors such as Falch *et al.,* (1993) and Laurence and Ingram (1971) report the terrestrial environment *Fischerella* genus, Crispim *et al.,* (2006) reported the genus present in historic buildings in Brazil, however, it is not a dominant genus. With microalgae, *Chlorella*, as shown in Figure 3, the amplified sequence is very close to *Chlorella sorokiniana*, making it the most likely species. In previous works (Kaur-Minhasa *et al.,* 2016) and with the use of universal molecular markers for eukaryotes, PCR products of 1.49 and 1.53kb have been obtained from microalgal isolates with 99% identity percentages, so this work verifies the efficiency in the use of 18S rRNA molecular markers as universal and functional for microalgae.

With the *Coelastrella*, a percentage of identity of 100% with the genus and a very close distance with two species are observed in the distance tree analysis: *C. terrestris* and *C. striolata* (Figure 4).

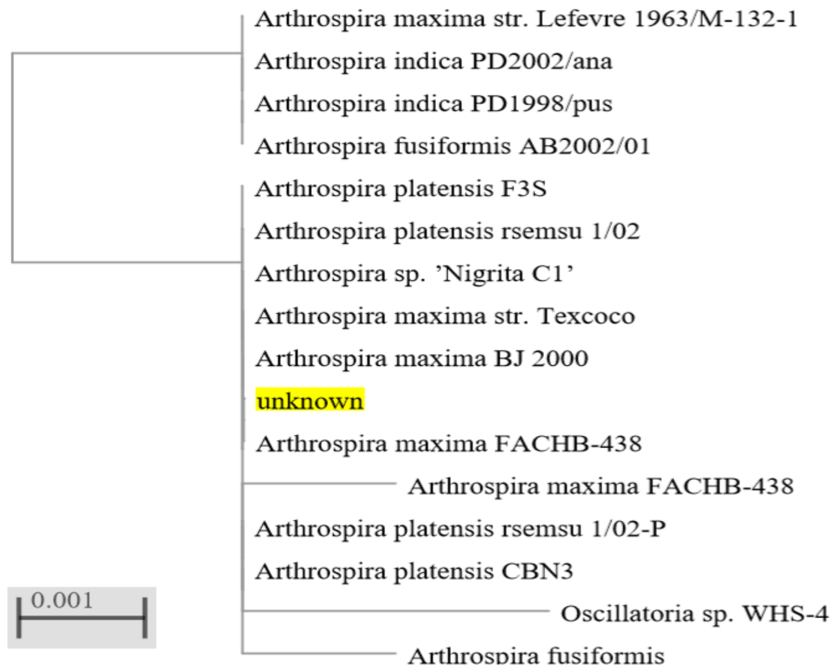
Authors such as Kaur-Minhasa *et al.,* (2016) have obtained larger PCR products (1.93 to 2.4 kb.) from different *Coelastrella* isolates using the 18S rRNA gene as a molecular marker, but with a lower percentage of identity (91 to 98%) than the one obtained in the present work, this could be due to the fact that the design of the primers is given so that they flank a sequence of a different size than the ones used here, so that by covering a greater number of nucleotides, the percentage of coverage (query coverage) will be less, which can cause the percentage of identity to decrease.

In the case of the *Chlorococcum* sp. at least four species were obtained that may correspond to these isolates (Table 1), since when performing the bioinformatic analysis in BLASTn more than 10 species were aligned with the same identity percentage, however, because the microscopic morphology was known of the strain it was possible to rule out some species that did not correspond to the morphology, either due to the shape or size of the cells, in addition to the fact that there was no morphological correspondence with the molecular one, since the genus identified molecularly was *Chlorococcum*, and as can be seen observe in Figure 5, the distance tree shows the molecular closeness with the species *C. vacuolatum*, so it is considered the most likely species.

