A cultivation-independent approach for the genetic and cyanotoxin characterization of colonial cyanobacteria

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ABSTRACT: To bypass the constraint of cyanobacterial strain isolation and cultivation, a combination of whole genome amplification (WGA) and enzyme-linked immunoassay (ELISA) for microcystin toxins (MCs) was tested on individual colonies of *Microcystis* and *Woronichinia*, taken directly from aquatic environments. Genomic DNA of boiled cells was amplified by multiple strand displacement amplification (MDA), followed by several specific PCR reactions to characterize the genotype of each colony. Sequences of 3 different housekeeping genes (*ftsZ*, *gltX*, and *recA*), of 3 MC biosynthesis genes (*mcyA*, *mcyB*, and *mcyE*), and the Internal Transcribed Spacer (ITS) were analyzed for 11 colonies of *Microcystis*. MCs were detected and quantified by ELISA in 7 of the 11 *Microcystis* colonies tested, in agreement with the detection of *mcy* genes. Sequence types (ST) based on the concatenated sequences of housekeeping genes from cyanobacterial colonies from Belgian water bodies appeared to be endemic when compared to those of strains described in the literature. One colony appeared to belong to a yet undiscovered lineage. A similar protocol was used for 6 colonies of the genus *Woronichinia*, a taxon that is very difficult to cultivate in the laboratory. The 16S rRNA sequences of 2 colonies were obtained and were quasi identical to that of *W. naegeliana* 0LE35S01. For one *Woronichinia* colony, the *mcyE* PCR gave a non-specific PCR product. The corresponding amino acid sequence was 50% identical to a *Microcystis* ketoacyl carrier protein transferase. This approach for the simultaneous detection and quantification of MCs with *mcy* genotyping, at single colony level, offers potential for the ecotoxicological characterization of environmental populations of cyanobacteria without the need for strain isolation and culture.

KEY WORDS: *Microcystis* · *Woronichinia* · Microcystins · ELISA · MDA · Colony-forming cyanobacteria

INTRODUCTION

Microcystis is a unicellular, colony-forming cyanobacterium present as blooms in nutrient-enriched, standing freshwaters. It can produce microcystins (MCs), cyclic heptapeptides that are potent hepatotoxins and tumor promoters. Over 90 structural MC variants have been characterized thus far, from a wide range of planktonic and benthic cyanobacterial

genera (e.g. Sivonen & Jones 1999, Metcalf & Codd 2012). *Microcystis* represents a good model organism for the study of MC production and is among the most commonly encountered MC-producing genera worldwide (Sivonen 2008). At the genetic level, the *mcy* gene cluster involved in MC biosynthesis consists of a combination of non-ribosomal peptide synthase (NRPS) and polyketide synthetase (PKS) genes. A recent multi locus sequence typing, carried out

mainly on strains from Asian water bodies, resulted in at least 8 different cryptic or panmictic lineages (Tanabe et al. 2009, Tanabe & Watanabe 2011).

So far, resolution of the comparative toxicological and corresponding genotypic characteristics within environmental populations of cyanobacteria has been limited by the technical need for strain isolation and cultivation. While this is possible with *Microcystis*, loss of the colony-forming habit almost invariably occurs (Visser et al. 2005). Moreover, a *Microcystis* bloom population can include several genotypes that may produce MCs or not, and evolve in space and time (Kardinaal & Visser 2005, Kardinaal et al. 2007). A further limitation of the strain isolation approach was shown by Fewer et al. (2009) in studies of a mixed *Anabaena/Nodularia* bloom in the Gulf of Finland, from which only certain genotypes may be selected during laboratory isolation and cultivation.

Uncertainty exists regarding the production of MCs by a second planktonic genus of cyanobacteria: *Woronichinia*. Although high concentrations of MCs were found in blooms dominated by *Woronichinia*, species of other established MC-producing genera were also present as minor members (Willame et al. 2005). *Woronichinia* belongs to the sub-family Gomphosphaerioideae in the family Merismopediaceae. This colony-forming genus is characterized by the binary fission of cells in 2 perpendicular planes in successive generations and the location of cells at the end of mucilaginous stalks which radiate from the center of the colonies (Komárek & Anagnostidis 1999). *Woronichinia* is frequently observed in European and Scandinavian lakes. It can dominate phytoplankton in oligotrophic, mesotrophic and eutrophic lakes and ponds (Rajaniemi-Wacklin et al. 2006). Little is known about the genus *Woronichinia* since only 2 strains, 0LE35S01 and 1ES42S01, have been isolated (Rajaniemi-Wacklin et al. 2006, Willame et al. 2006). Unfortunately, these strains could not be maintained in culture, although their 16S rRNA sequences were deposited in GenBank. Only one strain (1ES42S01) was tested for the presence of the *mcyE* gene and the result was negative (Willame et al. 2006).

