RESEARCH ARTICLE

Biogeography of thermophilic cyanobacteria: insights from the Zerka Ma'in hot springs (Jordan)

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Abstract

The thermal springs of Zerka Ma'in, with waters emerging at temperatures up to 63 °C, have been of interest to biologists already from the beginning of the 19th century. These waters, springing out from below ground and flowing into the hypersaline Dead Sea, form an isolated environment from a biogeographic point of view. We have investigated the molecular diversity of the cyanobacteria in the springs. The diversity discovered was large, defining operational taxonomic units (OTUs) by a cutoff of 97% similarity; 10 major OTUs were found, including an as yet unidentified cluster of cyanobacteria. The various patterns of similarities of our sequences to others obtained from different thermal environments worldwide led us to rethink the common theories in biogeography. Based on the data obtained, we suggest that there is no constant geographical separation of microorganisms; however, local speciation does occur at a rate dictated mainly by local community dynamics and the rate of entrance of new organisms into the ecosystem.

Introduction

Microbial biogeography has been a subject of intense discussion for the last two decades (Fenchel, 2003; Hughes-Martiny *et al.*, 2006). Baas-Becking (1934), later clarified by de Wit & Bouvier (2006), claimed that 'everything is everywhere BUT nature selects'. On the other hand, based on concepts of genetic speciation, Wright (1931, 1943) stated that given strong enough chemical/physical boundaries that prevent gene outflow, organisms will diverge genetically from their ancestors. However, although these theories contradict each other, the current literature provides evidence of the correctness of both.

Whitaker *et al.* (2003) have shown that populations of *Sulfolobus* sp. from different locations around the world differ from each other genetically. Reno *et al.* (2009) have taken this to the genome level, showing differences between seven isolates of the species *Sulfolobus islandicus* from three worldwide locations. Cho & Tiedje (2000) documented the biogeographic speciation of fluorescent *Pseudomonas* spp. originating from soil. Staley & Gosink (1999) have shown, although at the genus level only, that members of the same genera are found both in the Arctic and the Antarctic; however, they did not sample enough to reach conclusions on the species or the strain level. In freshwater bacteria, Zwart *et al.* (1998) found nearly identical clades of organisms in lakes in North America and in Europe.

In the case of cyanobacteria, Garcia-Pichel *et al.* (1996) have shown that *Microcoleus chthonoplastes* is a cosmopolitan cyanobacterium with a low worldwide diversity. Similar results were shown by Wilmotte *et al.* (1992) regarding marine Oscillatoriacean cyanobacteria. On the other hand, it was shown that for *Synechococcus* spp., a large variation already exists in the same geographic location (Castenholz, 1978; Ward *et al.*, 1990, 1998). This variation was later found to exist at a global scale, with certain groups of *Synechococcus* spp. appearing to be endemic to North America (Papke *et al.*, 2003). Using a large number of *Mastigocladus laminosus* isolates, Miller *et al.* (2007) have shown that despite the low 16S rRNA gene diversity within this species,

geographically related diversity can be demonstrated using multilocus analysis.

In general, studies supporting the 'everything is everywhere' theory have focused on marine and near-marine environments. Genetic speciation has been demonstrated mostly in isolated environments such as hot geothermal springs. Such environments may create an 'Island effect', leading to the divergence of its inhabitants from their ancestors (Papke *et al.*, 2003).

The Dead Sea Rift, being part of the Syrian–African Rift Valley, is rich in thermal springs. Some are freshwater springs, and others are saline or hypersaline. Examples are Hamat Gader (up to 52 °C) and the hot springs of Tiberias (up to 60 °C). The area of the Dead Sea, on the border between Jordan and Israel, contains many springs that differ in their physical and chemical properties. The eastern bank of the Dead Sea is especially rich in thermal springs. These include the springs of Zara on the Dead Sea shore (the ancient Kallirrhoe; Donner, 1963), with temperatures up to 59 °C, and the hot springs of Zerka Ma'in, 5 km inland, up to 63 °C (Abu Ajamieh, 1980; Ionescu *et al.*, 2007).

The thermal springs of Zerka Ma'in have attracted biological research in the past. Their biology was examined already in the beginning of the 19th century and later in the beginning of the 20th century (Seetzen, 1854; Blanckenhorn, 1912). Although the chemical and physical properties of the springs were analyzed recently (Abu Ajamieh, 1980, 1989; Swareih, 2000), the first recent biological survey was held in 2006 (Ionescu *et al.*, 2007), providing a microscopic description and a preliminary phylogenetic analysis of the cyanobacteria in the springs.

In this study, we attempted to gain a better understanding of the molecular diversity among the cyanobacteria in the Zerka Ma'in springs. Additionally, we suggest new ideas to solve issues in biogeography that arose during the Zerka Ma'in data analysis.

Materials and methods

Sampling site

The Zerka Ma'in springs are located 5 km inland on mountains on the east coast of the Dead Sea. The temperature of the various springs ranges between 39 and 63 °C. We collected samples from three different sites. Site A consists of two pools, the upper one flowing into the lower one through a pipe. The temperature of the upper one was 63 °C during all the sampling expeditions. The temperature of lower pool ranged between 62 and 63 °C. The upper pool is shallow (~50 cm) and is surrounded by rock walls. Green mats are found at and slightly above the water–air interface on the rocks. Submerged rocks are covered by mats as well. The lower pool is deeper, larger and surrounded by vegetation. Site B is located ~500 m west of site A. The water comes out through a series of pipes into a 50-m channel that ends in a waterfall. The temperature of the water is 59 °C along the entire channel. Green mats are found on the channels' earth banks as well as on submerged rocks. An orange mat is often found beneath the green mat. Site C is a stream located 50 m above site A. The main stream is a combination of two smaller ones at a temp of 25 and 51 °C. Over a distance of 25 m from the confluence point, the temperature of the stream reaches 39 °C. Site C was sampled only once.