Table.1 Molecular identification using genes *rrs*and *rns.*

Strain	Molecular ID	ID%	GenBank acces code
Arthrospira maxima	*Arthrospira maxima*	98%	GQ206141.1
Chlamydomonas reinhardtii	*Chlamydomonas reinhardtii*	99%	FR865574.1
***Oculatella* sp.**	*Oculatella coburnii*	99%	KF761586.1
	Oculatella mojaviensis	99%	KF761572.1
	Oculatella atacamensis	98%	KF761578.1
	Oculatella cataractarum	98%	KF761585.1
	Oculatella subterranea	98%	HQ917692.1
	Oculatella kauaiensis	98%	KF417431.1
***Mastigocladus* sp.**	*Westiellopsis* sp.	98%	MF066912.1
	Fischerella ambigua	98%	KJ768871.1
	Fischerella muscicola	97%	KJ768871.1
	Nostochopsis sp.	97%	AJ544081.1
	Hepalosiphon sp.	97%	KF761556.1
Chlorella sorokiniana	*Chlorella sorokiniana*	100%	KU948991.1
***Coelastrella* sp.**	*Coelastrella terrestris*	100%	JX513882.1
	Coelastrella striolata	100%	JX513880.1
	Coelastrella vacuolata	100%	MF580078.1
	Chlamydomonas moewusii	100%	FR865601.1
Chlorococcum vacuolatum	*Chlorococcum vacuolatum*	99%	KM020107.1
	Chlorococcum rugosum	99%	AB983621.1
	Chlorococcum minutum	99%	KP081402.1
	Chlorococcum isabeliense	99%	MG491510.1
***Desmodesmus* sp.**	*Desmodesmus pannonicus*	99%	KU291881.1
	Desmodesmus armatus	99%	KP281290.1
	Desmodesmus communis	99%	JX101327.1
	Desmodesmus komarekii	99%	AB818541.1
***Dicelulla* sp.**	*Chlorella sorokiniana*	99%	KU948991.1
	Micractinium sp.	99%	KF574393.1
	Chlorella thermophila	99%	KF661334.1
	Auxenochlorella pyrenoidosa	99%	KX752082.1
	Pseudochlorella pringsheimii	99%	KY364701.1
Klebsormidium flaccidum	*Klebsormidium flaccidum*	100%	FR717537.1
Chlorococcum minutum	Chlorellales (no cultivables isolated from soil)	99%	FR865687.1

Fig.1 Phylogenetic distance tree for *A. maxima*.



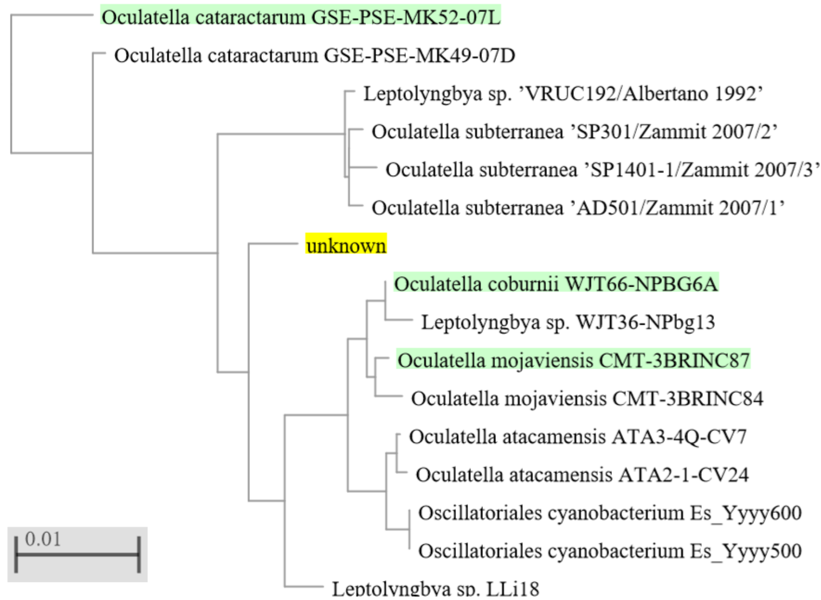


Fig.2 Phylogenetic distance tree for *Oculatella* sp.

