BABEŞ-BOLYAI UNIVERSITY
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PHD THESIS

INVESTIGATION OF BIODIVERSITY IN CYANOBACTERIAL MATS ASSOCIATED TO THERMOMINERAL SPRINGS FROM THE WESTERN PLAIN OF ROMANIA USING MOLECULAR TECHNIQUES

- summary -

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CLUJ-NAPOCA
2011

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Keywords:

- cyanobacterial mats
- thermomineral springs
- molecular biodiversity
- modern stromatolites
- 16S rRNA
- DGGE
- ARISA
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I. Introduction

The general idea of this PhD thesis was represented by biodiversity investigation using molecular techniques. The study targeted the cyanobacterial mats associated with certain geothermal (thermomineral) springs resulted after certain geological drillings in the Western Plain of Romania. These mats are an excellent model for studying the molecular diversity and the colonizing potential of cyanobacteria because they have precise spatial delimitation and homogenous conditions enforced by constant water temperature and chemistry. They are also a rich source of heat-resistant proteins (genes) which can be exploited in future studies. The project has focused on 5 cyanobacterial mats developed around 5 drillings with different temperature and other physio-chemical properties of the water: Ady Endre, Beltiug, Ciocaia, Marghita and Săcuieni.

II. The species concept for prokaryotes

The prokaryotes classification is the youngest and most dynamic branch in the field of taxonomic studies.

Among the species concepts described in the literature, two seem to be universal and could be used for the classification of all living organisms, including prokaryotes: PhSC (”Phenetic Species Concept”) and ESC (”Evolutionary Species Concept”) (Rosello-Mora and Amann, 2001).

The Phenetic Species Concept is a similarity concept based on characteristics which are not necessarily universal among the members of the taxa. This is the concept that was used for defining the prokaryotic species, seeming, until now, to be stable and operational.

The Evolutionary Species Concept was considered a highly theoretical evolutionary species concept. It is based on phylogenetic analysis of organisms based on several phylogenetic markers.

The current prokaryotic species concept resulted after enhancements of what was considered as a unit, the developement and upgrading of taxonomic classifications being linked to the usage of modern molecular techniques.

The phenetic species concept (PhSC) este the one generally used in defining the prokaryotic species.

The future of the prokaryotic species concept will be strongly influenced by the whole genome sequenciing. With hundreds of genomes being already sequenced and

more to coome, microbiolgy will be able to use huge databases, provind vauabe information that will have a great impact on today's species concepts.

III. Molecular phylogeny of bacteria based on comparative analysis of certain conserved genes

The comparative analysis of small subunit RNA plays a central role in the microbial identificaion and taxonomy even today, in the genomic era.

The use of 16S rRNA genes in the phylogenetic studies led to a revolutionary approach and finally to the re-organization of the living world in 3 domains: Archaea, Bacteria and Eukaria (Woese, 1987; Ludwig et al., 1993).

Even though the advantages of this marker are well known, with reference to their informational content and to the complexity of the 16S rRNA sequence database, it is generally accepted that this marker does not reflect in detail the evolutionary history. Additional phylogenetic markers should be taken into consideration for a better, more detailed phylogeny.

The existence of three life domains is well sustained by other genes also, with an increased level of conservation: Tu (1 alpha) elongation factor, heat-shock protein Hsp60, RNA polymerase subunits and some tRNA-synthases. If we admit that the F_1F_0 and V_1V_0 ATPases are paralog markers, then they can not be used in the evaluation of the three domains system because the eukaryotic and archaeal orthologous sequences are missing or are represented only by gene duplications with uncertain function (Ludwig and Schleifer, 2005).

Even though there are some differences between the overall and detailed topology of the phylogenetic trees, the universal view offered by the rRNA phylogeny can be truly changed only if the paralogous and lateral gene transfer issues of the alternative markers will be resolved. This is why, in the current level of knowledge, rRNA remains the most informative marker in phylogenetic analyses.

IV. Ancient and modern cyanobacterial mats

Microbial mats have dominated the Earth's surface for more than 3 billion years, prevailiing today in special environments like thermal springs, environments with incresed levels of salinity and in sulphurous waters. Recently, more and more researchers focus on life in hot springs because they ay that these environments are equivalent to those in which life started to develope on our planet.

In the few very well conserved fossils of ancient microbial mats (in Africa and Australia) a series of filamentous bacterial taxa were identified, most likely cyanobacteria belonging to the Oscillatoriales order (Walsh, 1992; Schopf, 1992, 2002; Wacey, 2009).

Modern microbial mats, developed in hot springs around the Globe, are characterized by a low diversity of cyanobacterial taxa, maybe due to the limitations enforced by the physico-chemical properties of the thermal springs.

Most of the thermophilic mats, growing at a temperature below 74°C, are dominated by filamentous cyanobacteria belonging to the Oscillatoriales order. Among the genera observed, *Phormidium*, *Leptolyngbya*, *Lyngbya*, *Mastigocladus*, *Oscillatoria* prevail. Therefore, based on the comparative studies of cyanobacterial biodiversity, an analogy between the ancient and modern (from hot springs) mats can be proposed.

V. Molecular methods for diversity investigation of microbial mats developed in several habitats

Over time, the determination of microbial diversity in natural and artificial ecosystems has proven quite difficult. The classical approach in determining the bacteria in field samples relied on enrichement-culture based techniques, along with several biochemical and physiological tests. These methods do not reflect the community structure, but rather the selectivity of the growth media. Also, they are time and resource consuming, the investigated organisms being able to turn its physiological and genotipic characteristics existing in natural populations; also, just a very small fraction of the bacterial population (0,1%-10%) is culturable in the laboratory.

Because most of the studies focused on DNA fingerprinting of microbial mats are using the rRNA gene, several techniques using this marker will be discussed further on.

Nucleic acid hybridization techniques

a) Hybridization analysis of total community DNA shows if 2 samples are formed of the same organisms, independently of the species composition.

b) Quantitative hybridization, based on fluorescent oligonucleotide probes. It can be a „dot-blot" hybridization (Rainey et al., 1994), in which a mix of nucleic acids

is investigated with the probes or it can be a whole-cell *in situ* hybridization, in which a fixed sample from a bacterial population is investigated (Amann and Fuchs, 2008).

ARDRA analysis (Amplified Ribosomal DNA Restriction Analysis)

- The 16S rRNA gene fragments amplified through PCR from a bacterial population DNA, even though they share approximately the same length, yet they present certain deifferences in the base pair composition.
- A possible way to detect these differences is to construct clone libraries, digest each clone with restrction endonucleases and compare the profiles (Mwrichia et al., 2010).

T-RFLP analysis (Terminal-Restriction Fragment Length Polymorphism)

- A fluorescent marker is added to one of the primers, which can be detected by a genetic analyzer.
- The PCR products obtained with 16S rDNA universal primers are digested with a restriction endonuclease and discriminated by capillary electrophoresis (Avaniss-Aghajani et al., 1996).

SSCP analysis (Single-Strand Conformation Polymorphism)

- In non-denaturing conditions, single-stranded DNA molecules give rise to secondary structure conformations by base pairing between nucleotides within a single strand
- A single nucleotide change may alter the conformation of a single strand DNA molecule and will allow two DNA fragments that differ in only one nucleotide to be distinguished when electrophoresed in non-denaturing polyacrylamide gels due to mobility difference between the molecules (Orita et al., 1989).

LH-PCR analysis (Length Heterogeneity PCR)

- This fingerprinting approach takes advantage of naturally occurring sequence length variations
- The typical protocol involves PCR amplification of a small part of the target gene with a labeled primer and then electrophoresis of the labeled product on an automated fluorescence-detection-based sequencing device (Suzuki et al., 1998)..
- If the marker used is the ITS region, then the technique is called ARISA (Amplified Ribosomal Intergenic Spacer Analysis) (Fisher and Triplett, 1999).

rep-PCR fingerprinting

- rep-PCR uses DNA primers complementary to naturally occurring, repetitive DNA sequences, dispersed throughout most bacterial genomes. The rep-PCR amplicons are resolved in a gel matrix, resulting in complex and highly specific genomic fingerprints.
- This method can not be applied directly to microbial mats, but only to identify species/strains isolated and cultured in the laboratory (Colwell et al., 1981; Muralitharan and Thajuddin, 2011)..

Analiza RAPD (Random Amplified DNA Polymorphism), AP-PCR (Arbitrarily primed-PCR), DAF (DNA Amplification Fingerprinting)

- The different names describe variations on a theme of PCR-based fingerprinting, which use short (10 nucleotides for RAPD, 18 nucleotides for AP-PCR or 608 nucleotides for DAF) nonspecific single primers at low annealing temperatures in order to generate genomic fingerprinting profiles.
- The primers anneal to multiple regions of the genome simultaneously. Essentially, the techniques scan genomes at a low stringency for small inverted repeats.

DGGE analysis (Denaturing Gradient Gel Electrophoresis)

- Using this technique, DNA fragments with identical or almost identical length, but with different nucleotide composition, can be separated electrophoretically.
- The separation is based on the changes in the electrophoretical mobility of DNA fragments which migrate vertically through a polyacrilamide gel with an increasing concentration of denaturing agents (formamide and urea) (Muyzer, 1993).

The studies combining the microbial molecular biodiversity studies with the measurement of some natural processes and parameters are more and more popular. These studies have the potential to link the communities' structure with the complex microbial functions and activities. However, the role of classical microbial ecology should not be underestimated. The molecular studies, alongside with the culture-based investigations, will help in the future to characterize the existing microorganisms in nature, their purpose being to discover the richness in the biodiversity of natural microbial mats.

VI. Methodological approach in the study of cyanobacterial biodiversity in the thermophilic mats from the Western Plain of Romania

The approach methodology of this study (fig. 1) consisted in collecting and studying the biologic material by light and electron microscopy, followed by DNA extraction for further analyses employing molecular biology methods: Automated Ribosomal Intergenic Spacer Analysis (ARISA), Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Denaturing Gradient Gel Electrophoresis (DGGE).