Sampling procedure

Cyanobacterial mats were collected from sites A, B and C on November 16, 2006 and June 3, 2007. Sampled mats were either submerged or in contact with water at the air–water interface. Samples were placed in 15-mL sterile tubes containing 2 mL of lysis buffer (8 M guanidine HCl, 20 mM EDTA, 20 mM MES and 50 mM β-mercaptoethanol), transferred to the lab in liquid nitrogen and placed at −80 °C until further processing.

DNA extraction

Two milliliters of buffered phenol solution (pH 7; Sigma) was added to each sample and the tubes were placed at 60 °C for 30 min with manual mixing every few minutes. The water phase was extracted with 2 mL of chloroform : isoamylalcohol (24 : 1) and phase separation was obtained by 5 min of centrifugation at 10 000 ***g***. The aqueous phase was transferred to a fresh tube and re-extracted with 4 mL of chloroform : isoamylalcohol (24 : 1), followed by centrifugation. The aqueous phase was transferred to a fresh tube and the DNA was precipitated with 1 volume of 2-propanol and 0.01 volume of 3 M Na-acetate (pH 5.5) for 30 min at −80 °C. Following a 30-min high-speed centrifugation, the liquid phase was discarded and the DNA pellet was washed with 500 μL of ice-cold 70% ethanol. The air-dried DNA was dissolved in 50 μL of autoclaved miliQ water.

PCR and cloning conditions

The PCR was carried out using a ready-made mastermix (Larova). The DNA was added to a final concentration of 1 ng μL^{-1}. The 16S rRNA gene cyanobacterial-specific primers 106F and 781a/bR (Nübel *et al.*, 1997) were added at a final concentration of 0.5 μM. The DNA was amplified in a BIOER thermocycler, starting with 5 min of initial denaturation at 95 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 60 °C and 45 s at 72 °C. The reaction was terminated with a 10-min final elongation step. The samples were analyzed on a 1.5% agarose gel. Positive amplicons were inserted into cloning vectors using the INSTAclone cloning kit (Fermentas) according to the manufacturer's instructions. Half of

each ligation reaction was used to check the success of the process. Successful reactions were sent for cloning and sequencing at the Genome Sequencing Center at Washington University, St. Louis, MO. To obtain reliable results, each clone was sequenced in both directions using the M13 forward and reverse primers. Each individual sequence used for the phylogenetic analysis is the result of two aligned sequences from the same clone.

Phylogenetic analysis

All sequences were compared with the NCBI nr database using the NetBlast application (available from NCBI). The top five hits as well as some additional relevant sequences were used for phylogenetic analysis. Sequences were aligned using the MUSCLE 3.6 software (Edgar, 2004).

We used the JMODELTEST software (Posada, 2008) to select the best-fitting model for distance calculation. Out of 88 possible combinations, the TNef model (Tamura–Nei with equal frequencies; Tamura & Nei, 1993) was found to be most suitable. Because JMODELTEST uses Phyml (Guindon & Gascuel, 2003), a maximum likelihood-based program for its analysis, and Tamura *et al.* (2004) have shown the accuracy of this method, we chose this method for the construction of trees. The maximum composite likelihood method, as implemented in MEGA 4.0 software (Tamura *et al.*, 2007), uses the TNef model for distance calculation, thus fulfilling the criteria of the JMODELTEST analysis. The validity of tree topology was evaluated using the bootstrap method (1000 replicates).

Rarefaction curves and operational taxonomic units (OTUs) were calculated with the DOTUR software (Schloss & Handelsman, 2005) using 301 sequences and the nearest-neighbor algorithm.

Results

Diversity analysis

Samples collected during two different expeditions yielded a total of 293 sequences. To analyze the extent of sampling, a distance matrix for these sequences together with eight isolated strains (Ionescu *et al.*, 2007, 2009) was calculated and rarefaction curves were constructed (Fig. 1). OTUs were defined at a cutoff of 97% similarity. This choice is supported by comparison of the BLASTN results obtained from the various sequences as well as by phylogenetic tree topologies obtained with various algorithms. At the chosen cutoff, a total of 15 OTUs were recognized (Table 1), of which 10 can be ascertained either by finding multiple clones or by the existence of isolates described previously in Ionescu *et al.* (2007, 2009). The remaining five OTUs, which all contain only one sequence with 85–97% similarity to the sequences in the NCBI nr database, were probably due to a shorter sequence and are not shown.