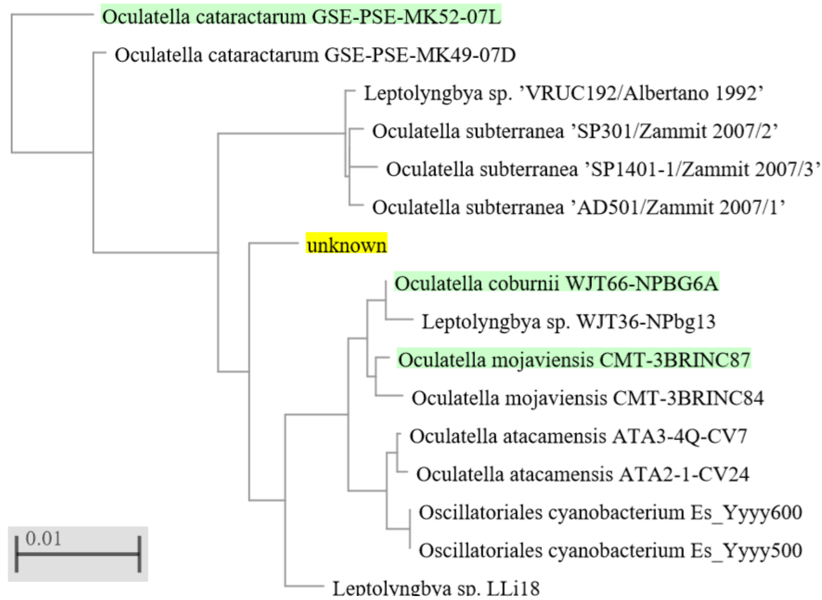


Fig.3 Phylogenetic distance tree for *Chlorella sorokiniana*.

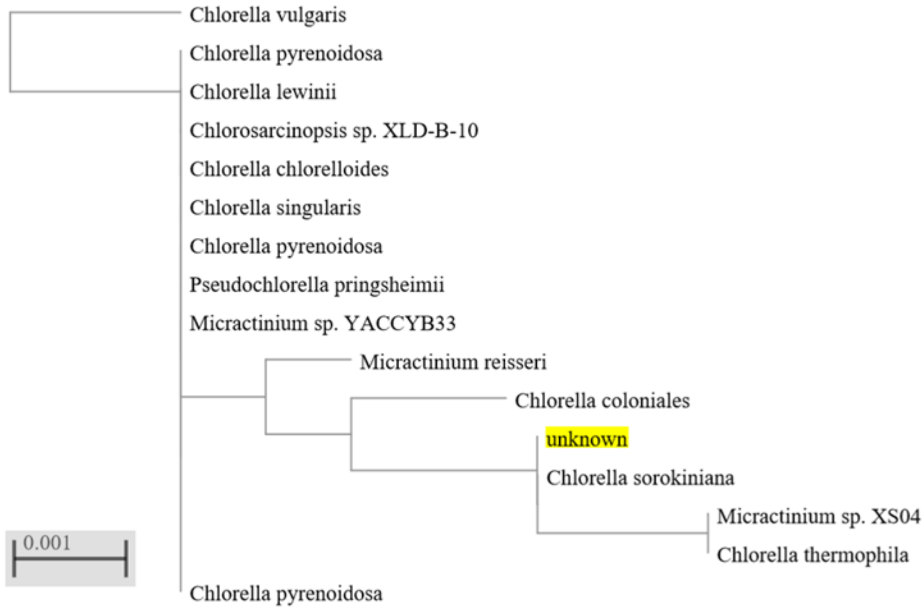


Fig.4 Phylogenetic distance tree for *Coelastrella*.

