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4 **Fingerprinting *Chamaesiphon* populations as an approach to**  
5 **assess the quality of running waters**  
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7 *Running title: Fingerprinting Chamaesiphon populations for monitoring rivers*  
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## Abstract

Cyanobacterial communities are highly diverse in freshwaters and respond rapidly to changing environments. Previous studies have highlighted variations in the structure and composition of epilithic cyanobacterial communities in response to eutrophication in watercourses. In the present study, changes in benthic cyanobacterial communities from Guadalix River (Spain) biofilms were examined using temperature-gradient gel electrophoresis (TGGE) in conjunction with microscopic examination of field-fixed samples, focusing on populations of one of the dominant cyanobacteria: *Chamaesiphon*. Environmental characteristics were determined in order to characterize the trophic status of the sampling sites. The presence of cyanobacteria in the river was determined from complex TGGE patterns, band extraction, and subsequent sequencing of 16S rDNA gene fragments. The microscopic observations revealed that the unicellular genus *Chamaesiphon* and the filamentous genus *Phormidium* were dominant in the studied locations. Within the 2 genera, 4 *Chamaesiphon* populations were identified (*Chamaesiphon fuscus*, *Chamaesiphon starmachii*, *Chamaesiphon subglobosus*, and *Chamaesiphon polymorphus*) and *Phormidium* was represented at the sampling sites by the *Phormidium autumnale* morphotype. TGGE banding patterns differed among samplings sites as a function of water quality. The genetic analysis revealed 4 phylotypes within the genus *Chamaesiphon* and 1 phylotype within the classic *P. autumnale* clade. *Chamaesiphon* phylotypes were not equally distributed in all the sampling locations. Some phylotypes were related to low nutrient concentrations, while others were associated with eutrophic conditions. Our results support the use of fingerprints of *Chamaesiphon* populations obtained by TGGE to examine changes in water quality.

46 Keywords: cyanobacterial populations, biomonitoring, eutrophication, EU Water  
47 framework directive, 16S rRNA gene, *Phormidium*, *Chamaesiphon*, TGGE.  
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## 1 | INTRODUCTION

Monitoring applications that use algae are based on information from studies of community composition and biomass, which have preferentially considered diatoms over other groups. However, since algae are a single assembly in which all groups are equally relevant and informative with respect to assessing the ecological status of rivers, cyanobacteria have been used to monitor and assess environmental contamination or long-term changes (Mateo, Fernández-Piñas, Perona, & Sabater, 2014; Mateo, Leganés, Perona, Loza, & Fernández-Piñas, 2015; Monteagudo & Moreno, 2016). *Chamaesiphon* populations are one of the dominant cyanobacteria in many streams and springs (Cantonati, Komárek, & Montejano, 2015; Eugen Rott & Pfister, 1988), and these populations have been used as indicators of pollution, principally eutrophication and acidification (Gutowski, Foerster, & Schaumburg, 2004; E. Rott & Schneider, 2014; Schneider & Lindstrøm, 2011). However, studies based mainly on the accurate taxonomic identification of the individuals found are often time-consuming, and difficulties frequently arise when trying to identify species that exhibit extreme morphological variability. Therefore, molecular methods have recently begun to be developed that can overcome many of these difficulties, and thereby enable the changes in the communities due to differences in water quality to be analysed.

Temperature-gradient gel electrophoresis (TGGE) is a PCR-based tool that has been used to analyse the diversity of microbial assemblages in different environments. Previous studies (Loza, Perona, & Mateo, 2013; Rodriguez, Aguirre de Cárcer, Loza, Perona, & Mateo, 2007) have used this technique to measure changes in cyanobacterial diversity along a pollution gradient in a river and compared the results with field-fixed and cultured samples obtained by optical microscopy. TGGE revealed a characteristic pattern of bands, or fingerprint, of each sampling site that implied a straightforward relationship

between cyanobacterial composition and water quality. The study reported here evaluates the broader applicability of the approach within the scope of a biomonitoring programme for ecological status evaluation, focusing on populations of one of the dominant cyanobacteria: *Chamaesiphon*. To this end, biofilms of benthic cyanobacterial communities from Guadalix River (Spain) were examined using TGGE in conjunction with microscopic examination of field-fixed samples. Environmental characteristics were measured to characterise the trophic status of the sampling sites. The presence of cyanobacteria in the river was determined from complex TGGE patterns, band extraction and subsequent sequencing of 16S rDNA gene fragments. The aim of this study was to test the fingerprinting of *Chamaesiphon* populations as an approach for assessing the quality of running waters.

## **2 | MATERIAL AND METHODS**

### **2.1 | Site description and sample collection**

The Guadalix River is a tributary of the larger Jarama River and is located in central Spain, near the city of Madrid, in the Tajo Basin. The geomorphological conditions of this basin encompass large granite-dominated highlands, dominated by uplifted blocks of siliceous materials. The river rises at an altitude above 1,790 m in the southern drainage area of the Guadarrama Mountains and flows into the Jarama River at an altitude of 600 m. The region experiences a typical continental Mediterranean climate, characterised in the high mountains by cold winters and high summer temperatures. At least 80% of the precipitation falls during the three winter months. The flow is about  $1 \text{ m}^3 \text{ s}^{-1}$  after the thaw and does not exceed  $0.60 \text{ m}^3 \text{ s}^{-1}$  the rest of the year. Sampling sites were selected above and below populated areas. Sampling site 1 (UTM 30TVL36841977) is unaffected by human influence and is located upstream at 1500 m, above a populated industrial area;

site 2 (UTM 30TVL40211599) 7 km from site 1, and site 3 (UTM 30TVL48260500), 22 km downstream, are near the confluence of the Guadalix and Jarama Rivers and receive industrial and domestic sewage from the nearby human settlements, San Agustín de Guadalix (Loza, Perona, et al., 2013; Munoz-Martin, Martinez-Rosell, Perona, Fernandez-Pinas, & Mateo, 2014).