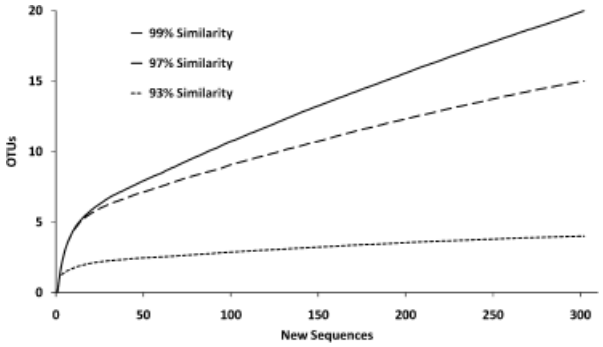

Fig. 1. Rarefaction curves at different similarity cut-off values. The distance matrix was calculated using the maximum composite likelihood method as implemented in the MEGA 4.0 software (Tamura *et al.*, 2007). The curves were calculated using the DOTUR software (Schloss & Handelsman, 2005).

Phylogeny

For a general overview of the phylogenetic groups represented in the Zerka Ma'in springs, we have plotted all obtained clones as a phylogenetic tree (Fig. 2). The five large OTUs (1–5 as shown in Table 1) can be seen by the large clusters of filamentous and unicellular cyanobacteria in the tree. Three clusters can be found within the *Oscillatoriales* and one large cluster in the *Chroococcales*. The phylogenetic affiliation of an additional cluster (marked as unknown) could not be assigned. This cluster is only 97% similar to three sequences of uncultured cyanobacteria (AF445667, AF445673 and AF445691) from Mammoth Hot Spring, Yellowstone National Park. The other OTUs were scattered throughout the tree. OTU 9 does not cluster with any other sequence, and its closest known relatives in the database are an uncultured organism from a Hawaiian lava mat and *Pseudanabaena tremula* UTCC 471, both with a maximal identity of 93%. OTU 10 clusters within a member of the *Leptolyngbya* group. OTU 8, represented only by isolates, forms a unique cluster with a newly identified unicellular cyanobacterial clone from a marine environment in Portugal and with *Chroogloeocystis siderophila*, an iron-dependent thermophilic cyanobacterium (Brown *et al.*, 2005).

As the *Synechococcus* group has been a subject for major discussion in terms of biogeography, we carried out an in-depth study of the phylogeny of these organisms in the Zerka Ma'in springs (Fig. 3). The majority of the sequences of *Synechococcus* spp. from the Zerka springs form a unique cluster within the *Synechococcus* sp. C1 (Ward *et al.*, 1990) lineage and contains representatives of similar organisms from Costa Rica and various parts of Asia. The C9 lineage of

Table 1. Zerka Ma'in OTUs resolved at a cutoff of 97% similarity out of 301 sequences

	Number of sequences					
OTU	Environmental	Isolates	Type of cell	Phylogenetic affiliation	Highest similarity to database (%)	T (°C)
1	108	0	Unicellular	*Synechococcales*	98	39–63
2	43	3	Filamentous	*Oscillatoriales*	98	59–63
3	29	0	Filamentous	*Oscillatoriales*	98	59–63
4	75	0	Filamentous	*Oscillatoriales*; *Pseudanabaenaceae*	97	59
5	32	0	Unknown	Unknown	97	59–63
6	1	2	Filamentous	*Stigonematales*	99	59–63
7	2	0	Unicellular	*Synechococcales*	99	59
8	0	2	Unicellular	*Chroococcales*	98	59
9	0	1	Filamentous	Unknown	93	59
10	0	1	Filamentous	*Oscillatoriales*; *Leptolyngbya*	99	59

Analysis was conducted using the DOTUR software (Schloss & Handelsman, 2005). Phylogenetic affiliation is shown to the point of confident identification. The highest similarity to the NCBI database refers to the similarity of at least one member of that specific OTU to known sequences in the database. The similarity values are rounded down to the closest integer value.

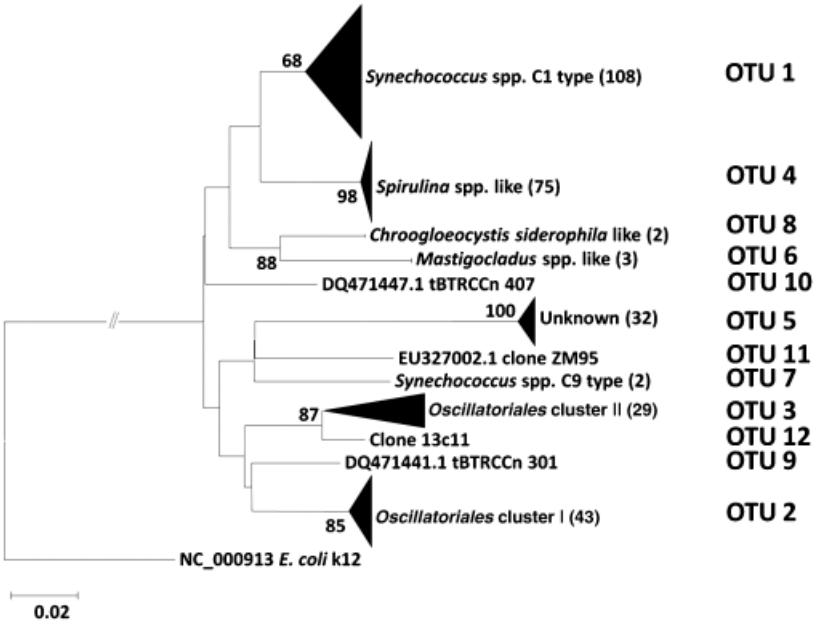

Fig. 2. A phylogenetic tree including all obtained sequences from Zerka Ma'in (isolates as well as environmental samples). The tree was rooted with *Escherichia coli* K-12. The topology of the tree was tested with the bootstrap method using 1000 replicates.