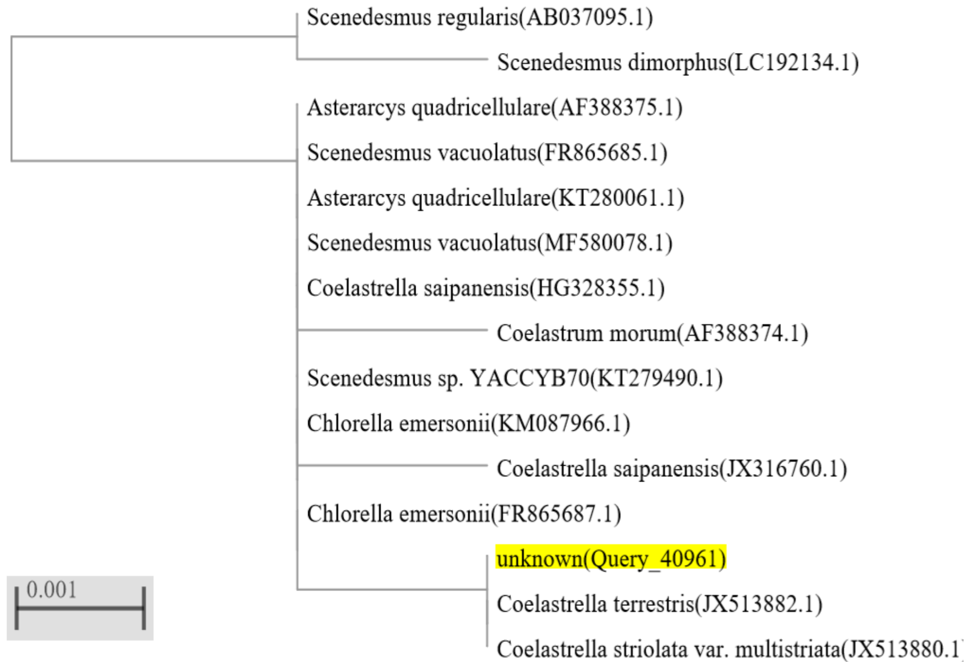


Fig.5 Phylogenetic distance tree for *Chlorococcum*.

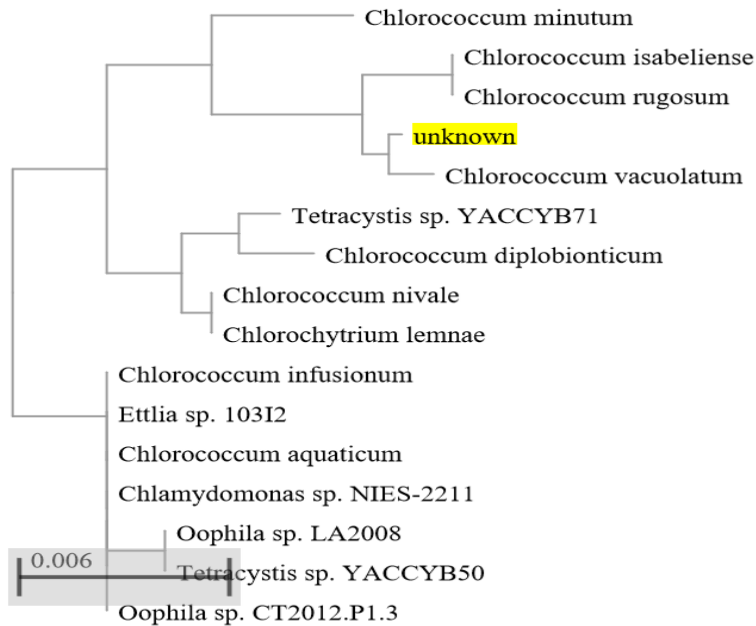


Fig.6 Phylogenetic distance tree for *Desmodesmus* sp.