Samples of water were taken in 1-l wide-mouthed polyethylene jars and kept cool in the dark. Epilithic cyanobacterial samples were collected from three stones at each sampling location. The attached cyanobacteria were removed by brushing the upper side of each stone. Subsamples were separated for microscopic examination (fixed with formaldehyde at a final concentration of 4%) and molecular analysis (frozen). Nutrient chemical measurements were made in the field within 1 h of collection using a DRELL 2010 portable laboratory (Hach Company). Water was sampled early in the morning, and each sample was analysed in triplicate.

## **2.2 | Water analysis**

Dissolved oxygen, temperature, pH and conductivity were measured *in situ* at every sampling site. The following electrodes were set up for use on the riverbank: conductivity, Crison CDTM-523 (Barcelona, Spain); temperature and dissolved oxygen, WTW OXI196 (Wissenschaftliche Technische Werkstetten, Weilheim, Germany) and pH, WTW pH-96.

Calcium and nutrient chemical concentrations ( $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ ,  $\text{PO}_4^{3-}\text{-P}$ ) were determined by colorimetric methods, adapted from Standard Methods for the Examination of Water and Wastewater (APHA, 1992), as previously described (Perona, Bonilla, & Mateo, 1999). Calcium was estimated volumetrically with a Cal Ver 2 Calcium indicator (detection limit 10 mg l<sup>-1</sup>), titrating with 0.08 M EDTA in alkaline medium.

Ammonium-N was measured by the Nessler method (estimated detection limit 0.05 mg l<sup>-1</sup>, precision ± 0.015 mg l<sup>-1</sup>), reading absorbance at 425 nm. When values were close to the detection limit, the salicylate method was used (detection limit 0.01 mg l<sup>-1</sup>), reading absorbance at 655 nm. NO<sub>3</sub><sup>-</sup>-N was analysed using a modification of the cadmium reduction protocol, using gentisic acid instead of 1-naphthylamine (estimated detection limit 0.5 mg l<sup>-1</sup>), reading absorbance at 500 nm. Samples below that detection limit were measured by a low-range method, which is an expanded modification of the former, using a chromotropic acid indicator (estimated detection limit 0.05 mg l<sup>-1</sup>, precision ± 0.01 mg l<sup>-1</sup>), reading at the same wavelength. NO<sub>2</sub><sup>-</sup>-N was determined colorimetrically using chromotropic and sulphanilic acids as indicators (estimated detection limit 0.01 mg l<sup>-1</sup>, precision ± 0.01 mg l<sup>-1</sup>), reading absorbance at 507 nm. PO<sub>4</sub><sup>3-</sup>-P was measured by a modification of the molybdenum blue procedure (estimated detection limit 0.01 mg l<sup>-1</sup>, precision ± 0.01 mg l<sup>-1</sup>), reading absorbance at 890 nm. All analyses were performed three times. The standard deviations of the pseudoreplicates remained within the typical precision ranges of each method.

### **2.3 | Microscopic observation**

Cyanobacteria from river biofilm samples were identified and morphologically characterised under a dissecting microscope (Leica, Leica Microsystems) and an Olympus BH2-RFCA photomicroscope equipped with phase-contrast, epifluorescence and video camera systems (Leica DC Camera, Leica Microsystems). Taxonomy was based on (Geitler, 1932; Komárek & Anagnostidis, 1999; Komárek & Anagnostidis, 2005; Whitton, 2011).

### **2.4 | Extraction of genomic DNA and amplification of the 16S rRNA gene**



Total genomic DNA was obtained from frozen field samples of cyanobacteria using a modification of previously described DNA extraction procedures (Rodriguez et al., 2007). A prior step was added at the beginning of the protocol: 1.5 ml aliquots of cell suspensions of each culture were homogenised and exposed to three freeze-thaw cycles, alternating immersion in liquid nitrogen and heating to 60°C. Cyanobacterial cells were lysed by a hot extraction procedure performed with 4% sodium dodecyl sulphate, 250 mM NaCl, 100 mM EDTA, and 350 mM guanidine isothiocyanate at 68°C, after vortex mixing for 1 min and sonication for 2 min. After incubation, the suspension was centrifuged for 15 min at 13,000 g (Eppendorf Minifuge), and 600 µl of the supernatant phase were mixed with 75 µl 5 M potassium acetate, 250 µl 40% polyethylene glycol 8000, and 10 µg of glycogen. Crude DNA extracts were partially cleaned using hexadecyltrimethylammonium bromide (CTAB), chloroform extraction, isopropanol precipitation and filtration with Microcon 100 microconcentrators (Amicon, Beverly, MA, USA).

Approximately 450 bp of the 16S rRNA gene fragments (corresponding to *E. coli* positions 358-805) were amplified using two sets of cyanobacteria-specific primers (Nübel, Garcia-Pichel, & Muyzer, 1997): CYA359F (with a 20-nucleotide GC clamp at the 5' end)-CYA781R(a) and CYA359F+GC Clamp-CYA781R(b), as described by (Boutte, Grubisic, Balthasart, & Wilmotte, 2006). Thus, two PCR products were amplified for each sample, and these were run on separate lanes in TGGE. The reverse primers CYA781R(a) and CYA781R(b), which differ by one base, amplify preferentially filamentous and unicellular cyanobacteria, respectively. Thermocycling was performed using a Perkin-Elmer GeneAmp 2400 PCR system and denaturation was carried out as described by (Rodriguez et al., 2007).