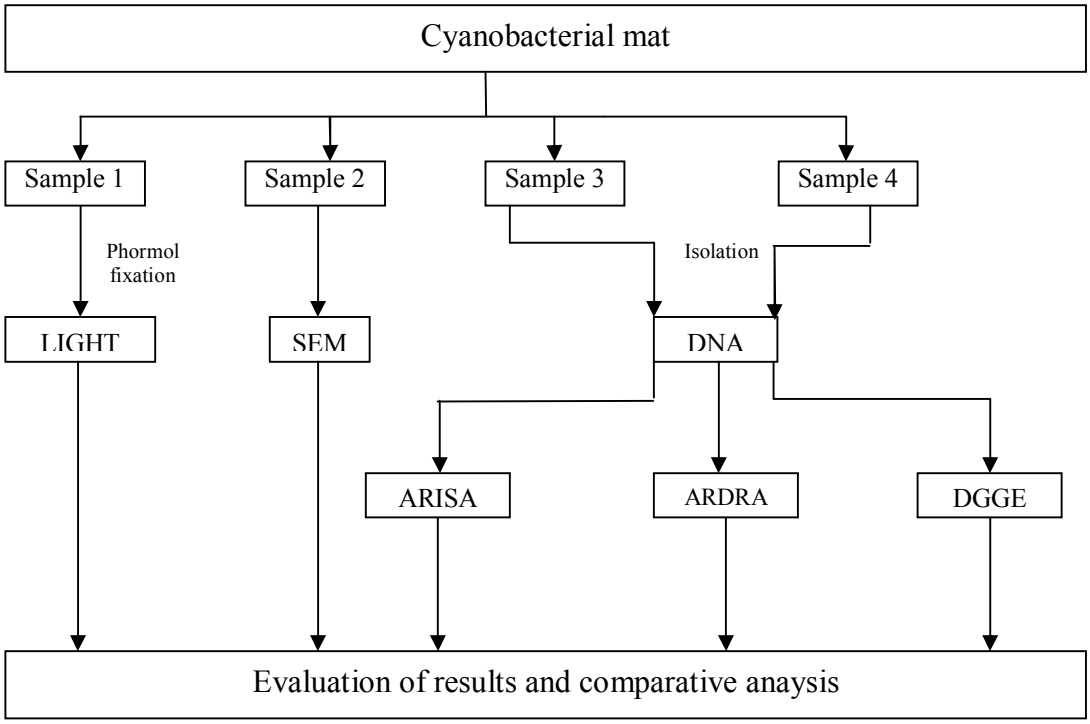

Fig. 1. Methodological approach in the study of cyanobacterial biodiversity in the thermophilic mats from the Western Plain of Romania

The cyanobacterial mats associated to thermomineral springs from The Western Plain of Romania display theoretically a low biodiversity because of the physico-chemical conditions of water. Because of this, we assumed that their investigation by using 3 of the previously described techniques (ARISA, ARDRA and DGGE) based on the 16S rRNA marker and ITS sequence, is enough for an overview of the diversity of cyanobacterial taxa that form these mats.

VII. Material and methods

Field sampling

Four distinct probes were sampled for light and electron microscopy, as well as for molecular analyses (DGGE, ARISA, ARDRA).

Morphological study

The morphologic investigation consisted both in the observation with an Olympus light microscope in order to identify the cyanobacterial taxons of the mat, and in a Scanning Electron Microscopy (SEM) analysis for the assessment of the spatial arrangement pattern of the mat.

DNA isolation

DNA isolation was accomplished with three individual techniques: a classic protocol developed in our lab, and two commercial kits.

Molecular approach

a) ARISA analysis

The principle of this method consists in the migration of certain DNA fragments tagged with a FAM fluorochrome through the capillary of a genetic analyzer, the machine being able to discriminate between different fragments based on their length. Thus, the cyanobacterial ITS region was amplified using a specific primer pair and the resulted DNA fragments were migrated through the capillary of an ABIPrism 310 genetic analyzer.

b) ARDRA analysis

The primer pair 27F and ITER was used to amplify the 16S rRNA+ITS fragment. The discrimination between them was accomplished by ligation of the amplicons in the plasmid vector pTZ57R/T (Fermentas), followed by sepparation through electroporation in *Escherichia coli* cells.

In order to identify the distinctive fragments, digestion with *Taq*I restriction enzyme was employed, followed by electrophoresis of the resulted DNA fragment

The different restriction profiles were assessed by cluster analysis based on the estimative length of the digested fragments.

The fragments with individual restriction pattern were sequenced, and the incomplete seqences were compared to the similar ones from the NCBI database by the BLAST method for taxon identification.

c) DGGE analysis

A fragment of the 16S rRNA gene was amplified by PCR directly from the genomic DNA isolated from the field samples, using a cyanobacteria-specific primer pair. The resulted amplicons were electrophoretically migrated in a polyacrylamide gel with urea and formamide as denaturing agents.

For the Marghita cyanobacterial mat, 8 fragments were isolated from the polyacrylamide gel, subsequent to DGGE. These fragments were used as DNA template for re-DGGE and profile comparing.

As long as the best part of the profiles were similar with the one they originated from, a clone library was constructed using the same primer pair for each fragment (5 clones/fragment, with a total of 40 clones). All the clones were migrated once again by DGGE, and their sequencing was attempted.

The fragments isolated from the gel, or the ones from the Marghita clone library were sequenced, and the resulted incomplete sequences were compared to the similar ones from the NCBI database by the BLAST method for taxon identification.

VIII. Results and discussion

Biotope description and morphological investigations

Geothermal springs (fig. 2) are stable biotopes and are ideal for biodiversity studies of cyanobacterial mats developed around them because they are not subject to seasonal temperature variation.

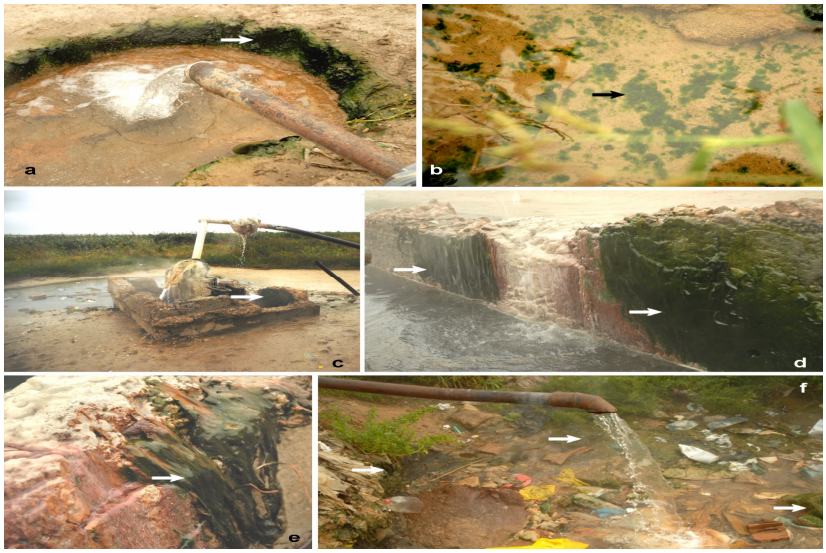

Fig. 2. The thermomineral drillings and the cyanobacterial mats as they were observed in the time of field sampling. **a-b.** Beltiug (Satu-Mare county), **c-e.** Ciocaia (Bihor county), **f.** Marghita (Bihor county). The arrows point to the cyanobacterial biomass („cyanobacterial mats").

Five cyanobacterial communities were selected for morphologic and molecular studies: Ady Endre, Beltiug, Ciocaia, Marghita and Săcuieni.

The water temperature in the 5 springs varies from 55°C to 67°C.

Using light microscopy, a low number of cyanobacterial taxa were observed in the 5 mats, most likely due to the physico-chemical properties of the thermomineral water.

Among the taxa identified, *Symploca thermalis, Mastigocladus laminosus, Phormidium janthiphorum* and *Symploca meneghineana* are typical in thermal springs.

SEM images (Marghita mat taken as an example; fig. 3) underlined the fact that the structure of the investigated mats is similar to the three-layered model proposed in the literature.

Fig. 2 (continued). The thermomineral drillings and the cyanobacterial mats as they were observed in the time of field sampling. **g-h.** Marghita (Bihor county), **i-j.** Ady Endre (Bihor county), **k-m.** Săcuieni (Bihor county). The arrows point to the cyanobacterial biomass („cyanobacterial mats").

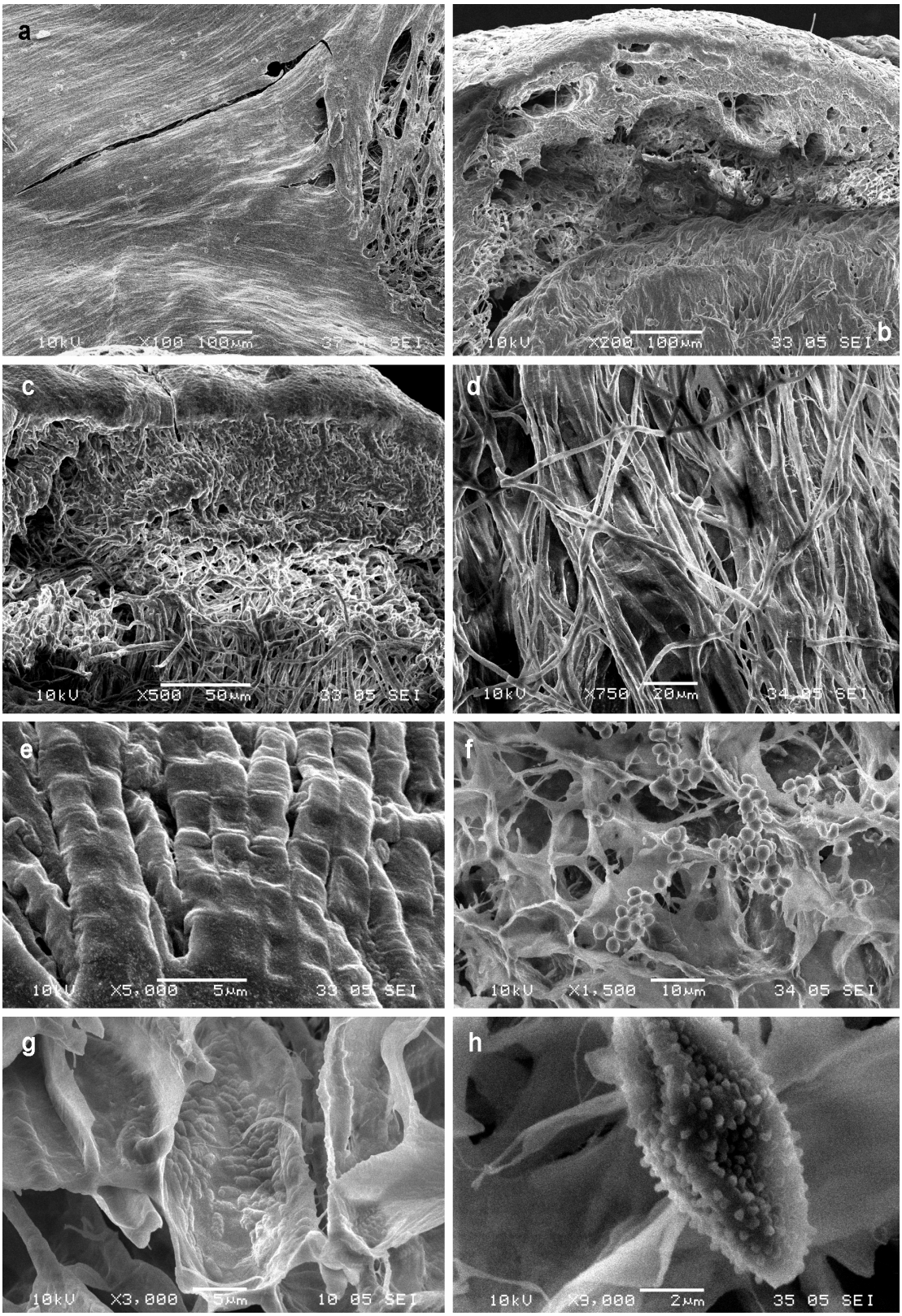

Fig. 3. The structure of the Marghita cyanobacterial mat as observed in Scanning Electron Microscopy (SEM). **a.** upper layer with a tight net of organized cyanobacterial filaments; **b-c.** view of the whole structure after fractioning; **d-e.** middle layer represented by a more relaxed net of cyanobacterial filaments and other microorganisms; **f-h.** inner layer presenting the distribution of bacteria.