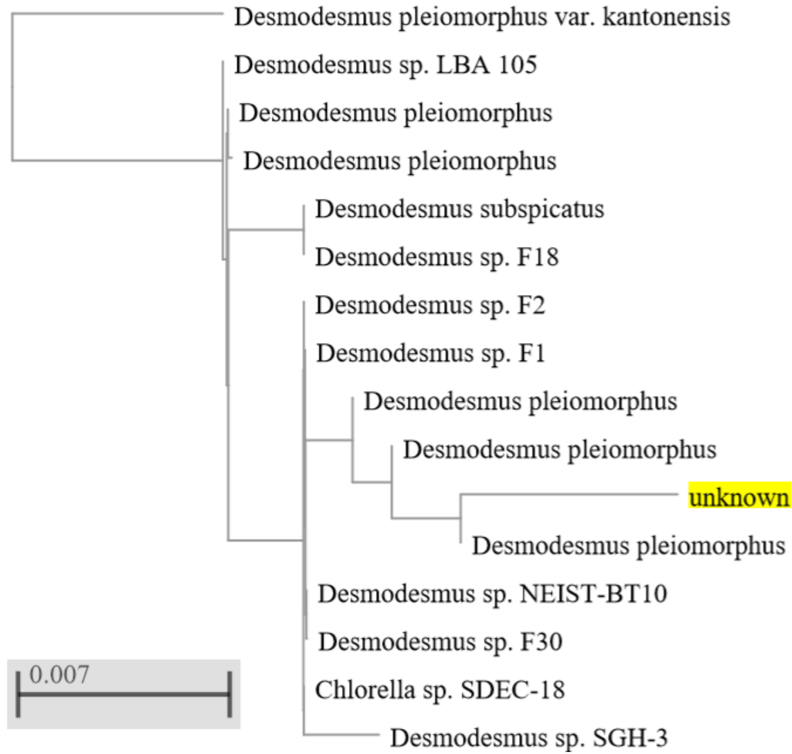
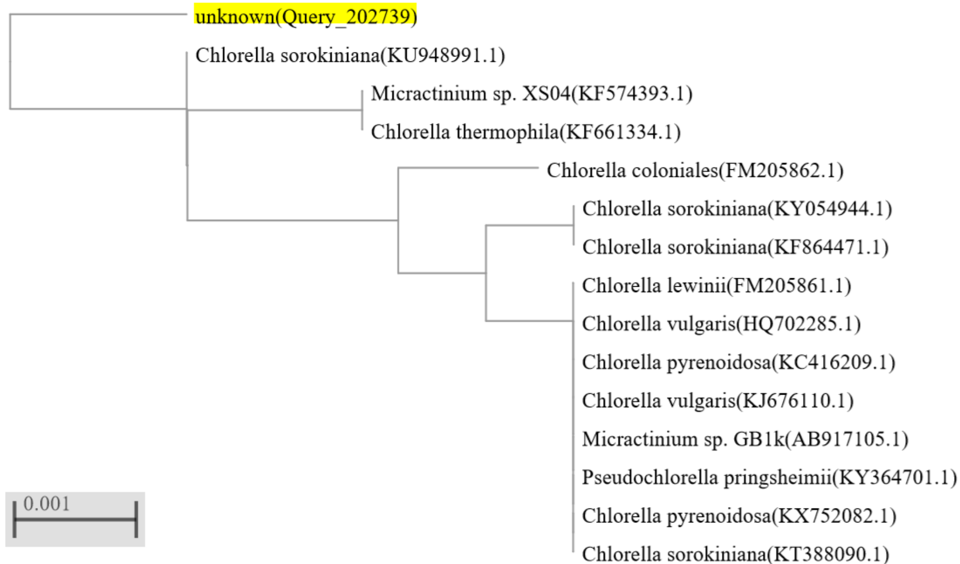


Fig.7 Phylogenetic distance tree for *Dicelulla* sp.



The 18S rRNA gene is separated from the 5.8S and 28S subunits by internal transcription spacer (ITS) regions, this region, due to the fact that they are introns, represents an area of high variability (Berdoulay and Salvado, 2009), however, these regions are not part of the nucleotide sequence flanking the EUF and EUR primers, so the primers used do not provide enough information to

differentiate between species of the same genus, since the amplified sequence does not have enough variability, these results are also demonstrated in 2006 by Crispim *et al.,* but using the 16S rRNA molecular marker for cyanobacteria.

In the case of *Desmodesmus* sp., there was a correlation between morphological and molecular identification with an identity percentage of 99%, however, it was not possible to identify at the species level. The molecular analysis shows at least six species with the same percentage of identity, so the distance tree analysis was used (Figure 6) where it is observed that there is no separation of genera by branches, however, the sequence amplified shows a closeness to the species *Desmodesmus communis*, however, the morphology of this species does not correspond to the morphology of the isolate, so molecular identification by amplification of the rns gene is ruled out.

In the case of the *Dicellula* sp. strain, there was no molecular correspondence with the morphological one, since the molecular analysis shows 99% identity with at least four different genera (Table 1), however, once the analysis was carried out by the distance tree (Figure 7) it was observed that all the species are distant, that is, there is no relationship with the amplified sequence.

When conducting a search for the genus in the AlgaeBase database (Guiry and Guiry, 2022) it was found that it was a recognized genus, with a last update in 2012, so the search for the genus in the NCBI database where no information regarding nucleotide sequences or genomes was found. This fact is very relevant, since the sequence obtained can contribute to the enrichment of gene databases and with this, provide more information that benefits the identification of this type of microorganisms, based on a greater number of elements.

For *Klebsormidium flaccidium*, correspondence with the molecular analysis of 100% was obtained, however, another four species of the genus obtained an identity percentage of 99%; morphologically all species are very similar, authors such as Premanandh *et al.,* (2006) showed that morphologically very similar strains can differ genotypically. This species has already been reported as cosmopolitan, it is from terrestrial and freshwater habitats and has a high percentage of dominance in biofilms (>50%) (Borderie *et al.,* 2012).