Recently, new approaches have been developed to study the genetic features of uncultured single cells including microbes, involving enzymes e.g. DNA polymerase Phi29 (Woyke et al. 2010). Phi29 is a strand displacement polymerase (Kvist et al. 2007), which amplifies DNA by isothermal multiple strand displacement (MDA). This whole genome amplification (WGA) technology has already helped characterize the metabolism of the uncultured unicellular nitrogen-fixing cyanobacterium UNCY-A (Tripp et al. 2010) and the apratoxin pathway of *Lyngbya bouillonii* (Grindberg et al. 2011).

Micromanipulation of *Microcystis* colonies directly isolated from the environment has already been performed to characterize their MC production by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) analysis and genotype by 2 to 3 PCR analyses (e.g. Janse et al. 2004, Via-Ordorika et al. 2004).

The aims of the present investigation were (1) to improve the genotyping of environmental colonies of *Microcystis* by increasing the amount of DNA material for multiple PCRs, to enable both genetic characterization and biochemical analysis (in this case, MC concentration); (2) to use this on colony-forming cyanobacteria that are, to date, difficult or impossible to cultivate such as *Woronichinia*. We therefore proposed to apply the WGA technology (before performing multiple PCRs) together with an immunochemical assay for MCs, on single colonies from natural populations, with portions of the same colony in each case being used for both WGA and immunoassay. We present a dataset obtained for 11 *Microcystis* and 6 *Woronichinia* colonies. We suggest that this approach can also be used on other colony-forming or filamentous cyanobacteria and can contribute to the study of cyanobacterial phylogeny.

MATERIALS AND METHODS

The experimental procedure was divided into 2 main steps (for full details, see the Supplement at www.int-res.com/articles/suppl/a069p135_supp.pdf). First, the environmental colonies of *Microcystis* and *Woronichinia* spp. were isolated under sterile conditions and washed in 100 µl sterile BG11 medium droplets to dilute potential co-occurring algal and bacterial contaminants present around the cyanobacterial colonies. The colonies were then photographed (100× to 400×) and suspended in 5 to 15 µl BG11 (Rippka et al. 1979). In the second step, the individual colonies were disrupted by boiling at 99°C for 1 min in a thermocycler. From the resulting suspensions, 0.5 µl subsamples were used as a template for MDA reactions. The remaining volumes of the suspension were used for MC quantification by enzyme-linked immunoassay (ELISA) with microcystin-LR (MC-LR) antibodies. The MDA reaction was performed with the QIAGEN REPLI-g Mini Kit following the 'Amplification of genomic DNA from blood or cells' protocol. ELISA was performed following Young et al. (2008). We then used multiple sets of *Microcystis*

and/or cyano-specific PCR primers (Table S1 in the Supplement) to estimate genome amplification efficiency. The success of the WGA is attributed to an effective dispersal and homogenization of the boiled colonies, as well as to the availability of a sufficient number of cells for the MDA reaction, shown to be critical by Rodrigue et al. (2009).

The sequences reported in the present study were deposited in the GenBank database, with accession numbers JN172589 to N172598 (Internal Transcribed Spacer [ITS] of *Microcystis*); JN172599 to JN172605 (*ftsZ*); JN172606 to JN172612 (*gltX*); JN172613 to JN172620 (*recA*); JN172621 to JN172622 (16S rRNA and ITS of *Woronichinia*); and JN172623 (clone *mcyE* amplicon of T2).