Biodiversity investigation using molecular techniques

a) Biodiversity investigation by DGGE

The DGGE profile (fig. 4) showed the existence of 3 cyanobacterial taxons in the Ady Endre mat, 4 in Beltiug and 6 in the mats from Ciocaia and Săcuieni.

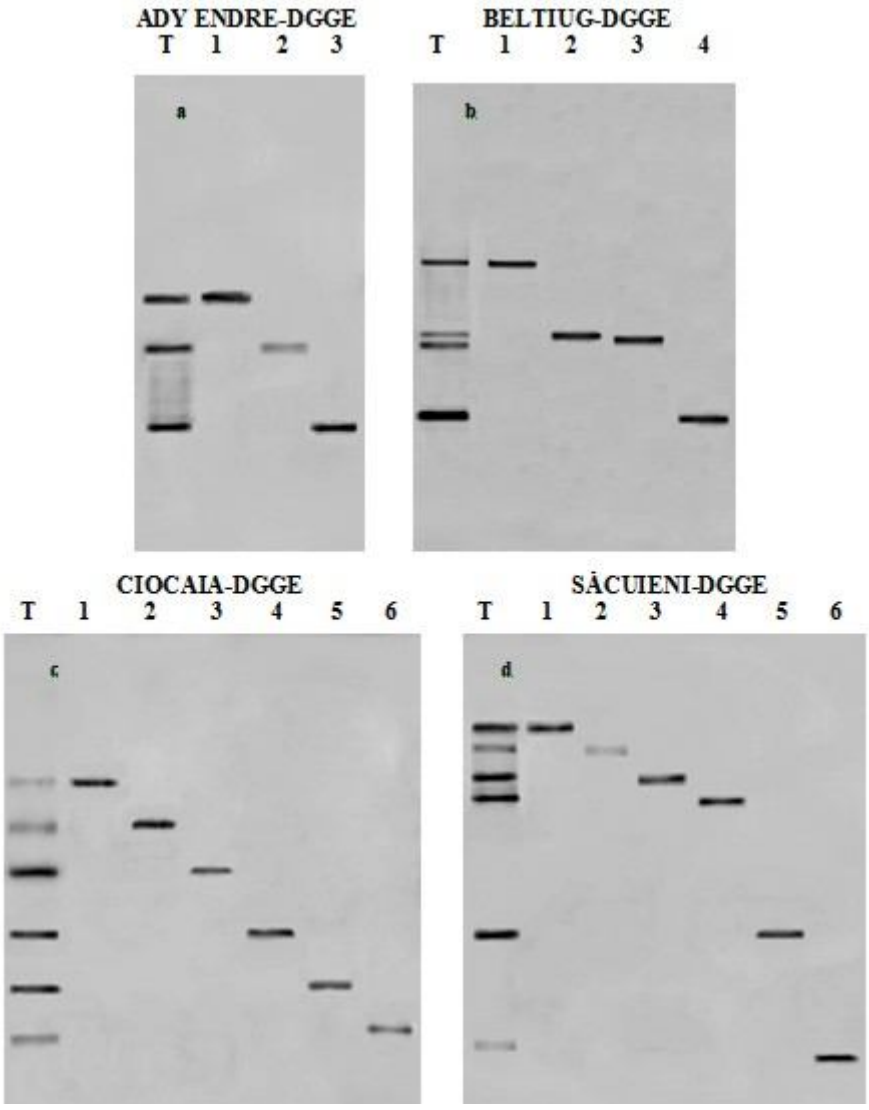

Fig. 4. DGGE profile of the 16S rDNA fragments amplified with the 359-781R(b) primers using DNA isolated from: **a** - Ady Endre; **b** - Beltiug; **c** - Ciocaia; **d** - Săcuieni. T - profile of the entire community; numbers represent the individual bands excized from the polyacrilamide gel and re-amplified.

In the case of Marghita (fig. 5; table 1), the denaturing gradient gel electrophoresis highlighted a series of limitations of this method (Coman et al.,2011), which can lead to an incorrect evaluation of microbial diversity in a given biotope. These limitations are: the existence of multiple operons in the same genome, the multiple melting domains in the same sequence and the hybridization among closely related sequences.

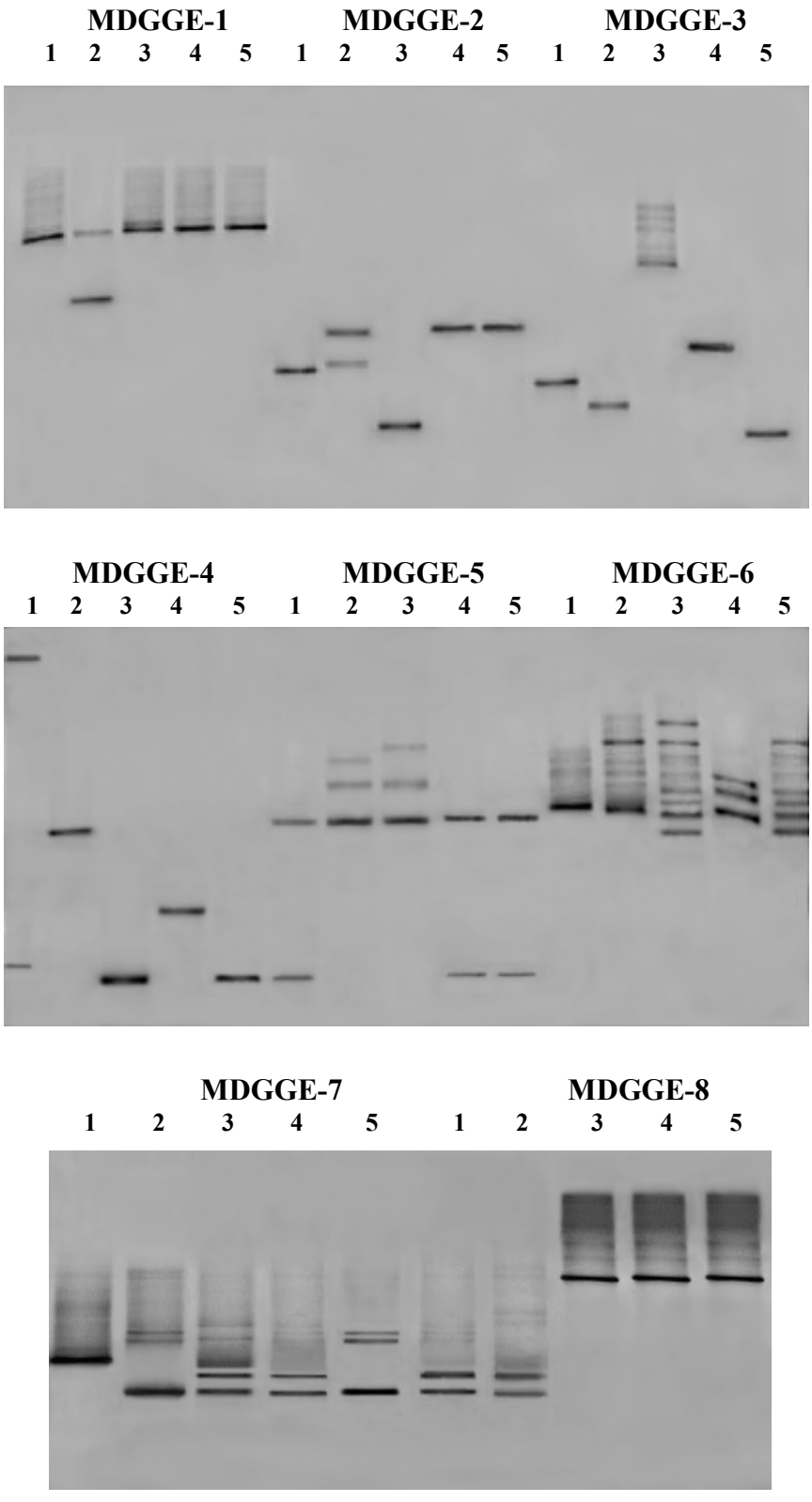

Fig. 5. DGGE profiles of the clone libraries constructed on the 8 fragments excised from the polyacrylamide gel after the initial DGGE analysis of the entire community DNA. The partial 16S rDNA fragments were amplified from the initial 8 excised bands using the 359F-781R(b) primer pair and a clone library was constructed for each of them (noted MDGGE1 to 8). Five clones (noted 1 to 5) were selected from every clone library and used as template for re-DGGE.

Table 1

Sequence similarity for the MDGGE clone libraries (fig. 4) from Marghita obtained by using the 8 individual fragments excized from the initial DGGE gel as template for the PCR.