Synechococcus sp. (Ward *et al.*, 1990) is represented only by two sequences. No representatives of the A/B lineage were found in the Zerka Ma'in springs; however, two sequences from Costa Rica and four sequences from Tibet cluster together with this group (Fig. 3).

The *Oscillatoriales* group in the Zerka Ma'in springs is highly diverse and contains three large clusters and some less commonly encountered organisms. To better observe the diversity of this group, we plotted it separately (Fig. 4). The cluster representing OTU 4, which contains only *Spirulina*-like sequences, is presented in Fig. 5. Additional sequences from this group either form individual clusters or show some similarities to members of the *Leptolyngbya* group (Fig. 5a). Cluster I of the *Oscillatoriales* (OTU 2) contains all three isolates obtained from this group and has some inner heterogenicity; Cluster II (OTU 3) is less diverse and shows no specific inner organization.

The *Spirulina*-like cluster of the *Oscillatoriales* (OTU 4) was plotted against all known *Spirulina*-like sequences from the databases (Fig. 5). It forms a separate cluster, with the exception of two sequences located apart from the main cluster. An inner clustering can be observed in this group as well (data not shown); however, it is not as pronounced as in cluster I of the *Oscillatoriales*.

The *Mastigocladus*-like group represented by two isolates and one environmental sequence were plotted against

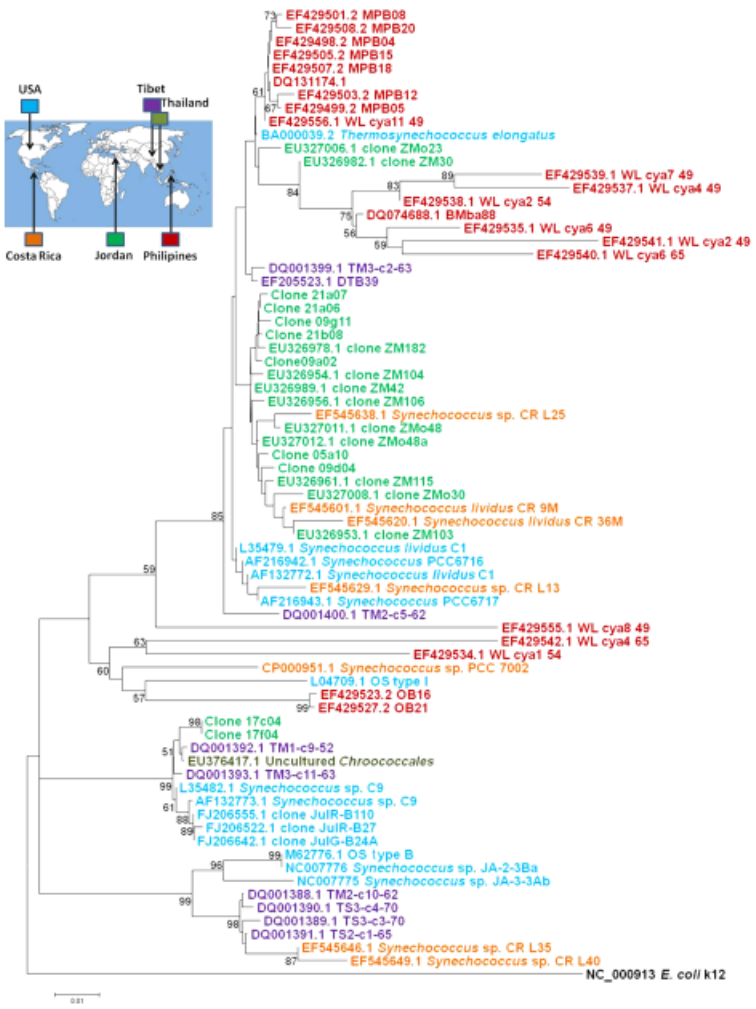

Fig. 3. A phylogenetic tree including all obtained *Synechococcus* sp. sequences from Zerka Ma'in together with relevant sequences from the database. The tree was rooted with *Escherichia coli* k12. The various sequences are color coded according to their origin as described in the global map legend. The topology of the tree was tested with the bootstrap method using 1000 replicates.

similar sequences from Costa Rica (DQ786169-172), which represent the closest BLASTN match, as well as other sequences previously grouped by a multilocus analysis into seven distinct groups (Miller *et al.*, 2007). Interestingly, the isolates clustered with group IV, while the environmental sequence, together with a sequence from Costa Rica, which is not among the BLASTN top hits for the isolates, form a separate group, which we designated group VIII (Fig. 6).

All phylogenetic trees in Figs 3–6 are color coded according to the place of origin of the sequence. To create clearer tree images, identical sequences have been removed or clustered wherever possible.

Discussion

Our current phylogenetic analysis of the cyanobacteria in the springs reveals a diverse community of filamentous and unicellular types and sheds new light on the identity of the cyanobacteria that could not be obtained by culture-dependent studies.

Previous studies in thermal springs have shown an increasing diversity with decreasing temperature (Kullberg, 1968; Brock, 1978; Castenholz, 1978; Miller & Castenholz, 2000; Sompong *et al.*, 2005). As far as cyanobacteria are concerned, this appears not to be the case for the Zerka Ma'in springs (Table 1). With the exception of the *Spirulina*-like OTU 4, all the major clusters (OTUs 1, 2, 3 and 5) are cosmopolitan with respect to temperature. With the exception of temperature, the chemical composition of the different sampling sites does not differ considerably (Ionescu *et al.*, 2007). This may be due to the common source of the waters or due to the fact that the springs may merge through underground passages. If so, given the slightly higher elevation of site A (63 °C), organisms surviving there may be constantly delivered to site B (59 °C), thus masking any temperature speciation.