## **2.5 | Temperature-Gradient Gel Electrophoresis (TGGE)**

The TGGE Maxi System (Biometra) was used for sequence-specific separation of PCR products. Electrophoresis was performed by loading 6 ml of PCR product in a gel containing 5% acrylamide/bisacrylamide, 8 M urea, 2% glycerol, 20% formamide and 1% TAE, as described by (Rodriguez et al., 2007), with a gradient ranging from 38.5 to 49.0°C. A TGGE ladder was prepared by mixing equal amounts (3 µl) of the PCR products obtained from the DNA extracted from reference strains (Loza, Berrendero, Perona, & Mateo, 2013; Loza, Perona, et al., 2013). This marker consisted of a PCR-mix of 16S rRNA gene fragments from six cyanobacterial strains previously isolated from the field samples (*Aphanocapsa muscicola* UAM 385, *Chamaesiphon investiens* UAM 386, *Aphanocapsa rivularis* UAM 390, *Leptolyngbya boryana* UAM and *Cyanobium* sp. UAM 406). The gels were then stained following a routine silver-staining protocol (Sanguinetti, Dias Neto, & Simpson, 1994).

Digital images of the gels were analysed using the Quantity One software package (BioRad Laboratories; Hercules, CA, USA). TGGE banding patterns were characterised by the previously described computer-assisted method (Fromin et al., 2002; Loza, Berrendero, et al., 2013). A total of 200 bands were carefully excised from TGGE gels using an autoclaved surgical scalpel. Excised bands were resuspended as described by (Sanguinetti et al., 1994) and reamplified with the same primers without a GC clamp. The products were purified using a Real Clean Spin kit (Real) and commercially sequenced in both directions. The nucleotide sequences obtained in this work have been deposited in the GenBank database (KT983911-KT983960).

## 2.6 | Phylogenetic analysis

16S rRNA gene sequences were aligned using the ClustalW program (Thompson, Higgins, & Gibson, 1994). Pairwise similarities were calculated with the EzTaxon server, a

freely available web-based tool (<http://www.ezbiocloud.net>). The alignment was checked and corrected manually with Bioedit (version 7.0.5.3) (Hall, 1999). The Mallard program (Ashelford, Chuzhanova, Fry, Jones, & Weightman, 2006) was used to identify chimeras and other anomalies in 16S rRNA gene sequences within multiple sequence alignments. Sequences were subjected to a BLAST search (<http://www.ncbi.nlm.nih.gov/blast>) and representative sequences ascribed to known cyanobacteria taxa were added to the alignment. The phylogenetic tree was computed using the neighbour-joining (NJ) (Tajima and Nei model) analysis conducted in Mega 5.0. (Tamura et al., 2011). Complete deletion handling of gaps and confidence levels were calculated by bootstrapping (Felsenstein, 1985) using a resampling number of 1,000.

## **3 | RESULTS**

### **3.1 | Environmental characteristics**

The physicochemical parameters of the water at the Guadalix River sampling sites are shown in Table 1. The environmental conditions featured mild temperatures and moderate acidity to neutral pH. Sites 1 and 2 presented low ionic content and a high percentage of oxygen saturation in most seasons. Nutrient content ( $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N,  $\text{NH}_4^+$ -N,  $\text{PO}_4^{3-}$ -P) revealed an increasing downstream eutrophication gradient, which was due to agricultural, domestic and industrial disposal. The highest values were observed at sites 2 and 3 in autumn and summer. According to these results, the trophic status of the three sampling locations was defined with respect to the phosphorus classification limits described by (Kelly & Whitton, 1998): site 1 (upstream) was oligo-mesotrophic, site 2 was meso-eutrophic (eutrophic status during autumn and summer) and site 3 (downstream) was eutrophic most of the hydrological year.

### 3.2 | Microscopic analysis of cyanobacterial populations

The microscopic observations showed the unicellular genus *Chamaesiphon* A. Braun et Grunow in Rabenhorst 1865 and the filamentous genus *Phormidium* Vaucher ex Gomont to be dominant in the studied locations. Four *Chamaesiphon* populations were identified: *C. fuscus* (Rostafinski) Hansgirg 1888, *C. starmachii* Kann 1972, *C. subglobosus* (Rostafinski) Lemmermann 1910 and *C. polymorphus* Geitler 1925. *Phormidium* was represented by a single morphotype, *P. autumnale* C.A. Agard ex Gomont 1892, in all the sampling sites (Figure 1). *C. fuscus* showed elongated cells, club-shaped to narrowly ovoid, 2.5-4 µm wide, 6-9 µm long, usually featuring one exocyte as wide as the cell (Figure 1a-c). *C. starmachii* had usually pear-shaped or ovoid, slightly narrowed towards the top cells, 4-6 µm wide, 5-7 µm long, with spherical exocytes (Figure 1d-h). Cells of *C. subglobosus* were spherical or ovoid to oval, 3-5 µm wide, 3-6 µm long, with usually one spherical or slightly flattened exocyte (Figure 1i-m). *C. polymorphus* showed irregular rows of cells enveloped by mucilage. Cells were subspherical but often becoming almost cylindrical, 3-6 µm wide, 3-9 µm long, Exocytes usually remained within the common mucilage contributing to the growth of the colony (Figure 1n-p).

The spatial distribution of *Chamaesiphon* populations differed according to the nutrient concentrations at each location (data not shown). *C. fuscus* and *C. starmachii* were observed mainly at a site 1. *C. subglobosus* was observed at all sampling sites, but in small amounts, and *C. polymorphus* was found mostly in eutrophic downstream sites. *P. autumnale* appeared in all sampling sites, although populations were more abundant at downstream sites. Other cyanobacteria observed were *Leptolyngbya boryana* Anagnostidis et Komárek 1988, which was present at all sites in autumn but only at site 3 in winter, and *Aphanocapsa rivularis* (Charmichel) Rabenhorst 1865, which was present principally at downstream locations.