RESULTS AND DISCUSSION

Microcystis

To characterize the genotypes corresponding to different morphotypes present in several ponds, we used 11 colonies from water bodies in Belgium (Fig. 1). The colonies were characterized by microphotography, ELISA for MCs and by using 5 to 9 genetic markers, except for the last 3 colonies which were not photographed. Six colonies were selected to represent the 2 morphotypes observed in Ixelles Pond I (Brussels): 3 *Microcystis aeruginosa*-like (X5, X38, X39), 2 *M. ichthyoblabe*-like (X8, X11), and a small colony without morphotype attribution (X40). The colonies F13 and T1 were respectively isolated from 2 other water bodies, Lake Falemprise (Silenrieux) and Tervuren Pond (Leuven). It was difficult to distinguish whether the morphotype of F13 corresponded to *M. aeruginosa* or *M. viridis* (Fig. 1h) and the vertical position taken by colony T1 in the liquid hindered its identification (Fig. 1g). A further 3 *M. aeruginosa*-like colonies (We1, We2, We3) which were not photographed were isolated from Westveldpark Pond (St Amansberg) (see Table S2 in the Supplement for the list of characterizations performed).

Microcystins were detected in 7 of 11 colonies tested by ELISA (Table 1).

The estimated toxin content per colony, based on cross-sectional area measurement (Young et al. 2008), ranged from 70.15 to 854.9 pg. *Microcystis* colony cross-sectional area as an indicator of colony volume was preferred as a more reliable indicator than mathematical models, which tend to be specific for the populations from which they are drawn (Morrison 2005). The MC concentrations per colony area ranged from 0.36 to 31.37 ng mm^{-2}. The highest concentration of MCs was found in colony F13, which was 3 times higher than found by Young et al. (2008). MC concentrations (ng mm^{-2}) in *M. aeruginosa* (X5, X38, X39) and X40 from Ixelles pond vary by a 1.16 to 4.61 ratio . These variations are within the range which can be due to differences in gene expression, as was shown for *Microcystis* PCC7806 (Kaebernick et al. 2000).

For genotypic characterization, the MDA products were used as a template for *mcy* operon detections, ITS denaturing gradient gel electrophoresis (DGGE) analysis and amplification and direct sequencing of *ftsZ*, *gltX*, and *recA*. The *mcyA*, *mcyB*, and *mcyE* genes, 3 genetic loci involved in MC biosynthesis
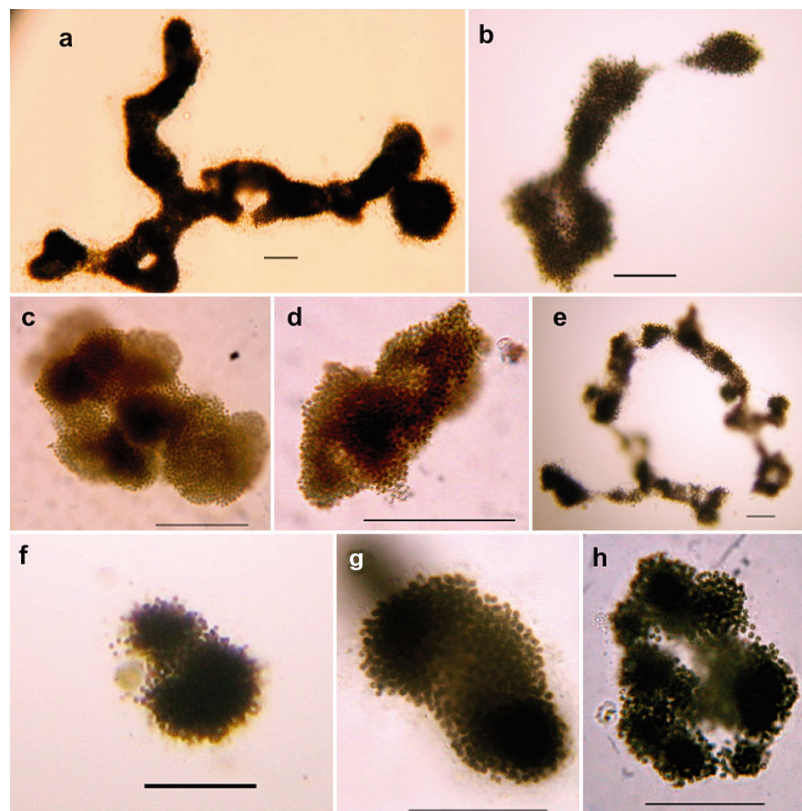

Fig. 1. *Microcystis.* Colonies taken from water bodies in Belgium (see Table 1): (a) X5; (b) X38; (c) X8; (d) X11; (e) X39; (f) X40; (g) T1; (h) F13. Scale bars = 100 μm