Clona	Cea mai apropiată potrivire GenBank (NCBI)
MDGGE1-1	Uncult. bact. Geyserite B1 (identical to M1-3); ns.*
MDGGE1-2	*Leptolyngbya* sp. CCMEE6116
MDGGE1-3	Uncult. bact. *Geyserite* B1
MDGGE1-4	Uncult. bact. *Geyserite* B1
MDGGE1-5	Uncult. bact. Geyserite B1 (identical cu M1-3);ns
MDGGE2-1	*Phormidium pseudopristleyi* ANT.ACEV5.4
MDGGE2-2	*Phormidium* sp. NIVA-CYA202
MDGGE2-3	*Leptolyngbya* sp. 0BB30S02
MDGGE2-4	Uncult. cyanobact. clone R8-R60
MDGGE2-5	Uncult. cyanobact.clone R8-R60 (identic to M2-4);ns
MDGGE3-1	*Plectonema* sp. HPC-49
MDGGE3-2	*Leptolyngbya* sp. 0BB30S02
MDGGE3-3	*Oscillatoriales* cyanobact. BC007
MDGGE3-4	*Phormidium* sp. NIVA-CYA202
MDGGE3-5	*Leptolyngbya* sp. 0BB30S02
MDGGE4-1	Uncult. cyanobact. clone 02D2Z20
MDGGE4-2	*Leptolyngbya* sp. 0BB30S02
MDGGE4-3	*Leptolyngbya* sp. 0BB30S02
MDGGE4-4	*Phormidium animale* CCALA140
MDGGE4-5	*Leptolyngbya* sp. N62DM (identic to M4-3); ns
MDGGE5-1	*Leptolyngbya* sp. 0BB30S02 (ident. M7-2+M7-3);ns
MDGGE5-2	Uncult. bact. *Geyserite* B1
MDGGE5-3	Uncult. bact. Geyserite B1(identical to M10-2); ns
MDGGE5-4	*Leptolyngbya* sp. 0BB30S02 (ident. M7-2+M7-3);ns
MDGGE5-5	*Leptolyngbya* sp. 0BB30S02 (ident. M7-2+M7-3);ns
MDGGE6-1	Uncult. bact. Geyserite B1
MDGGE6-2	Uncult. bact. Geyserite B1
MDGGE6-3	Uncult. bact. Geyserite B1
MDGGE6-4	Uncult. bact. Geyserite B1
MDGGE6-5	Uncult. bact. Geyserite B1
MDGGE7-1	*Leptolyngbya* sp. 0BB30S02
MDGGE7-2	*Leptolyngbya* sp. 0BB30S02
MDGGE7-3	*Leptolyngbya* sp. 0BB30S02
MDGGE7-4	*Leptolyngbya* sp. 0BB30S02 (identical to M7-3); ns
MDGGE7-5	*Leptolyngbya* sp. 0BB30S02 (identical to M7-2); ns
MDGGE8-1	*Leptolyngbya* sp. 0BB30S02 (identical to M7-3); ns
MDGGE8-2	could not be sequenced
MDGGE8-3	Uncult. bact. *Geyserite* B1
MDGGE8-4	Uncult. bact. Geyserite B1 (identical to M8-3); ns
MDGGE8-5	Uncult. bact. Geyserite B1 (identical to M8-3); ns

*ns- not sequenced

After DGGE, sequencing and BLAST investigation (table 2), the main conclusion is that the bacterial taxa observed are all filamentous cyanobacteria, from the Oscillatoriales group. The following genera were identified: **Ady Endre**: *Leptolyngbya*, *Phormidium*; **Beltiug**: *Oscillatoria*, alte Oscillatoriale; **Ciocaia**: *Arthrospira*, *Oscillatoria*, *Lyngbya*, *Phormidium*; **Marghita**: *Leptolyngbya*, *Phormidium*, *Plectonema*, other Oscillatoriales; **Săcuieni**: *Leptolyngbya*, *Phormidium*, *Geitlerinema*, *Microcoleus*.

Table 2

The closest GenBank match (DNA sequence) of the partial 16S rDNA fragments amplified from the excized DGGE gel bands.

Mat	No. fragm. DGGE obs.	Fragment	Closest GenBank match (NCBI)	Identity (%)
Ady Endre	3	AE DGGE-1	*Leptolyngbya compacta* GSE-PSE28-08A	98
		AE DGGE-2	*Phormidium corium* 0416	98
		AE DGGE-3	Uncut. *Leptolyngbya* sp. isol. DGGE band 3-5	98
Beltiug	4	B DGGE-1	Uncult. cyanobacterium clone TDNP-wbc97_251_1_123	96
		B DGGE-2	*Oscillatoria* sp. LEGE06018	96
		B DGGE-3	*Oscillatoria* sp. MMG-3	97
		B DGGE-4	*Oscillatoriales* cyanobacterium BC007	97
Ciocaia	6	C DGGE-1	*Arthrospira platensis* Sp_11	98
		C DGGE-2	*Oscillatoria* sp. LEGE06018	99
		C DGGE-3	*Lyngbya birgei* CCC 333	95
		C DGGE-4	*Phormidium* sp. 195_A12	97
		C DGGE-5	Uncult. bacterium clone clone JW56-B11	94
		C DGGE-6	Uncult. bacterium clone clone JW56-B11	96
Săcuieni	6	S DGGE-1	*Leptolyngbya* sp. CR_10M	99
		S DGGE-2	*Leptolyngbya* sp. GSE-PSE28-08A	96
		S DGGE-3	Uncult. cyanobacterium clone Mat-CYANO-S22	100
		S DGGE-4	*Phormidium* cf. *formosum* P-FW	98
		S DGGE-5	*Geitlerinema* sp. CR_13M	98
		S DGGE-6	*Microcoleus* sp. SAG2212	99

b) Biodiversity investigation by ARISA

The cyanobacterial diversity was determined by counting the peaks in fig. 6 resulted after capillary electrophoresis. Thus, 5 ITS fragments were observed in Ciocaia, 6 in Săcuieni, 7 in Ady Endre, 8 fragments in Beltiug and 13 in Marghita. The length of the ITS fragments varied from 310 bp to approximately 780 bp.

c) Biodiversity investigation by ARDRA

After evaluating the restriction profiles and sequencing (table 3) it was observed that the majority of taxa identified are filamentous cyanobacteria from the order Oscillatoriales.

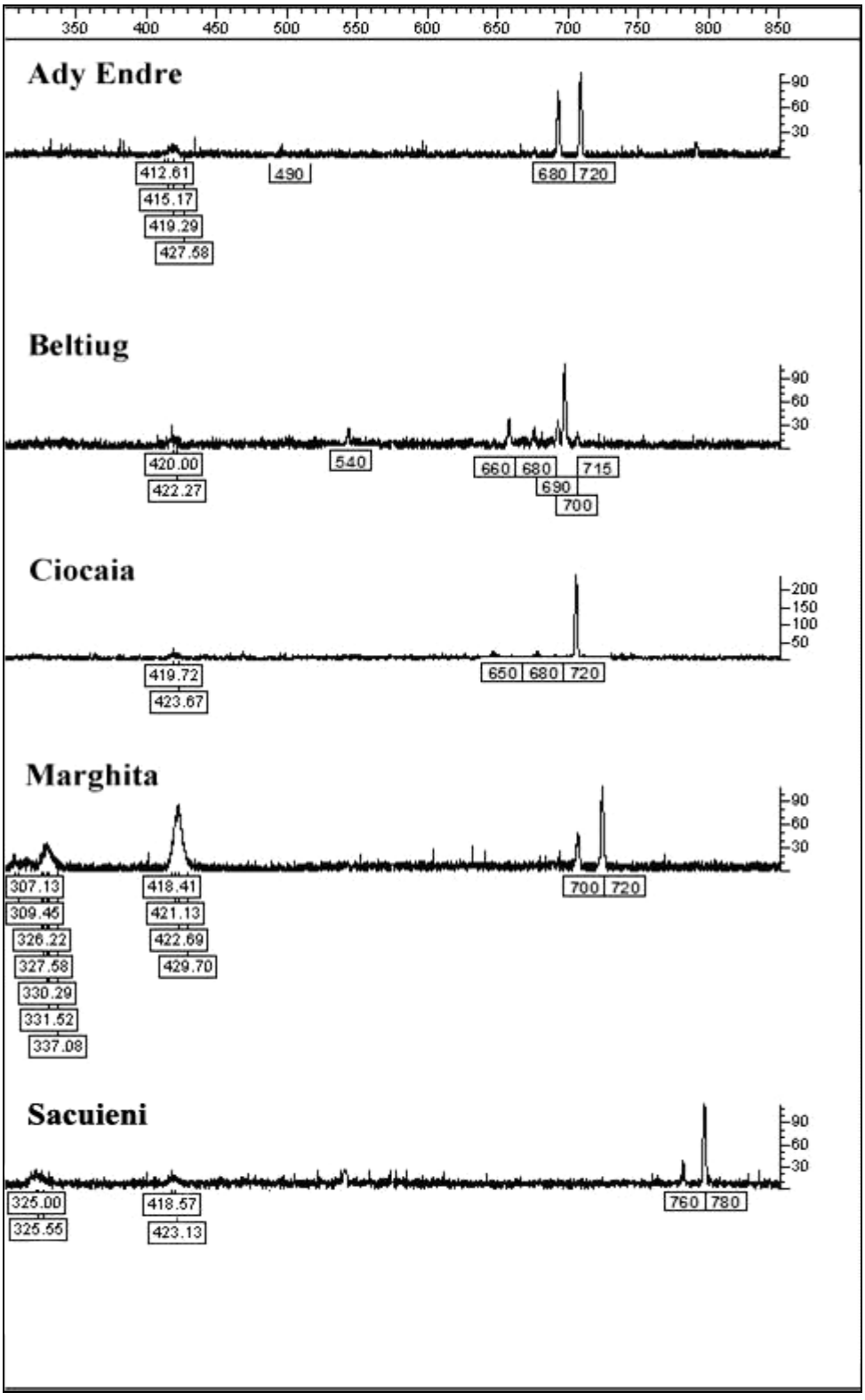

Fig. 6. The ARISA electrophoretic profile for the cyanobacterial ITS fragments amplified from the 5 investigated mats.

Table 3

The closest GenBank matches for the partial 16S rDNA sequences obtained after aplying the ARDRA analysis for all the 5 investigated mats.