### 3.3 | TGGE fingerprinting and phylogenetic analyses

In order to evaluate fingerprint differences among the sampling sites, 12 TGGE gels were run. These gave similar results for each sampling site and season over the year. Four representatives of these are shown in Figure 2. In the gels, sampling sites 1, 2, and 3 were represented by three replicates (a, b, and c) that corresponded to three stones collected at each sampling point, and the two primers used (Ra and Rb). A total of 49 bands were successfully sequenced from a total of 100 excised and reamplified bands. These were phylogenetically analysed. As shown in Figure 2, distinct banding patterns were detected in all the samples from different locations. 16S rRNA gene sequences from the excised bands were compared with those of cyanobacterial strains and field samples from the Guadalix and other Spanish rivers that had previously been genetically analysed, and with other sequences obtained from GenBank. The phylogenetic tree comprised five principal clusters, in which sequence similarity within representatives of these clusters was always  $\geq 96\%$  (Figure 3). Cluster A was represented by sequences belonging to the genus *Phormidium*, which showed a sequence similarity between 99.5% and 100%. This cluster included 18 bands from all the sampling sites and one band from a *P. autumnale* mat from this river, as well as two sequences from the GenBank database identified as *P. autumnale*. Cluster B was associated with the genus *Chamaesiphon*, in which several subclusters were distinguished. The similarities within these subclusters ranged from 96.7% to 99.2%. Subcluster I included bands mainly from site 3, subclusters II and III comprised bands only from sampling site 1, and subcluster IV included bands mainly from site 1 and one from site 2. Subcluster II also included a sequence of *C. subglobosus* PCC 7430 from GenBank with a sequence similarity of 99.5%. Cluster C comprised two TGGE bands from site 2 and three sequences from the database, corresponding to *A. rivularis*

and *Synechocystis* (99.2% to 100% similarity). Cluster D included novel populations represented by a small number of bands with sequence similarities ranging from 97.8% to 100% with no clear matches identified from the BLAST search of the database. Cluster E corresponded to plastid sequences.

## 4 | DISCUSSION

### 4.1 | Morphological versus phylogenetic analysis

Similar results were obtained with the microscopic and molecular approaches, whereby hits obtained from the BLAST analysis corresponded to the genera identified by microscopy: *Chamaesiphon* and *Phormidium* were the dominant genera recorded at the sampling sites. However, assignment of band sequences to the species level was not always possible, since database matches were not found for many of the sequences (principally those corresponding to the genus *Chamaesiphon*; see below). Sequences of TGGE bands corresponding to *Phormidium* (Cluster A) were almost identical, matching *P. autumnale* from the databases, in agreement with the microscopic analysis. Similarly, sequences corresponding to *Aphanocapsa* (Cluster C) had a high percentage similarity with *Aphanocapsa rivularis* from our previous study in the Guadarrama River (Loza, Berrendero, et al., 2013), although *Synechocystis* sequences were also included in this cluster since the genus *Aphanocapsa* is not recognised by some authors, who included the two genera in *Synechocystis* (Herdman, Castenholz, Iteman, Waterbury, & Rippka, 2001; Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979). The morphotype corresponding to *Aphanocapsa/Synechocystis rivularis* was also recorded in microscopic examinations. However, sequences corresponding to *Chamaesiphon* only gave one match with a sequence of *C. subglobosus* from the database, whereas the other 23 had low sequence similarities (<98%) with the GenBank sequences. A large number of 16S rRNA

gene sequences from planktonic cyanobacteria are available in databases, which facilitates the phylogenetic classification of taxa in this habitat (e.g., (Touzet, McCarthy, & Fleming, 2013). However, there were fewer cyanobacteria sequences from running waters; for some taxa, such as *Chamaesiphon*, numbers are particularly low. The present study distinguished four subclusters in the *Chamaesiphon* cluster. The microscopic observations also revealed four dominant morphotypes. The most frequent and abundant species of *Chamaesiphon* were *C. fuscus*, *C. starmachii*, *C. subglobosus* and *C. polymorphus*. Therefore, it might be inferred from the morphological and genetic analysis that the four phylotypes found within the genus *Chamaesiphon* may correspond to the four morphotypes found in the microscopic analysis. Phylotype I included 13 sequences in subcluster I, which were retrieved only from downstream eutrophic sites. This could correspond to *C. polymorphus*, which was clearly dominant at those locations. Phylotype II, with a sequence similarity of 99.5% with *C. subglobosus* in the database could be assigned to this taxon. Phylotypes III and IV, which only occurred in clean upstream waters, may correspond to *C. fuscus* and *C. starmachii*. Further studies with isolated strains from these species, and the corresponding genetic characterisation, are needed to confirm these assignments.