The springs of Zerka Ma'in represent an interesting site for cyanobacterial biogeographical observation as they are completely isolated from their environment. The source of the water is local within the rift valley and is not expected to

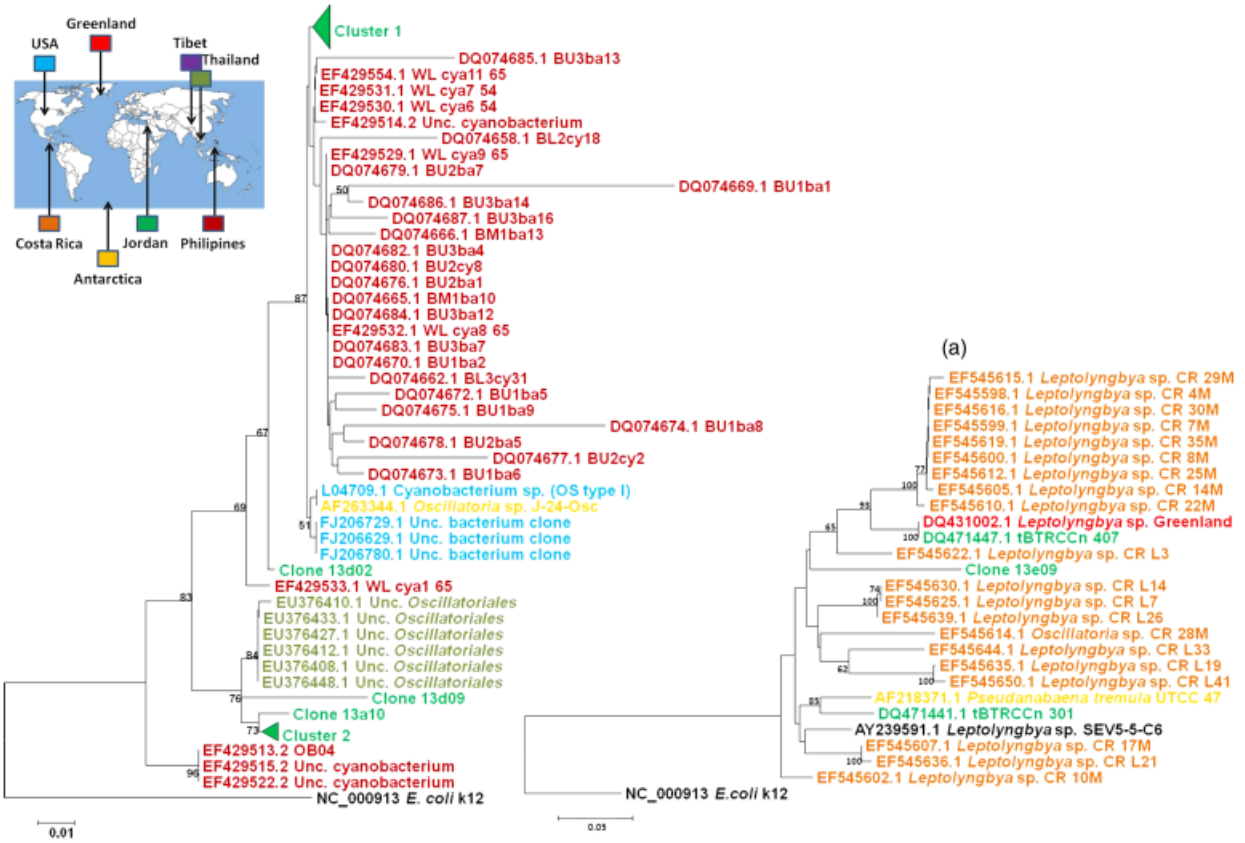

Fig. 4. A phylogenetic tree including all obtained *Oscillatoriales* sequences that do not match *Spirulina*-like sequences from Zerka Ma'in, together with relevant sequences from the database. The tree was rooted with *Escherichia coli* k12. The various sequences are color coded according to their origin as described in the global map legend. The topology of the tree was tested with the bootstrap method using 1000 replicates. The inset (a) shows the phylogeny of OTUs 9 and 10, together with relevant sequences from the database.

carry phototrophs from other springs. The waters flow into the extremely saline Dead Sea, which, besides not providing these organisms with a suitable environment for survival, is a terminal lake not connected to any other water basin. Dust storms that occur in desert areas are known to carry up to 10^{18} microorganisms per year globally (Griffin *et al.*, 2002). Transport of organisms from the Zerka Ma'in springs through the air is possible; however, as these cyanobacteria are probably not particle-associated organisms, but can only become airborne following water evaporation, their distribution distance is believed to be limited to ~1 km (Bovallius *et al.*, 1980; Kellog & Griffin, 2006).

The cyanobacteria identified by us in the Zerka Ma'in springs do not follow a single pattern when compared with similar organisms from different thermal sites in the world. Some clones and isolates showed low similarities to those from other environments (85–97%), while others showed over 99% similarity to their counterparts.