Clona	Taxonul cu cel mai mare grad de similaritate	Identitate %
	ADY ENDRE	
AE1	*Leptolyngbya compacta* GSE_PSE28_08A	99
AE3	*Leptolyngbya laminosa* ETS_08	98
AE21	*Spirulina laxissima* SAG 256.8	97
AE23	*Leptolyngbya* sp. 0BB32S02	97
	BELTIUG	
B1	*Leptolyngbya* sp. CR_L26	96
B2	*Oscillatoria* sp. LEGE06018	95
B4	*Oscillatoria acuminata*	97
B5	*Oscillatoriales* cyanobacterium BC007	96
B11	*Gemmatimonas aurantiaca* T-27	95
B13	Uncultured *Gemmatimonas* sp. A1631	96
B26	Uncultured *Gemmatimonas* sp. A1631	95
	CIOCAIA	
C1	*Oscillatoria earlei* NTAP016	92
C2	*Arthrospira platensis* Sp-11	97
C3	*Leptolyngbya* sp. LEGE 07319	99
C7	*Phormidium animale* PMC239.04	99
C16	*Phormidium* sp. 195-A12	99
	MARGHITA	
M1	*Gemmatimonadetes* bacterium 100M2_B10	90
M2	*Leptolyngbya antarctica* TM1FOS73	93
M4	*Phormidium pseudopristeleyi* ANT.ACEV5.3	98
M5	*Phormidium* sp. MBIC10025	96
M6	*Oscillatoriales* cyanobacterium BC007	96
M12	Uncultured bacterium GBII-5	92
M21	*Phormidium* sp. 195-A12	98
M24	*Gemmatimonas aurantiaca* T-27	92
M29	*Microcoleus steenstrupii* 148-2A	97
M36	*Leptolyngbya* sp. CR_L14	95
M40	Uncultured *Gemmatimonas* sp. A1631	92
M43	*Microcoleus* sp. HTT-U-KK5	95
M44	Uncultured *Gemmatimonas* sp. A1631	92
	SĂCUIENI	
S1	*Leptolyngbya* sp. CR_10M	96
S2	*Phormidium pseudopristleyi* ENCB-AD17	97
S3	*Leptolyngbya* sp. GSE-PSE28-08A	97
S5	*Microcoleus* sp. HTT-U-KK5	99
S6	*Gemmatimonas aurantiaca* T27	93
S16	*Leptolyngbya* sp. BX10	95
S20	*Geitlerinema* sp. CR_13M	97

Thus, the following genera were observed after ARDRA: **Ady Endre**: *Leptolyngbya*, *Spirulina*; **Beltiug**: *Leptolyngbya*, *Oscillatoria*; **Ciocaia**: *Oscillatoria*, *Arthrospira*, *Phormidium*; **Marghita**: *Leptolyngbya*, *Phormidium*, *Microcoleus*; **Săcuieni**: *Microcoleus*, *Phormidium*, *Leptolyngbya*, *Geitlerinema*.

Table 4

The number of taxa and the identity of the cyanobacterial genera observed in the investigated mats

Comunitatea	**Număr taxoni cianobacterieni identificați**		**Genuri cianobacteriene identificate**
Ady Endre	DGGE	3	*Leptolyngbya*, *Phormidium*, *Spirulina*
	ARDRA	4	
Beltiug	DGGE	4	*Leptolyngbya*, *Oscillatoria*
	ARDRA	4	
Ciocaia	DGGE	4	*Arthrospira*, *Oscillatoria*, *Lyngbya*, *Phormidium*
	ARDRA	5	
Marghita	DGGE	8	*Leptolyngbya*, *Phormidium*, *Microcoleus*, *Plectonema*
	ARDRA	8	
Săcuieni	DGGE	5	*Microcoleus*, *Phormidium*, *Leptolyngbya*, *Geitlerinema*
	ARDRA	6	

d) Conclusions

The molecular approach consisted in the analysis of the 16S rRNA gene and the ITS region independently of the culture-based techniques.

Using the 3 techniques (DGGE, ARISA and ARDRA) in the same study led to an increased number of cyanobacterial taxa identified in the investigated mats (tab. 4).

Even though the mats started to develop only in the 1970s, the taxa richness is similar to other cyanobacterial mats from thermal springs, sometimes even greater.

The cyanobacterial taxa encountered in the 5 mats belong to the order Oscillatoriales, prevailing the *Phormidium* and *Leptolyngbya* genera. *Mastigocladus laminosus*, some *Symploca* species, as well as *Geitlerinema thermale*, identified initially by morphological approach, were not observed in the molecular results, questioning once again the accuracy of the classical approach in the microbial biodiversity studies.

Species from the *Leptolyngbya* genus have quite a narrow distribution in hot springs worldwide. They were encountered only in Greenland, Mexico and Thailand, at a temperature ranging from 40°C to 74°C and a pH from 7 to 9. In the mats from

the Western Plain of Romania, this genus was observed in Ady Endre, Beltiug, Marghita and Săcuieni.

Phormidium genus is much more abundant, its species being observed on all continents. In Romania, thermophilic species of Phormidium were identified in Ady Endrre, Ciocaia, Marghita and Săcuieni.

Species of *Arthrospira* are quite uncommon in hot springs first of all because of the harsh conditions that prevail in these environments and second of all because they are planctonic organisms. In the case of the Ciocaia mat, identifying a partial 16S rDNA belonging to *Arthrospira platensis* can be associated with the existence of this taxon in the cyanobacterial mat. Given that this is the first evidence of a *Arthrospira* taxon in a hot spring, this statement has to be confirmed by further studies.

The only hot springs in which species of *Spirulina* were encountered are in Argentina and Mexico, at 35°C-38°C and a pH higher than 9. In this study, a taxon with a high degree of similarity on a partial 16S rDNA fragment with *Spirulina laxissima* was observed in the Ady Endre mat. This is, propably, the first evidence of this taxon in a hot spring in Europe, alongside with other species, belonging to the genera *Lyngbya* (Ciocaia), *Plectonema* (Marghita) and *Geitlerinema* (Săcuieni).

In the case of the cyanobacterial genera from the mats in the Western Plain of Romania which were mentioned for the first time in Europe or in the world, the springs should be monitored in the future in order to confirm the results.

Microcoleus steenstrupii is known to be rather a desert cyanobacterium, but it has previously been encountered also in warm springs in Iceland. Due to the 96% identity of partial 16S rDNA sequences, it is most likely to inhabit the Marghita mat also, this being the second evidence of this taxon in a hot spring around the world.

IX. Are geothermal spring cyanobacterial mats involved in the formation of modern stromatolites?

Modern microbial mats play a key role in evolutionary studies. Sometimes, they can be considered analogue systems to those existing on this planet's surface billion years ago.

In the Western Plain of Romania, in the geothermal spring from Ciocaia, were discovered modern, laminated sedimentary structures, most likely modern stromatolites (fig. 7). This is an important step in the evolutionary and early-life

studies because it offers the possibility to study the mineralization and fossilization processes in a microbial mat developed in an environment other than marine.

To confirm the stromatolitic nature of the structures, 3 samples were taken (S1, S2, S3) (fig. 7) for chemical, morphological and cyanobacterial diversity investigations.

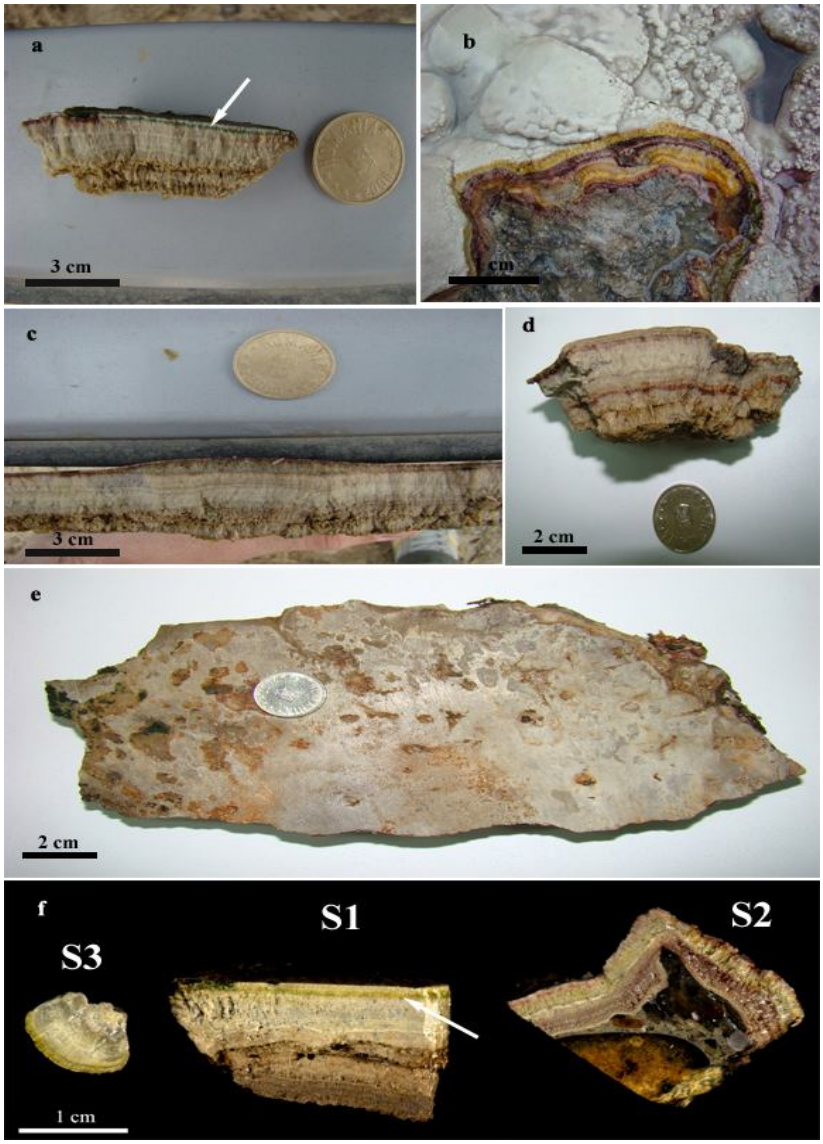

Fig. 7. The structure of the samples taken from the geothermal spring in Ciocaia. **a** - cross-section; the arrow points to a biofilm, most likely cyanobacterial, which is starting to be covered by $CaCO_3$; **b** - photo of a stromatolite as it appears in the field after fracturation; c-e - field samples; **c-d** - cross-sections; **e** - upper view; **f** - the structure of the stromatolites as it appears in section; the arrow points to the cyanobacterial biofilm covered in $CaCO_3$. The red circles indicate the areas from which DNA was extracted.

The light microscopy images of thisn sections show a laminated, multi-layered structure, the thickness of the layers ranging from a few µm to hundreds of µm, in all 3 samples (fig. 8 and 9).