Table 1. *Microcystis.* Summary characterization of 11 colonies taken from water bodies in Belgium. Morphotypes were identified as *M. aeruginosa* (*aer*) and *M. ichthyoblabe* (*icht*). MC-LR per colony is the concentration of microcystin-LR measured. Apparent areas were measured using the AXOVISION software from Zeiss. *mcy* gene detection was carried out by PCR targeting *mcyA*, *mcyB*, and *mcyE*. The results are indicated by '+' for the presence of a PCR band of suitable length and '–' for its absence. Sequence typing: for each locus, the Roman numerals indicate a unique sequence type (ST). na: not available; nd: not detected

Colony	Morphotype identification	MC-LR per colony (pg colony^{-1})	Apparent area (mm^2)	*mcy* gene detection			Sequence typing			Conca-tenated sequences
				mcyA	*mcyB*	*mcyE*	*ftsZ*	*gltX*	*recA*	
Ixelles Pond I										
X5	*aer*	558.6	0.268	+	+	+	na	na	I	**na**
X8	*icht*	nd	0.036	–	–	–	II	III	IV	**IV**
X11	*icht*	nd	0.01	–	–	–	III	II	III	**III**
X38	*aer*	442.3	0.046	+	+	–	II	I	II	**II**
X39	*aer*	854.9	0.174	+	+	+	I	I	I	**I**
X40	na	98.9	0.012	+	+	+	I	I	I	**I**
Lake Falemprise										
F13	na	658.7	0.021	+	+	+	V	V	VI	**VI**
Tervuren Pond										
T1	na	nd	0.018	–	–	–	IV	IV	V	**V**
Westveldpark Pond										
We1	*aer*	nd	na	–	–	–	VI	VI	VII	**VII**
We2	*aer*	83.6	na	+	+	+	VII	VII	VIII	**VIII**
We3	*aer*	70.1	na	+	+	+	VII	VII	VIII	**VIII**

(Nonneman & Zimba 2002, Hisbergues et al. 2003, Rantala et al. 2004), were detected for colonies X5, X39, X40, and F13, where significant MC concentrations were measured. None of these 3 loci was detected in colonies X8, X11 and T1. The detection of *mcy* genes agreed with the ELISA results. The *mcyA* and *mcyB* genes were detected in colony X38, but not *mcyE* (nor the ITS locus). In this case, the WGA step may have been incomplete due to a low number of template cells, which could bias PCR efficiency (Rodrigue et al. 2009). Indeed, the thick mucilage surrounding this colony could have impaired colony disruption and release of cells into the suspension, so that less template would be available for the MDA reaction.

It was possible to amplify the ITS sequences of colonies F13, T1, X8, X11, X39, X40, We1, We2, and We3 with a single PCR, whereas 2 successive PCRs were necessary for X5, which appeared identical to X39 and X40. However, it was not possible to amplify the ITS from colony X38. The number of bands (from 1 to 10) in the DGGE gel varied among the colonies. The presence of heteroduplexes and artifacts was controlled (Fig. S1a,b in the Supplement) and only the correct sequences were used for phylogenetic analysis (Fig. 2a). Two ITS sequences were obtained for colonies F13, T1 and X8, as was the case for several strains of *Microcystis* in GenBank (e.g. NIES44, NIES98, NIES101, PCC7941). A careful comparison

showed an indel of 4 to 5 nucleotides, 17 nt after the tRNA^{ILE} end and 14 nt before the box B region in both colonies and strains. Interestingly, colony F13, contained the only pair of ITS sequences that does not hold such indel occurrences. Phylogenetic analysis of partial sequences of ITS (460 nt) (Fig. 2a) showed that the 2 band sequences from the same colony were closely related except for colony F13, where the 2 band sequences were far apart. Moreover, the full sequence of band 2 showed only 99.4% identity with the ITS of NIES90, although the partial sequences are identical in the tree. In addition, the ITS sequence of X39 was identical to X40 and the ITS of We2 was identical to We3.

We suggest that all these variations are evidence of intragenomic differences between the 2 ribosomal operons in *Microcystis* genomes, although the 2 copies in NIES843 were identical. This may lead to reconsideration of the use of the ITS locus for genotyping analysis of *Microcystis* in environmental populations.