The primers used provided information for the taxonomic identification of the study strains. however, one of them (CYACR and CYAF), being a highly conserved region, did not provide information at the species level, so the use of other regions is suggested (Crispim and Gaylarde, 2004; Emami *et al.,* 2015). It has also been reported in literature reviews that the results may vary depending on the genera of cyanobacteria (Crispim and Gaylarde, 2004).

Much of the information available in gene databases is limited to the use of the 16S-ITS-23S rRNA regions and very few species contain information on other genes, such as the ATPase gene, the nitrate transporter gene (nrtP) or heat shock proteins (Hsp90 gene), even, there are no complete genomes of any species of the genus *Oculatella*.

For microalgae, and with the information available in gene databases, two of the study strains do not have complete genomes available (*Chlorococcum vacuolatum* and *Desmodesmus pleiomorphus*) but there is more gene information, compared to cyanobacteria, since in addition to the ITS and 18S rRNA regions, there are complete mitochondrial and chloroplast genomes, and tufA genes and genes that code for RuBisCO, although not for all the study strains. Due to this, it is important to highlight that special attention should be paid to the production of DNA databases in order to accelerate the availability of information on the species present in biofilms (Crispim and Gaylarde, 2004), since the percentages of identity are low, as well as the distance between species in the phylogenetic tree, due to the fact that different ecological groups are formed and the species reported in the database correspond to the marine or freshwater groups.

The previous results confirm the need to relate the taxonomy based on morphological characters and studies that involve the use of molecular markers to achieve a more complete and reliable taxonomic identification, using a greater number of elements (morphological and genetic) for what is convenient.

It should be noted that the molecular characterization does not replace the morphological characterization because, despite the fact that there is more information on the sequences of the 16S rRNA and 18S rRNA genes, it is necessary to complement and enrich the databases with other molecular markers that provide variability between species of the same genus., as with the use of primers ITS1 and ITS4, so it is necessary to perfect the technique of amplification and purification of bands from the gel, for subsequent sequencing. The findings of this study agree with the work carried out by Pineda-Mendoza *et al.,* (2011).

In the case of the biodiversity of species found, there are numerous studies on the analysis of communities that develop on the surface of historical monuments; between them, the study by Macedo *et al.,* (2009) is very useful. They review the diversity of cyanobacteria and green algae in Mediterranean monuments, analyzing 45 studies published between 1976 and 2009 with 172 taxa that include 37 genera. of cyanobacteria and 48 of green algae. Among the most reported genera are *Chlorella* and *Chlorococcum*, which were found in the present study in the archaeological zone of Malinalco in the State of Mexico and in Yohualichan in Puebla. These genera were found colonizing granite, sandstone, marble, and limestone substrates in monuments in Italy, Spain, Portugal, and Greece. Both genera have also been reported in the Mayan Uxmal archaeological zone, in Yucatan, Mexico (Ortega-Morales, 2006). In another study, Gaylarde and Gaylarde (1998) studied and identified the composition of photosynthetic microorganisms in monuments in Latin America (Argentina, Mexico, Bolivia, Brazil, and Peru), identifying more than 46 genera of algae, where the most common was *Chlorella* with a percentage of occurrence >68%.

Many are the studies carried out about the communities that develop on monuments and buildings of historical importance, however, very few of them use molecular methods for the identification of microorganisms and in a smaller number those that compare the morphological identification with molecular data, however, correlating these characters can provide greater taxonomic resolution in these study groups. The results of the present study agree with those obtained by Premanandh *et al.,* (2006).

In this way, the results obtained in the present study contribute to the enrichment of the gene databases, because despite the fact that most of the isolates were identified, the phylogenetic trees show a certain distance with the probable species, so the identity in the nucleotide sequence is not 100% each time, moreover, in the case of *Dicellula* sp. there is no information in the gene bank. It was shown that phenotypic plasticity plays a very important role in the identification of these microorganisms, since it can lead to errors in the traditional identification based on morphological characters. In addition, there is very little information in gene databases regarding the species that develop in this type of habitat.

The molecular markers 16 srRNA and 18 srRNA allowed the generic identification of all the strains studied, but only some of them provided identification at the species level. Molecular characterization does not replace identification based on morphological characters but must be complementary.

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