## **4.2 | TGGE banding pattern and phylogenetic analyses**

Differences in the banding pattern were found between sampling sites, which, in turn, differed in their trophic status, showing a clearly increasing eutrophic gradient downstream. As stated above, our TGGE and phylogenetic analysis showed that sequences of bands corresponded to dominant populations observed by the microscopic analysis, and therefore these TGGE gels are representative of the cyanobacterial diversity found. Denaturing gel electrophoresis fingerprinting techniques are very effective for

characterising bacterial community structures and have been applied to environmental samples in recent decades (Fromin et al., 2002; Gerard Muyzer & Smalla, 1998). They are consequently well suited to monitoring whole communities, focusing on phylotypes for which the occurrence and/or the relative frequency are affected by any environmental change (Díez, Pedrós-Alió, Marsh, & Massana, 2001; Fromin et al., 2002). Our previous investigations revealed that the genetic profiles of TGGE reflected changes in the structure and composition of cyanobacterial communities in the Guadarrama River. As the nutrient content increased downstream, some phylotypes were associated with low nutrient concentrations, while others were associated with eutrophic-hypertrophic conditions (Loza, Perona, et al., 2013; Rodriguez et al., 2007). In this study, 18 bands from the TGGE gel were affiliated to *P. autumnale* with a high percentage of similarity, but with no clear association between the banding pattern and the sampling site; although there was a trend towards there being more bands of this phylotype at downstream sites, probably in relation to the greater abundance of this cyanobacterium, as noted in the microscopic analysis. A difference in only one nucleotide can give rise to different melting behaviour (G Muyzer, de Waal, & Uitterlinden, 1993), and the more individuals there are in these locations, the greater is the probability of finding these small sequence variations. Therefore, this phylotype is not a good candidate for monitoring changes in water quality. However, bands corresponding to distinct phylotypes of *Chamaesiphon* can be differentiated by their position in the banding pattern; we found clear differences in the phylotypes at different sampling locations. A problem noted by some authors is that different sequences can have identical melting points, leading to the same level of migration in denaturing gel electrophoresis. Thus, different organisms can produce bands at the same position (Casamayor, Schäfer, Bañeras, Pedrós-Alió, & Muyzer, 2000; Sekiguchi, Tomioka, Nakahara, & Uchiyama, 2001). In the present study, as we found in our previous study



(Loza, Perona, et al., 2013), and consistent with the findings of others (de Figueiredo et al., 2007; Schiaffino et al., 2009), there was no evidence of the comigration of different sequences, since comigrating bands had identical or almost identical sequences. Therefore, these phylotypes are suitable for monitoring changes in water quality in this river, as described below.

#### 4.3 | Profiling *Chamaesiphon* populations

Our results revealed substantive changes in *Chamaesiphon* phylotypes between locations of contrasting trophic status. Some bands were present only upstream, whereas others appear only downstream. Bands corresponding to phylotype I were found mainly at site 3, and also at site 2 in some seasons, where high levels of nutrients were recorded. On the other hand, bands corresponding to phylotypes III and IV were found mainly at oligotrophic site 1. Previous studies of the Guadalix River also showed high phosphatase activity of *Chamaesiphon* populations at site 1 in relation to low bioavailable P at this location (Munoz-Martin et al., 2014). Bands of phylotype II, ascribed to *C. subglobosus*, were comparably rare, as has been described with respect to the occurrence of populations of this species in German rivers (Gutowski, Foerster, Doege, & Paul, 2015). Therefore, banding patterns and phylogenetic analysis showed that dominant species of *Chamaesiphon* were replaced by other species at some sampling sites.

*Chamaesiphon* species are used to assess the ecological quality of streams and rivers according to the European Water Framework Directive (WFD; 2000/60/EC). In a recent study, (Gutowski et al., 2015) analysed the records of several species of *Chamaesiphon* from 429 sampling sites over six years, concluding that among others, *C. fuscus* and *C. starmachii* occur at significantly lower nutrient levels and are therefore characteristic of unimpaired sites, whereas *C. polymorphus* prefers nutrient-rich sites.

Other studies showed similar patterns of occurrence (Mateo et al., 2015, and references therein), although Stancheva, Fetscher, & Sheath (2012) found *C. polymorphus* to be associated with low total nitrogen in streams of California, and Fetscher et al. (2014) further evaluated this species as indicator of low total phosphorus in the development of indices of biotic integrity for stream bioassessment in southern California. These contrasting results could arise from the high genetic diversity found in cyanobacteria, with some genera found paraphyletic, such as *Leptolyngbya* (Moro et al., 2010), whereby identical morphotypes could correspond to different genotypes, and therefore, similar morphotypes corresponding to *C. polymorphus* from Europe could be genetically distinct from those found in North America, responding in a different way to trophic conditions. Further species specific molecular studies with these geographically distant *Chamaesiphon* populations are needed to confirm this possibility.

Results from morphological, TGGE banding-pattern and phylogenetic analyses suggest that the four phylotypes found corresponded to the four morphotypes observed, and that their distribution depends on the water quality. This supports the use of these fingerprints from *Chamaesiphon* populations as a sentinel or “early warning” detector of the presence of pollutants in this river. The development of molecular tools for monitoring running waters has led to the use of new technical approaches for detection and identification of bioindicator species. The monitoring of the whole algal community has been traditionally based on conventional methods, mainly involving microscopic identification. However, these traditional techniques are often labor-intensive, with training needs of taxonomic expertise, while molecular methods are much faster and often more accurate (Fromin et al., 2002; Rodriguez et al., 2007). Molecular methods, such as the TGGE used in this work, supplement established traditional methods as they allow quick and easy comparison of profiles from related microbial assemblages, providing a cost-

effective means of examining and comparing a large number of samples in a short time (Fromin et al., 2002; Kumari, Srivastava, Bhargava, & Rai, 2009; Gerard Muyzer & Smalla, 1998). They have the other advantages of being reliable and reproducible, and that selected bands can be sequenced, and the presence of a particular phylotype, such as the *Chamaesiphon* phylotypes in this study, can thereby be monitored in environmental samples (Díez et al., 2001). As a consequence, these techniques are now used in many ecological studies (Bukowska, Bielczyńska, Karnkowska, Chróst, & Jasser, 2014; Echenique-Subiabre et al., 2015; Rigonato et al., 2013; Touzet et al., 2013). In addition, the use of computer-assisted characterisation of profiles (Fromin et al., 2002), which avoids the subjective interpretation of fingerprint patterns, is important for the handling and analysis of data. In this study, computer-assisted data analysis allowed us to determine the fingerprints of *Chamaesiphon* populations. Our results support the use of fingerprints of these populations obtained by TGGE to examine changes in water quality.