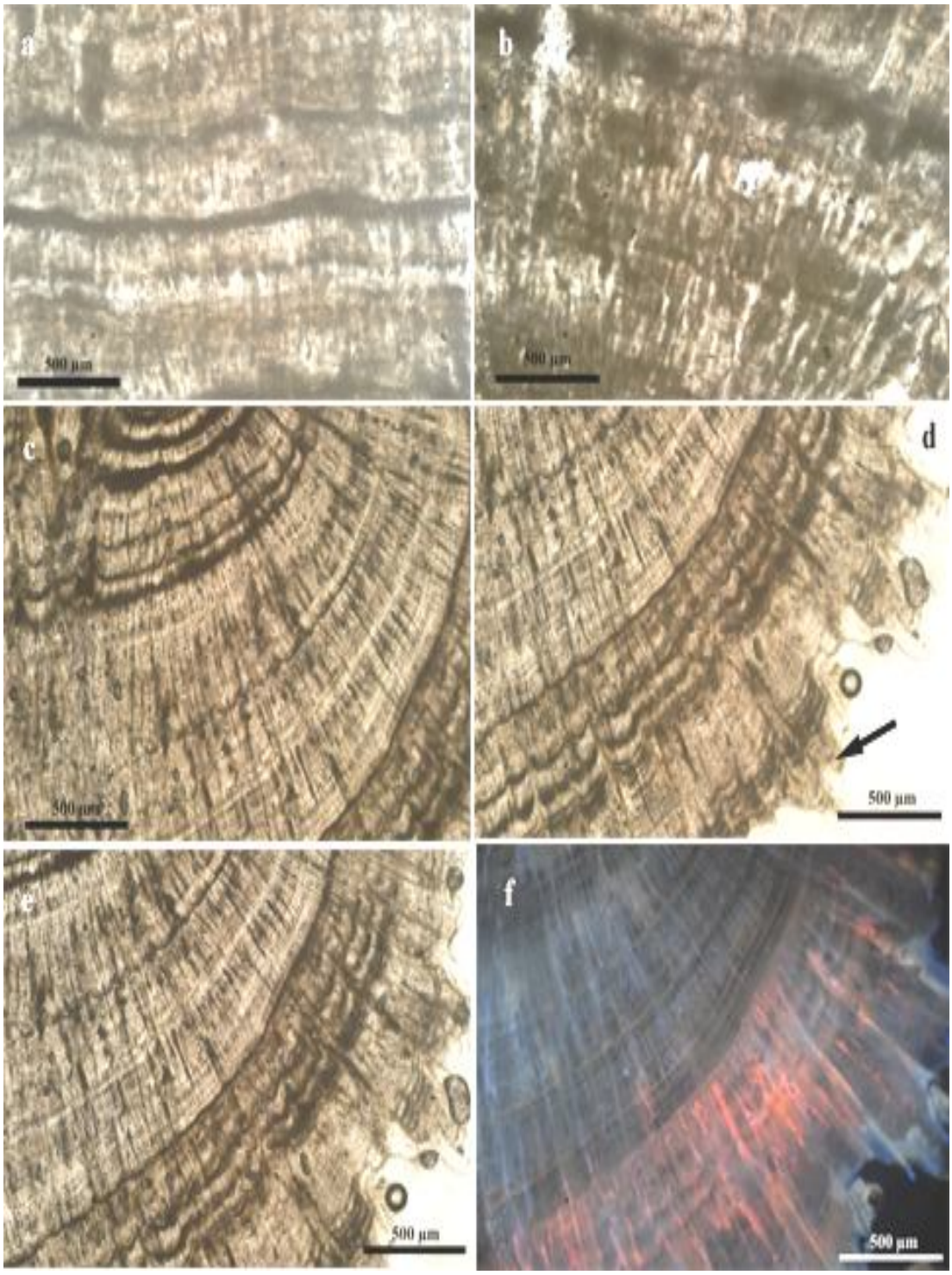

Fig. 8. Light microscopy images of thin sections taken from the stromatolitic samples S1 (**a-b**) and S3 (**c-f**). A compaction of the inner layers can be observed, the development of the stromatolite taking place in the upper layer, at the nucleation centers (as the one pointed by the arrow in fig. **d**). Epifluorescence light microscopy (**f**) shows traces of chlorophyll, most likely from cyanobacteria.

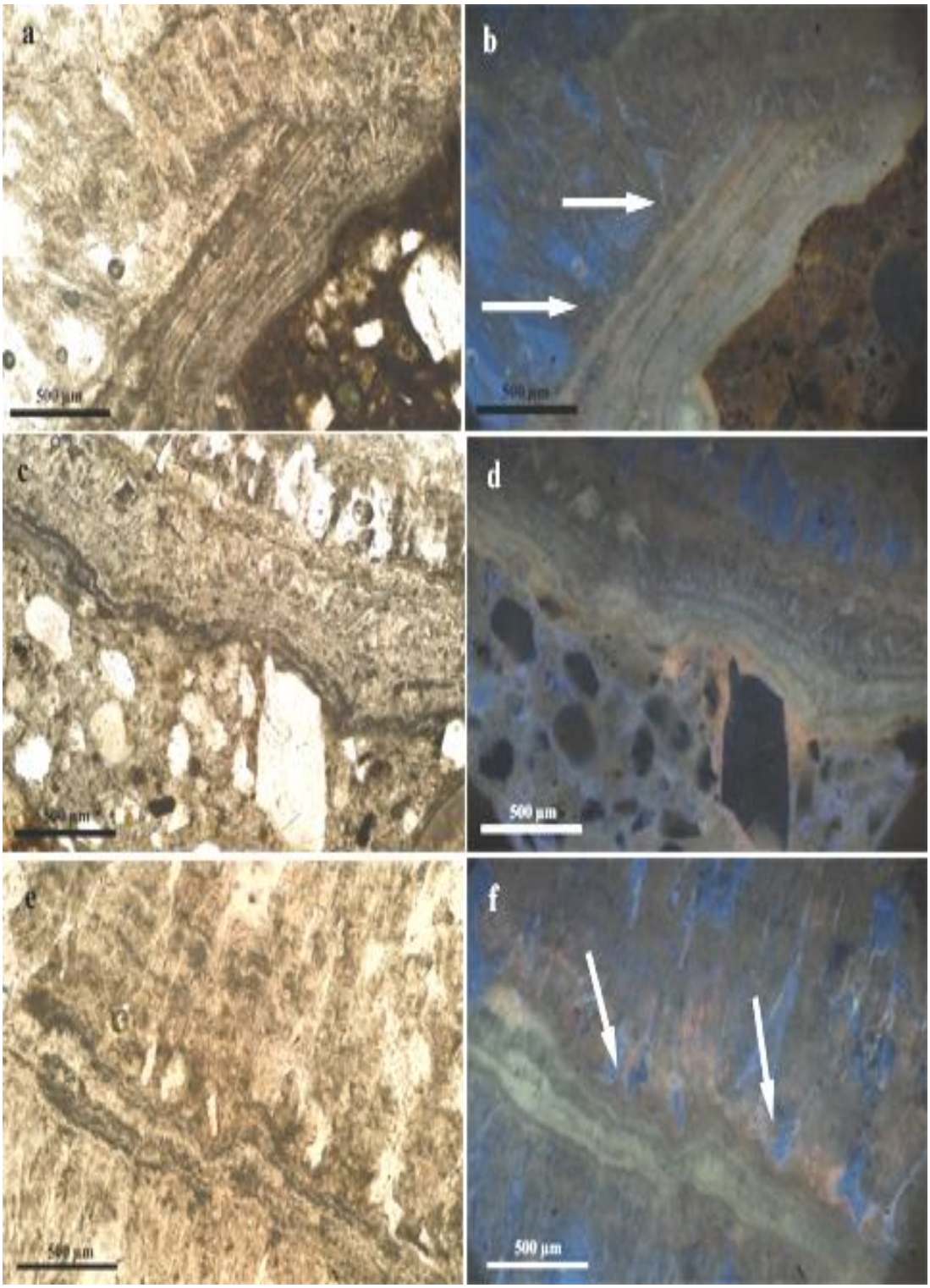

Fig. 9. Light microscopy images of thin sections taken from the stromatolitic sample S2. A compaction of the inner layers can be observed, the development of the stromatolite taking place in the upper layer, at the nucleation centers (pointed by arrows). Epifluorescence light microscopy (**f**) shows traces of chlorophyll, most likely from cyanobacteria.

The EDX (”Energy Dispersive X-ray analysis”) results show that O (între 41,1%-50,4%), C (între 15,8%-33,6%) and Ca (între 16,5%-30,1%) prevail in the samples, in the upper layer, as well as in the inner layers. Other elements were identified also, but with less than 5% predominance: Ba, Sr, Zr, Fe, Na, Mg, S.

Based on these results, we can say that the sedimentary structure is most likely formed by $CaCO_3$ precipitation in the form of the calcite and aragonite groups: calcite - $CaCO_3$; magnesite - $MgCO_3$; siderite - $FeCO_3$; strontianite - $SrCO_3$; witherite - $BaCO_3$).

The bacterial biofilm has a paralell orientation in regards to the $CaCO_3$ crystals, sometimes wrapped around them (fig. 12-14). The cyanobacterial filaments are around 2.5 μm in diameter and tens of μm in length (fig. 12g), sometimes forming a fibrous net (fig. 12h).

Together with cyanobacteria, other bacteria were observed, also, some being filamentous (less than 1 μm diameter) (fig. 12f-g; fig. 14g-h), other are rod-shaped (fig. 12h; fig. 14h).

Based on the morphological results we can say without a doubt that the microbialites discovered at Ciocaia are modern stromatolites („living stromatolites”), these kind of structures being encountered in just a very few places on Earth. This is a msjor discovery because these stromatolites are very important in the studies focused on the organomineralization process and on the evolution of life on our planet.

Even though the 5 mats are similar in regards to the cyanobacterial composition, an organomineralization process was observed only in Ciocaia. Therefore, we can say that, alongside with cyanobacteria, an important role in the mineralization process is held by the abiotic factors, in the case of Ciocaia, the HCO_3^- (7 g/l) și Ca^{2+} (55 mg/l) ion concentrations.

We observed a decrease in the cyanobacterial diversity in the Ciocaia stromatolites, only the *Leptolyngbya* genus being observed and only in the S1 sample.