One of the best-documented genera of thermophilic cyanobacteria is the genus *Synechococcus* (Ward *et al.*, 1990, 1998; Miller & Castenholz, 2000; Ramsing *et al.*, 2000; Papke *et al.*, 2003). Papke *et al.* (2003) described the global distribution of the *Synechococcus* sp. lineages C1, C9 and A/B. The C9 lineage was the only lineage found in most sampling sites, with the exception of northern Italy, while the A/B cluster was found only in North America. The *Synechococcus* spp. from Zerka Ma'in cluster mostly with the C1 lineage, together with some other clones from Asia and Costa Rica, but forming a separate group from the Octopus springs isolates (Fig. 3). The C9 lineage was found in the springs, albeit in low numbers, supporting its global distribution (Fig. 3). Additional clones from Costa Rica and the Philippines cluster within the A/B lineage (Fig. 3). Although forming a separate group within this cluster, the existence of organisms from the A/B can no longer be attributed solely to North America. No physical or chemical data are available for the 'Las Lilas' geothermal spring from which these sequences originate, and therefore no comparison can be made between the two ecosystems.

Synechococcus spp. are not the only group of sequences that show high (> 99%) similarity to isolates from the Rincon de Vieja volcano area (Costa Rica). Isolates tBTRCCn 101 and tBTRCCn 403 (Ionescu *et al.*, 2007) and clone ZM 21e12 (OTU 6) belonging to the *Stigonematales*

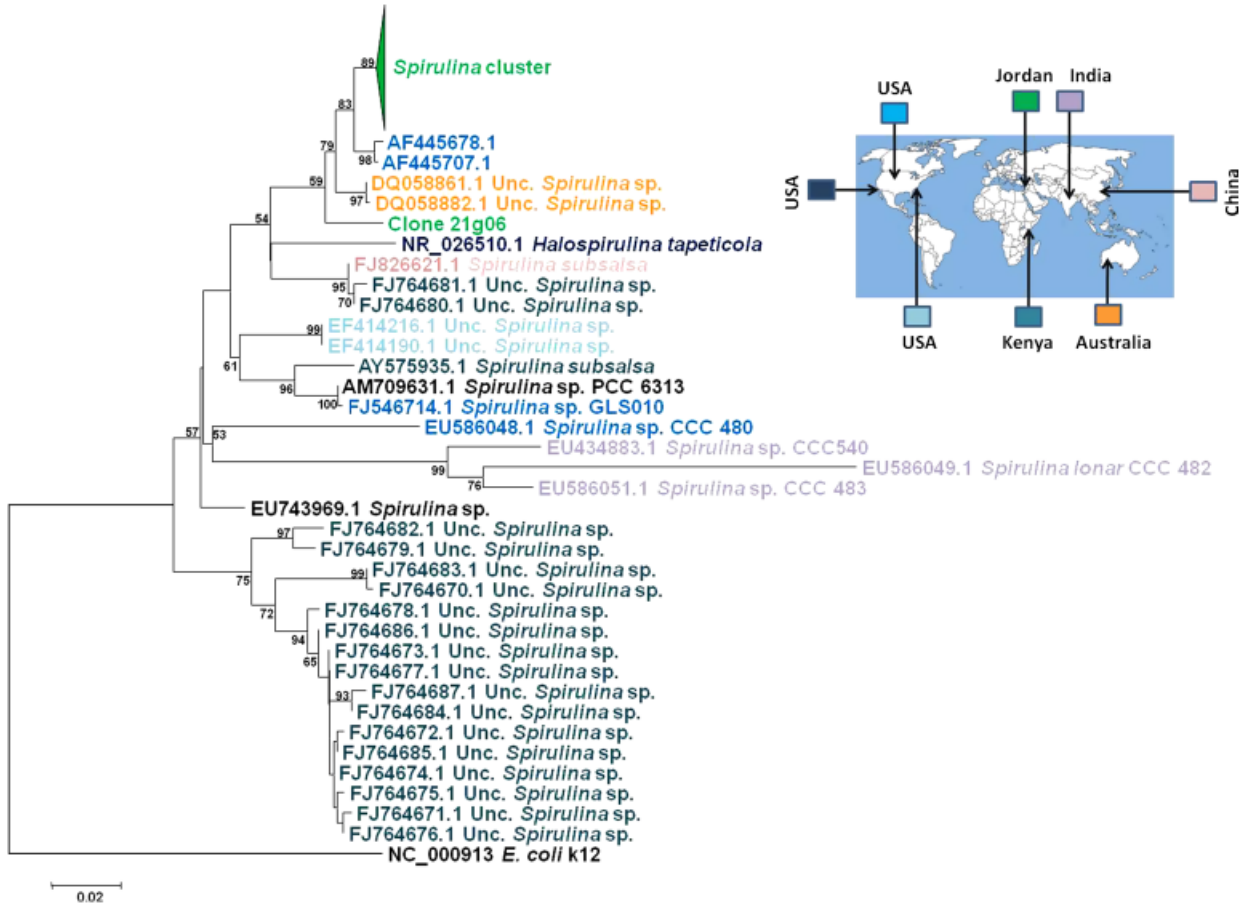

Fig. 5. A phylogenetic tree including all obtained *Spirulina*-like sequences from Zerka Ma'in, together with relevant sequences from the database. The tree was rooted with *Escherichia coli* K-12. The various sequences are color coded according to their origin as described in the global map legend. The topology of the tree was tested with the bootstrap method using 1000 replicates.