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## FIGURE LEGENDS

**Figure 1.** Microphotographs of dominant cyanobacteria in the biofilms of the Guadalix River. (a-c) *C. fuscus*, (d-h) *C. starmachii*, (i-m) *C. subglobosus*, (n-p) *C. polymorphus* (q) *P. autumnale*. (c, h, f, j, l, o, p, q) Bright light micrographs (a, b, d, e, g, i, k, m, n) Autofluorescence Scale bar = 5 µm.

**Figure 2. (a)** - Comparative analysis of TGGE genetic profiles of PCR-amplified fragments of 16S rRNA genes obtained from epilithic biofilms collected from the Guadalix River. Three sampling points (1, 2 and 3) with three replicates (stones a, b and c) during the different climatological seasons are represented in the gels. **Ra**: using the primers CYA359F-CYA781R(a); **Rb**: using the primer set CYA359F-CYA781R(b). A mixture of PCR products derived from six cyanobacterial strains isolated from the epilithon was applied as a reference marker (M) (from top to bottom: *Aphanocapsa muscicola* UAM 385 (JX413492), *Chamaesiphon investiens* UAM 386 (JQ070061), *A. cf. rivularis* UAM 390 (JQ070058), *Leptolyngbya boryana* UAM 391 (JQ070062), *Phormidium* sp. UAM 361 (JN382219) and *Cyanobium* sp. UAM 406 (JQ070060). **(b)** - Diagram of the TGGE banding profiles after computer-assisted characterisation and phylogenetic analyses of excised bands (see Figure 3).

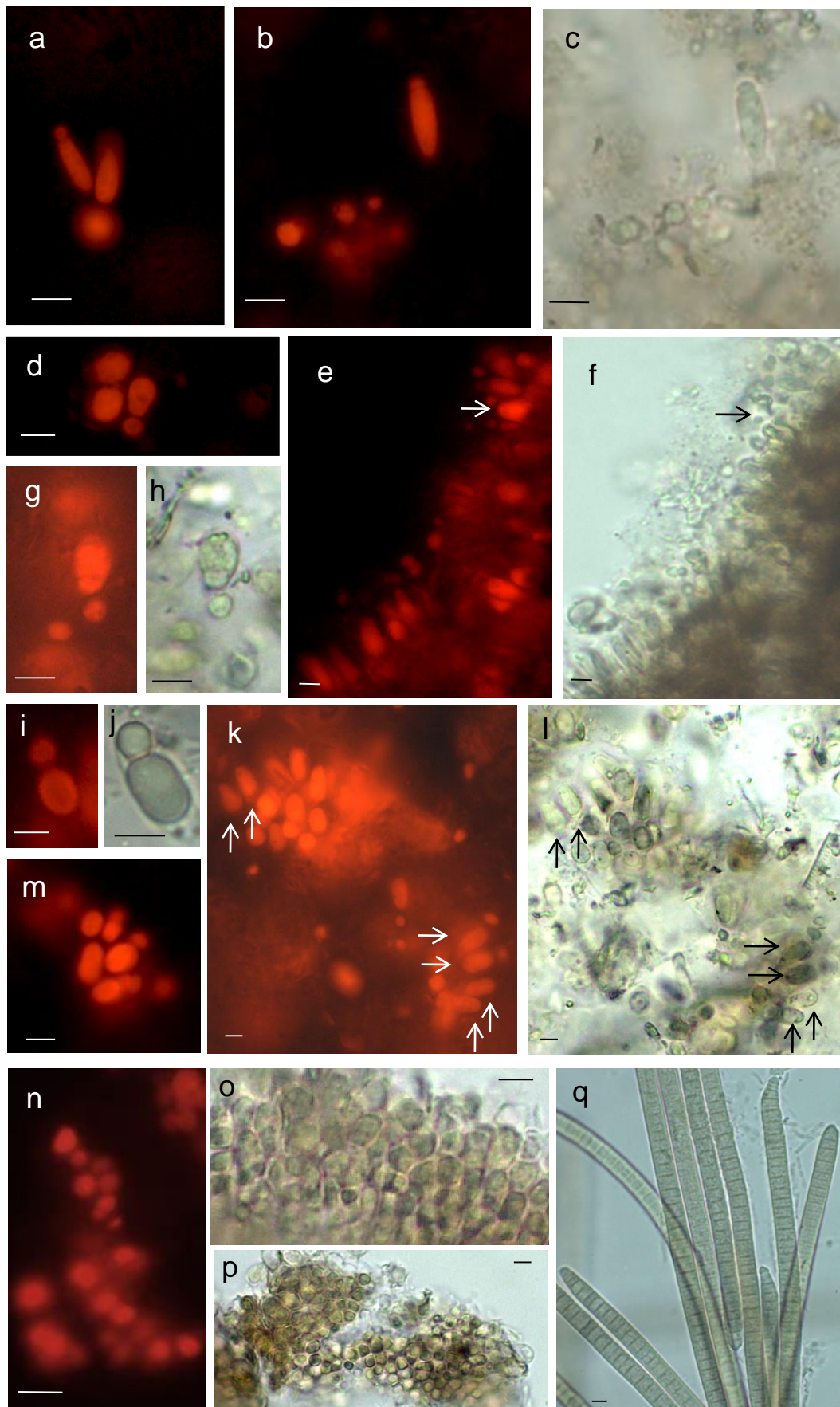
**Figure 3.** Neighbour-joining (NJ) tree based on the analysis of the 16S rRNA gene, showing the TGGE band sequences obtained in the present study (in bold). Numbers near nodes indicate bootstrap values ≥ 50% in NJ. TGGE bands were labelled according to the climatological seasons (A, autumn; W, winter; Sp, spring; S, summer), sampling sites (1, green; 2, orange; 3, red) and replicates (stones a, b and c).