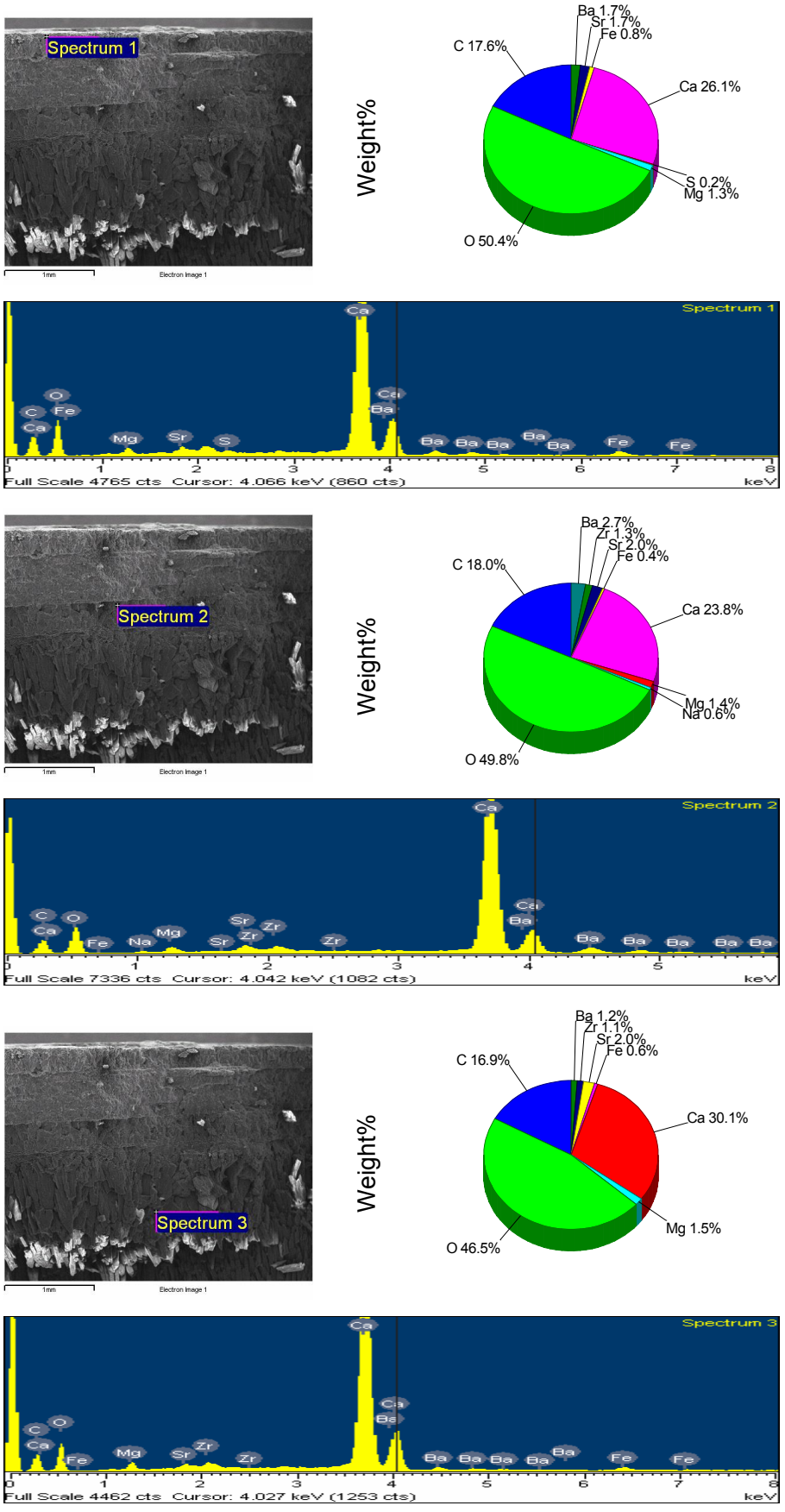

Fig. 10. Elemental distribution (EDX analysis) in the S1 sample, taken from the Ciocaia geothermal spring.

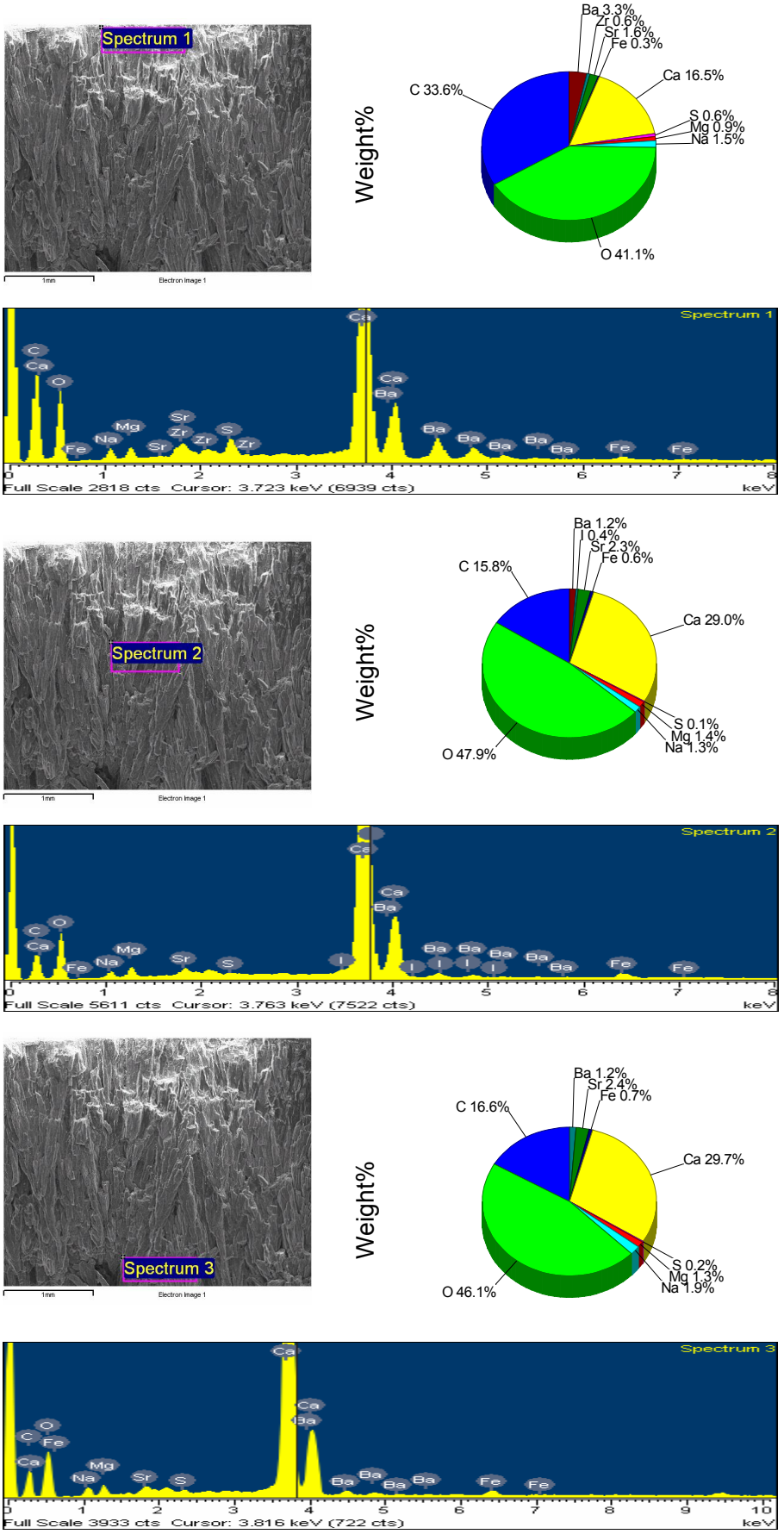

Fig. 11. Elemental distribution (EDX analysis) in the S2 sample, taken from the Ciocaia geothermal spring.

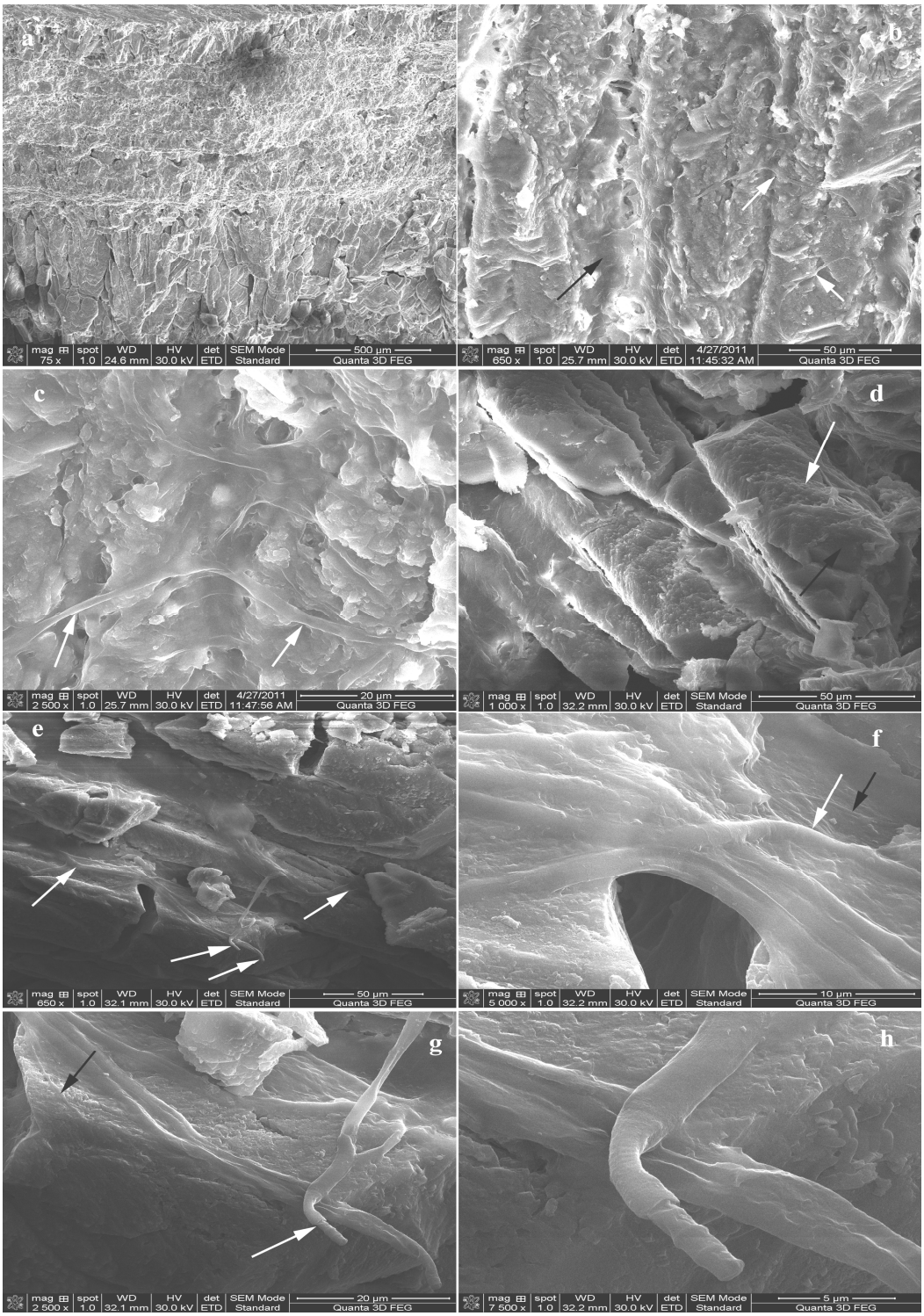

Fig. 12. The S1 stromatolite structure as observed in SEM: **a** - cross-section; **b-d** - biofilm/filaments (white arrows) fossilized around the $CaCO_3$ crystals (black arrows); **e-f** - cyanobacterial/bacterial filaments surrounded by extrapolymeric substances (EPS) and fossilized; **g-h** - cyanobacterial filament covered in EPS.