show the best BLASTN matches with *Fischerella* sp. from the same area (Finsinger *et al.*, 2008). This group is not represented in the microscopic analysis of the microbial mats from the springs (Ionescu *et al.*, 2007) and is poorly covered by the clone library. However, a large number of the enrichment cultures set up from the springs have been rapidly taken over by members of this group. We have plotted our clone and isolates together with the matching sequences from Costa Rica as well as all the isolates used for the biogeographic analysis by Miller *et al.* (2007). Although Miller *et al.* (2007) have used multilocus analysis for the validation of the seven different *M. laminosus* groups, all clusters were obtained using only 16S rRNA gene sequences (Fig. 6). The Costa Rican sequences obtained from Finsinger *et al.* (2008) form a separate cluster (IV-a) close to group IV as identified by Miller *et al.* (2007). It is important to mention that the sequences used by Miller *et al.* (2007) are shorter, and therefore the Costa Rican isolates may still belong entirely to group IV. Other sequences from Costa Rica obtained in different studies fall mostly within group VI, while one forms a new group together with Clone 21e12. Looking at the geographic location of the isolates from group IV, as well as that of the organisms in the other groups, they do not originate from one unique location and furthermore, cannot be, separated into subgroups using multilocus analysis (Miller *et al.*, 2007). The addition of sequences from the isolated springs of Zerka Ma'in as well as springs from Costa Rica to these groups and others weakens the support for a site-specific speciation of *M. laminosus*. Furthermore, Miller *et al.* (2007) have used isolates for their analysis. It has been shown in the past both for bacteria in general (Liesack & Stackebrandt, 1992; Barns *et al.*, 1994) and for cyanobacteria (Ward *et al.*, 1990, 1994; Ferris *et al.*, 1996) that cultivated organisms do not represent the natural diversity in the environment. This is also demonstrated by the difference between the *Mastigocladus*-like isolates obtained from Zerka Ma'in (tBTRCCn 101, 403) and the clone obtained from the environmental samples (Fig. 6).

The *Oscillatoriales* in the springs are extremely diverse with three OTUs (Figs 2 and 4). Cluster II (Fig. 2) and the