TABLE 1. Physicochemical characteristics of water at sampling locations on the Guadalix River. Data are presented as mean  $\pm$  SD and range.

	Autumn			Winter			Spring			Summer		
	1	2	3	1	2	3	1	2	3	1	2	3
Water temperature ( $^{\circ}\text{C}$ )	7.4 $\pm$ 0.06 7.0-7.5	7.2 $\pm$ 0.07 7.2-7.3	7.5 $\pm$ 0.07 7.5-7.6	6.3 $\pm$ 0.1 6.3-6.5	4.8 $\pm$ 0.2 4.7-5.1	6.2 $\pm$ 0.07 6.2-6.3	12.3 $\pm$ 0.32 12.0-12.6	12.6 $\pm$ 0.5 12.3-13.2	13.9 $\pm$ 0.15 13.8-14.1	13.5 $\pm$ 0.1 13.4-13.6	17.0 $\pm$ 0.15 16.9-17.2	21.3 $\pm$ 0.1 21.2-21.4
Conductivity ( $\mu\text{S cm}^{-1}$ )	91.0 $\pm$ 1.6 90-92	197.6 $\pm$ 1.1 197-199	493.5 $\pm$ 0.7 493-494	106.3 $\pm$ 1.5 106-108	152.6 $\pm$ 2.3 150-154	301.3 $\pm$ 1.5 300-303	75.3 $\pm$ 2.5 73-78	124.3 $\pm$ 4.0 120-128	518.0 $\pm$ 2.6 515-520	107.5 $\pm$ 0.7 107-108	279.0 $\pm$ 1.4 278-280	663.0 $\pm$ 1.4 662-664
Dissolved oxygen (% saturation)	103.7 $\pm$ 1.25 102.3-104.7	101.5 $\pm$ 0.7 101-102	93.4 $\pm$ 1.0 92.7-94.1	103.7 $\pm$ 0.2 103.5-104	100.3 $\pm$ 0.6 100-101	104.6 $\pm$ 0.6 105-104	102.9 $\pm$ 0.23 102.8-103	101.6 $\pm$ 0.9 100.8-102.6	90.2 $\pm$ 0.55 89.7-90.8	95.8 $\pm$ 0.3 95-96	97.3 $\pm$ 5.2 93-101	95.7 $\pm$ 0.2 95-96
pH	7.2 $\pm$ 0.08 7.1-7.2	7.5 $\pm$ 0.05 7.5-7.6	7.9 $\pm$ 0.4 7.6-8.1	6.9 $\pm$ 0.07 6.7-6.8	7.3 $\pm$ 0.05 7.3-7.4	8.0 $\pm$ 0.13 7.9-8.1	6.4 $\pm$ 0.08 6.3-6.5	7.2 $\pm$ 0.06 7.1-7.3	7.8 $\pm$ 0.05 7.8-7.9	6.6 $\pm$ 0.2 6.5-6.8	6.7 $\pm$ 0.05 6.7-6.8	7.8 $\pm$ 0.05 7.8-7.9
$\text{NO}_3^-$ -N (mg l $^{-1}$ )	0.13 $\pm$ 0.03 0.11-0.15	0.09 $\pm$ 0.03 0.07-0.12	0.08 $\pm$ 0.03 0.06-0.1	0.13 $\pm$ 0.03 0.1 $\pm$ 0.15	0 <sup>a</sup> -	1.20 $\pm$ 0.14 1.1-1.3	0.23 $\pm$ 0.06 0.2 $\pm$ 0.3	0.65 $\pm$ 0.07 0.6-0.7	1.26 $\pm$ 0.07 1.2-1.3	0.55 $\pm$ 0.07 0.5-0.6	1.75 $\pm$ 0.07 1.7-1.8	0.35 $\pm$ 0.07 0.3-0.4
$\text{NO}_2^-$ -N (mg l $^{-1}$ )	0.005 $\pm$ 0.004 0.003-0.008	0.005 $\pm$ 0.001 0.004-0.006	0.04 $\pm$ 0.02 0.03-0.06	0.035 $\pm$ 0.01 0.03-0.04	0.01 $\pm$ 0.00 0.005-0.1	0.03 $\pm$ 0.00 0.02-0.03	0.002 $\pm$ 0.006 0.002-0.003	0.04 $\pm$ 0.003 0.036-0.04	0.06 $\pm$ 0.003 0.063-0.06	0.04 $\pm$ 0.01 0.03-0.04	0.05 $\pm$ 0.01 0.05-0.06	0.04 $\pm$ 0.01 0.03-0.05
$\text{NH}_4^+$ -N (mg l $^{-1}$ )	0.2 $\pm$ 0.03 0.18-0.22	0.33 $\pm$ 0.01 0.32-0.34	0.57 $\pm$ 0.03 0.55-0.59	0.02 $\pm$ 0.01 0.02-0.03	0.05 $\pm$ 0.01 0.04-0.05	0.15 $\pm$ 0.01 0.15-0.16	0.08 $\pm$ 0.006 0.08-0.09	0.14 $\pm$ 0.05 0.1-0.2	0.18 $\pm$ 0.02 0.17-0.2	0.23 $\pm$ 0.01 0.22-0.24	0.27 $\pm$ 0.04 0.25-0.3	0.25 $\pm$ 0.02 0.24-0.27
$\text{PO}_4^{3-}$ -P (mg l $^{-1}$ )	0.035 $\pm$ 0.007 0.03-0.04	0.16 $\pm$ 0.01 0.15-0.17	0.34 $\pm$ 0.014 0.33-0.35	0.03 $\pm$ 0.0 0.02-0.03	0.06 $\pm$ 0.0 0.05-0.06	0.23 $\pm$ 0.01 0.22-0.25	0.03 $\pm$ 0.006 0.03-0.04	0.07 $\pm$ 0.006 0.07-0.08	0.43 $\pm$ 0.11 0.4-0.5	0 <sup>a</sup> -	0.32 $\pm$ 0.03 0.3-0.34	0.5 $\pm$ 0.01 0.49-0.51
Hardness (mg $\text{CaCO}_3$ l $^{-1}$ )	15 $\pm$ 4.24 12-18	29 $\pm$ 2.8 27-31	130 $\pm$ 2.8 128-132	6.5 $\pm$ 2.12 5-8	27.0 $\pm$ 1.41 26-28	59.2 $\pm$ 1.81 57.5-61	11.15 $\pm$ 0.92 10.5-11.8	18.8 $\pm$ 0.42 18.5-19.1	152.5 $\pm$ 2.1 151-154	17.05 $\pm$ 0.21 16.9-17.2	41.7 $\pm$ 0.14 41.6-41.8	146.5 $\pm$ 3.54 144-149