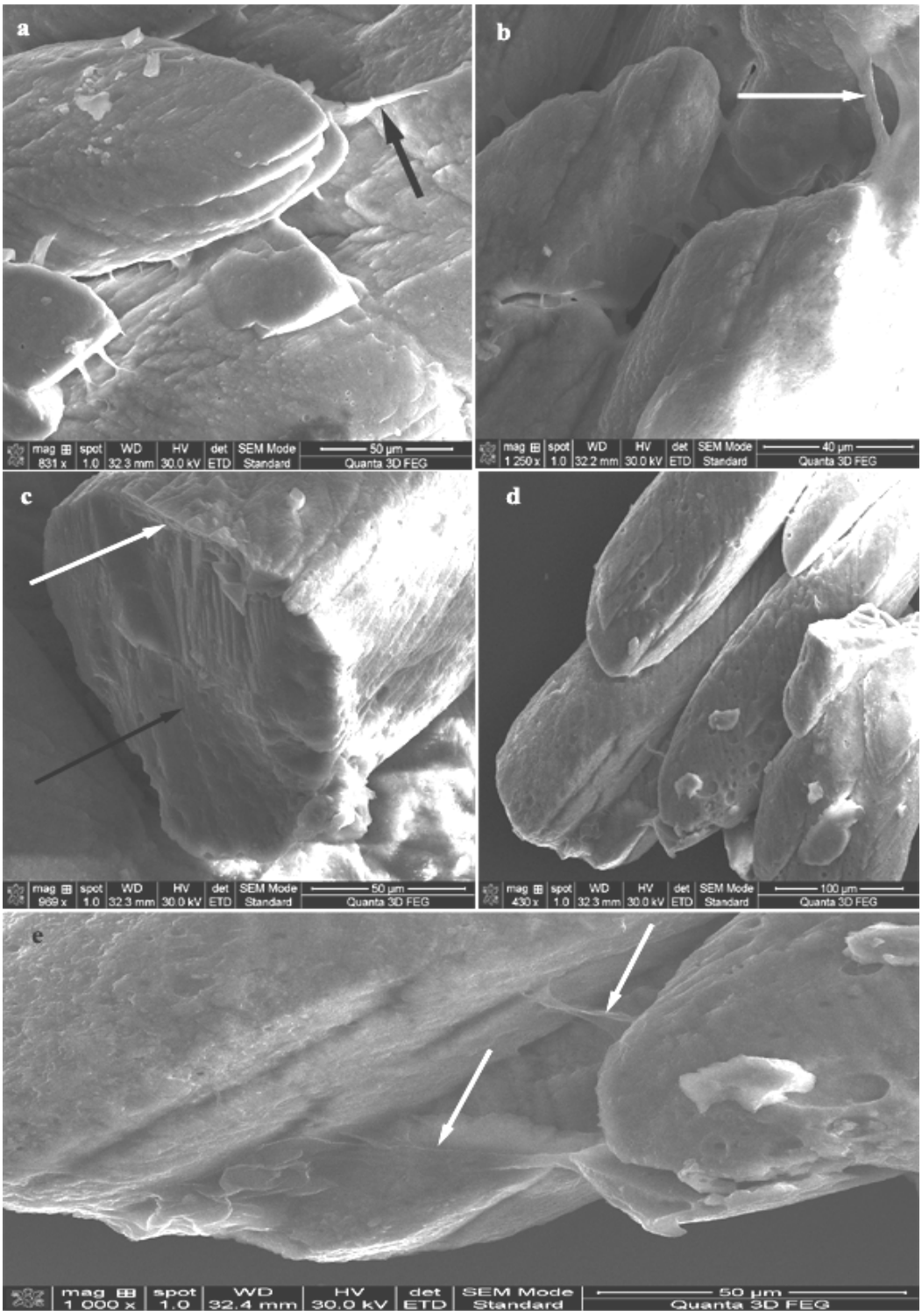

Fig. 13. The S2 stromatolite structure as observed in SEM: **a** - the arrow points to a biofilm rich in EPS and fossilized; **b-c** - biofilm/filaments (white arrows) fossilized around the $CaCO_3$ crystals (black arrows); **e** - arrows showing fossilized microbial biofilms.

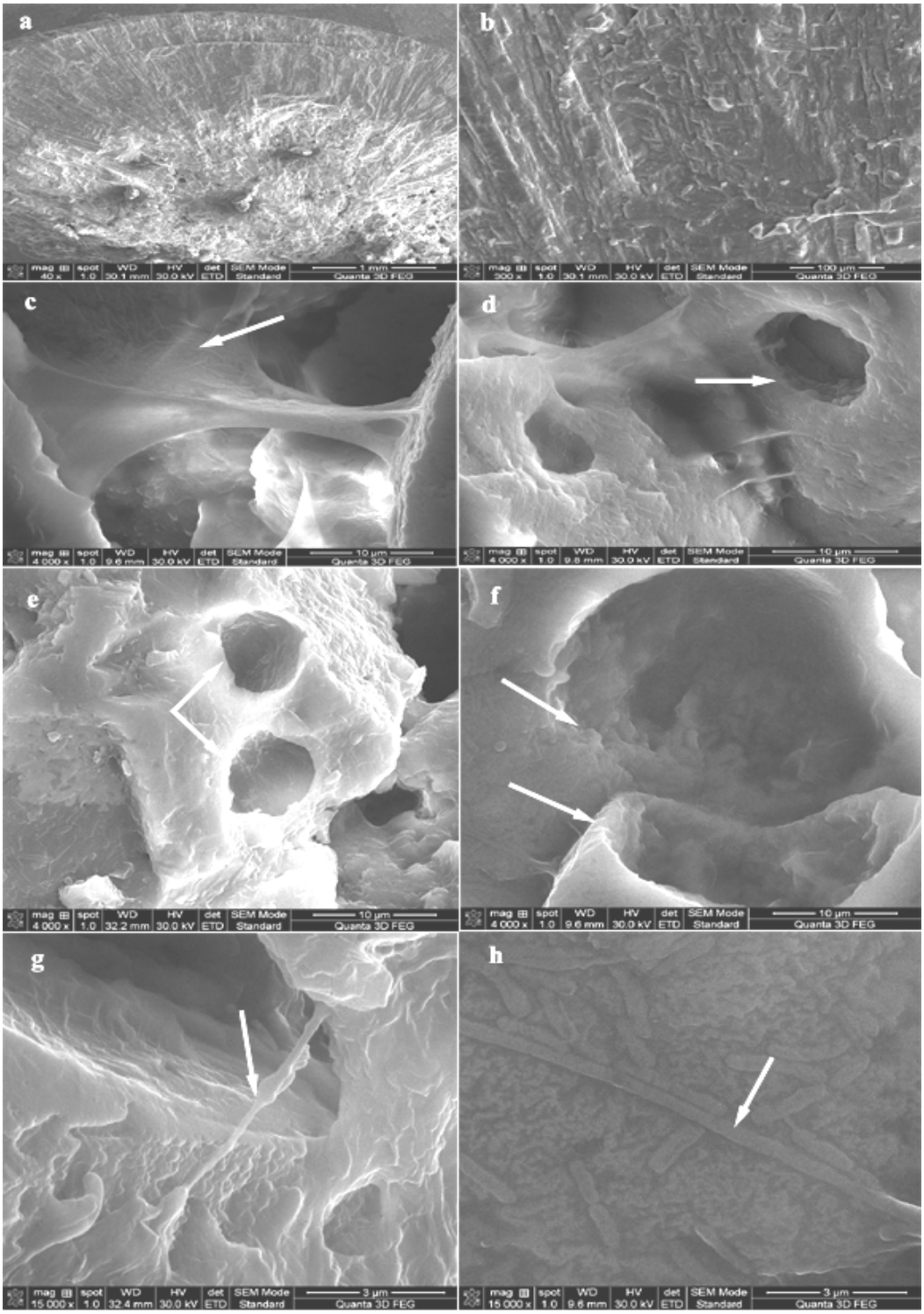

Fig. 14. The S3 stromatolite structure as observed in SEM. **a-b** - sross-section; **c** - fossilized microbial biofilm, rich in EPS; **d-f** - fossilized EPS sorrounding the $CaCO_3$ crystals (removed during sample preparation); **g-h** - filamentous bacteria.

X. Limitations of the study and future work

This study can be continued, following these recommandations:

1) More geothermal springs from the Western Plain of Romania to be taken into consideration for future studies in order to get a better understanding regarding the microbial diversity in time and space.

2) To use other molecular techniques for a better resolution:methods of clone library construction, by the use of group specific primers and by *in situ* studies (for example, by FISH - Fluorescence *In Situ* Hybridization).

3) Physiological studies should be performed using enrichment cultures obtained from these mats in order to see the effect of UV, chemical compounds and other physical or chemical factors on the growth process.

XI. Conclusions

The general idea of this PhD thesis was represented by biodiversity investigation using molecular techniques. The study targeted the cyanobacterial mats associated with certain geothermal (thermomineral) springs resulted after certain geological drillings in the Western Plain of Romania

The results obtained led to the following conclusions:

a. Scanning electron microscopy revealed a multi-layered structure of the investgated mats, with an upper layer consisting in a tight net of cyanobacterial filaments, an inner layer represented by a more relaxed net of filaments and an inner layer, with very few cyanobacteria and an increased number of bacteria.

b. The cyanobacterial diversity in the investigated mats is quite low, with a maximum of 8 cyanobacterial taxa in the Marghita mat; using the 3 techniques (DGGE, ARISA and ARDRA) in the same study led to an increased number of cyanobacterial taxa identified in the investigated mats in comparison with the use of an individual method.

c. Partial 16S rDNA sequences obtained revealed that the majority of cyanobacterial taxa belong to the order Oscillatoriales, the following genera being observed: *Phormidium*, *Oscillatoria, Lyngbya, Leptolyngbya, Plectonema, Geitlerinema* şi *Microcoleus*.

d. The microbialites from Ciocaia (Bihor county) are modern stromatolites, having a major role in the organomineralization and evolutionary studies.

e. The cyanobacterial diversity in microbialites is very low, only the *Leptolyngbya* genus being identified.

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