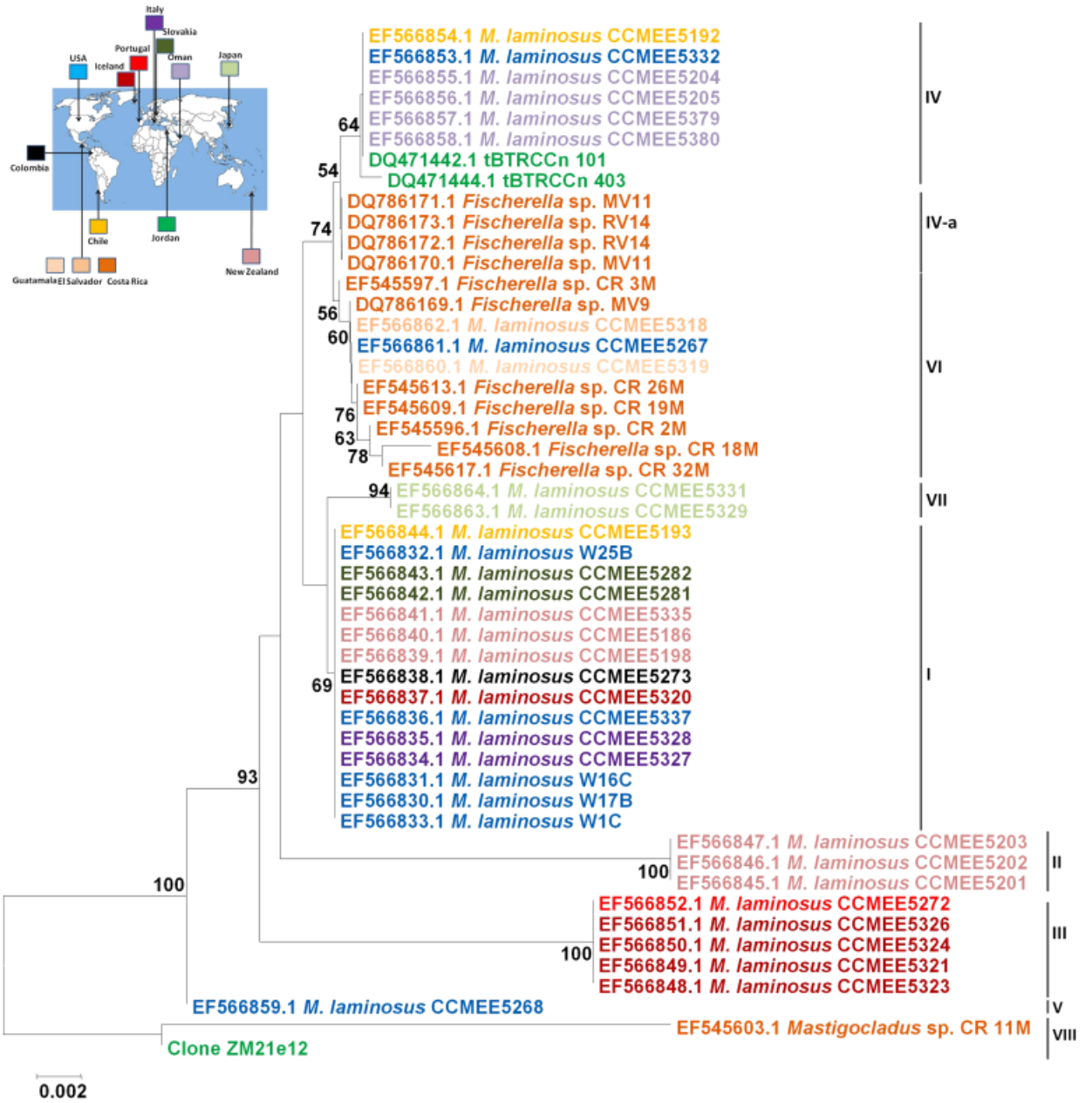

Fig. 6. A phylogenetic tree including all obtained *Mastigocladus*-like sequences from Zerka Ma'in, together with relevant sequences from the database. The various sequences are color coded according to their origin as described in the global map legend. To reduce confusion, all sequences deriving from the USA were colored with the same color. The topology of the tree was tested with the bootstrap method using 1000 replicates.

Spirulina sp. group (Fig. 5) show a very low inner diversity and have no isolated representative. Cluster I, on the other hand consists of two groups, each containing both isolates and uncultured organisms. Although this group forms a separate branch, it clusters together with sequences from the Philippines and from Yellowstone National Park. Additional sequences representing OTUs 9 and 10 cluster with Arctic and Antarctic organisms, respectively, and belong to the ambiguous *Leptolyngbia* group. Cluster III, representing *Spirulina*-like organisms, shows only 97% similarity to known sequences in the database, and clusters separately from any known *Spirulina*-like organisms. Several other isolates and clones that do not fall into the large clusters show high similarity to sequences from other thermal locations in the world (Figs 4 and 6).

The last group of organisms represents a newly emerging group of isolates that clusters with *C. siderophila* (Brown *et al.*, 2005). Although not represented in our clone library,

this group was dominant in the slow-flowing part of site B during our preliminary survey in 2006 (Ionescu *et al*., 2007). As only four sequences from this group are now present in the database, insufficient data exist for a biogeographic analysis of this group. One member of this group (FJ589716) originates from a mesophilic, marine environment, while the other members are freshwater thermophiles. Among the latter, *C. siderophila* was isolated from an iron-rich spring and requires 30 μM iron for growth (Brown *et al*., 2005), while the Zerka Ma'in springs contain iron levels ranging from 0.4 μM (Khoury *et al*., 1984) to 2.2–3.6 μM (Rimawi & Salameh, 1988). Given the wide range of environments covered by this group, on the one hand, and the small numbers of representatives found so far, on the other, this group may represent a generalistic cyanobacterium that grows in low abundance together with more specialized organisms.

The isolated springs of Zerka Ma'in pose a biogeographic conundrum hosting both organisms highly similar to others in thermal environments in the world and endemic species with only distant relatives. Looking back on the theories of Baas-Becking (1934) and of Wright (1931, 1943), it is our opinion that the nature of the cyanobacteria found in the Zerka Ma'in springs can only be explained by a combination of both theories. In general, the distribution of microorganisms around the globe should not be considered in terms of the present situation, but rather based on the ability of each organism to be relocated to a new environment by any means at a given time (past, present or future). Once an organism is present in the respective environment as a cryptic species, its speciation will depend on several factors: (1) the separation of the new environment from the original source, affecting the rate of supply of new 'original-like' organisms and (2) the local microbial (cyanobacterial) dynamics, affecting the time an organism has to evolve.

The case of the Zerka Ma'in springs is a good example of relocation of organisms. The 1–3% genetic divergence of some of the species from their known relatives suggests a physical separation of 50–150 million years, considering that a rough value of 1% difference equals 50 million years (Ochman *et al*., 1999). However, the geological history of the area does not support these numbers. The Dead Sea itself was formed only 3 million years ago (Katz & Starnisky, 2009; Torfstein *et al*., 2009), after which the Zerka Ma'in area, located currently at ∼–70 m b.s.l., was submerged for a long period. The exact period when the Zerka Ma'in springs were formed is not known; however, it is unlikely that they existed before or soon after the formation of the Dead Sea as the topographic depression of the basin was just starting to form (A. Torfstein, pers. commun.). Therefore, although the organisms found in the springs of Zerka Ma'in may have been separated from their ancestors over 50 millions of years ago, they were moved to their present location at a later stage.

Throughout the course of time, organisms have several chances of being relocated to new environments, thus eventually reaching all corners of the planet. Once in a new environment, as long as the organism manages to leave the state of cryptic species, it will start multiplying, accumulating random neutral or beneficial mutations. In case the rate of multiplication is slower than the new arrival of organisms from the original source, some speciation may still occur within the new 'local' community, but it may be undetectable. Finally, the local dynamics of the microbial community may lead to a noncontinuous growth pattern of the 'new' community and a similar speciation rate. The sum of all these processes will appear upon sampling as groups inhabiting the same ecosystem, but behaving differently from a biogeographic point of view. We lack sufficient information regarding the uniformity of the acquisition of mutations among different microorganisms, a process that surely affects the final picture obtained at the time of sampling.

In conclusion, we suggest, based on our analysis of the complex community of the Zerka Ma'in springs, a combined theory to explain the nonuniform biogeographic patterns occurring within an individual sampling site.

Acknowledgements

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