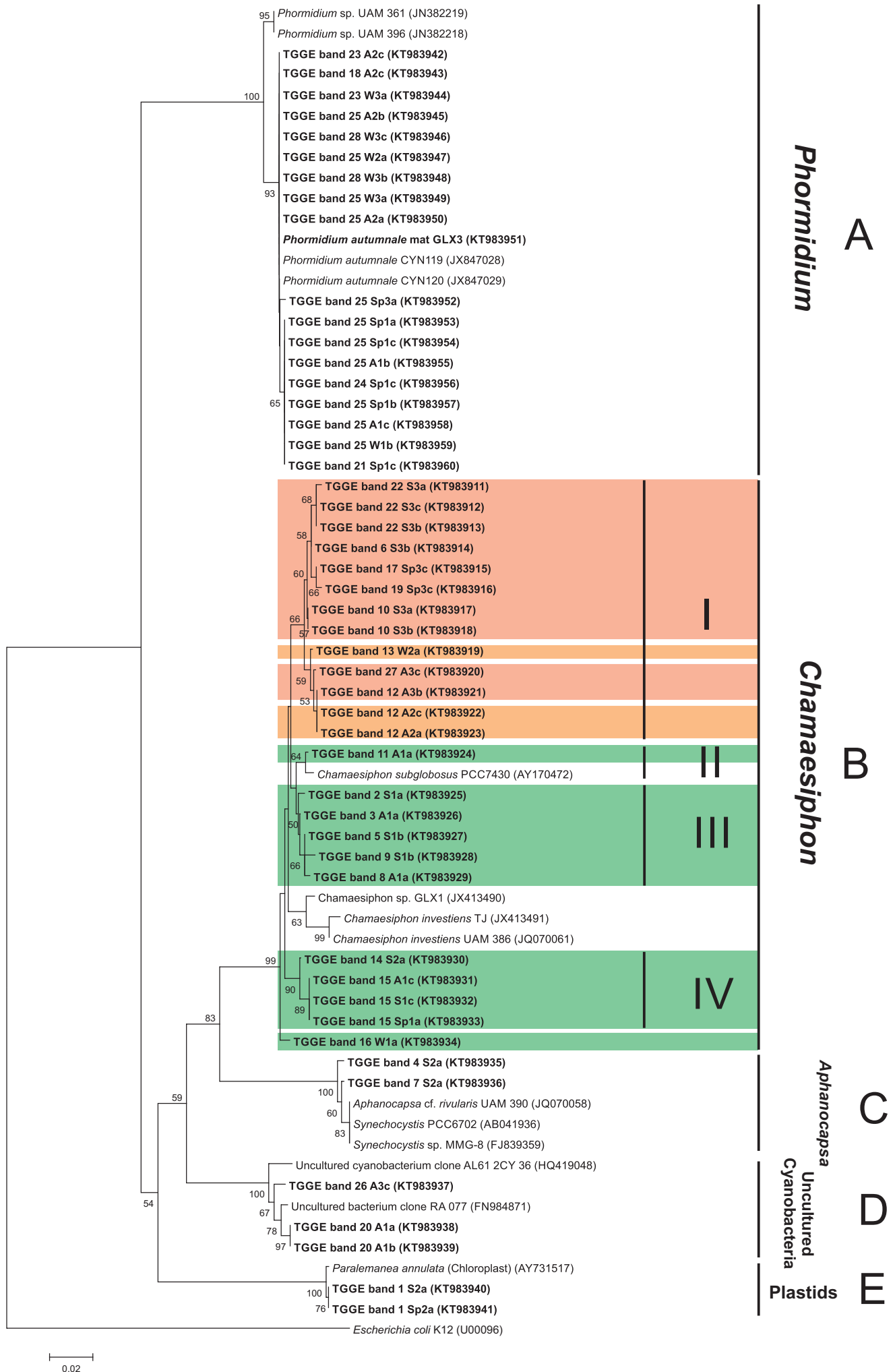
<sup>a</sup> Zero values indicate levels beneath detection limits; see Materials and Methods





**Fig. 1**





**Fig.3**