To compare our environmental colonies to the GenBank dataset of Asian *Microcystis* strains, we used 3 of the 7 markers used by Tanabe et al. (2007). The *ftsZ* locus was chosen for its ability to cluster the group G of Tanabe et al. (2009), whereas the 2 other markers were randomly chosen. The 3 (*ftsZ*, *gltX*, *recA*) gene loci were present in only 1 copy in the genome of *Microcystis* NIES843 (Kaneko et al. 2007).
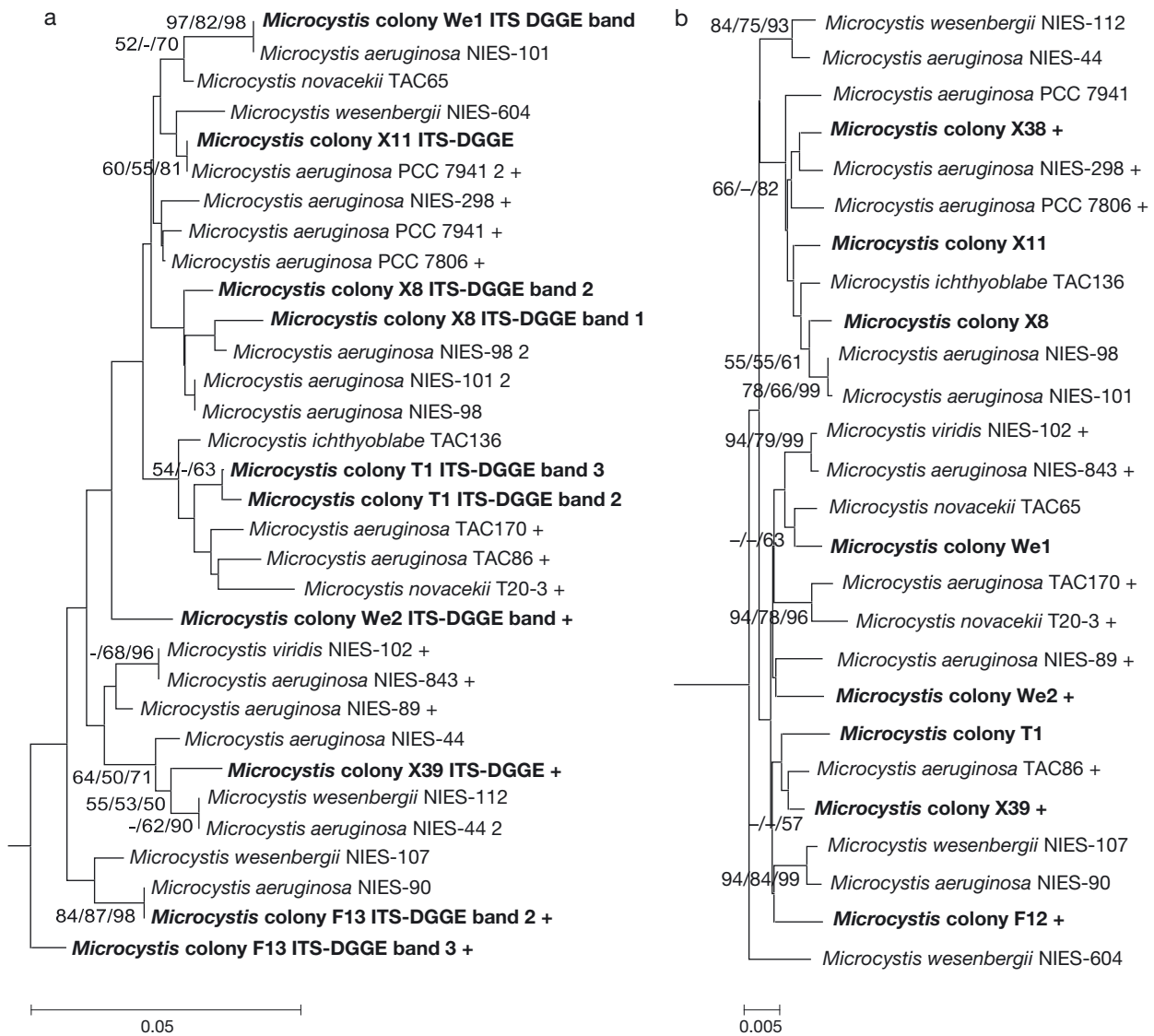
Fig. 2. *Microcystis*. (a) Distance tree of ITS sequences using the neighbor-joining method. Total alignment length was 460 nt. (b) Distance tree of concatenated sequences (*ftsZ*, *gltX* and *recA*) using the neighbor-joining method implemented in MEGA5 (Tamura et al. 2011). The total concatenated alignment contained 409 nt positions for *ftsZ* gene, 341 nt for *gltX* gene, and 446 nt for *recA* gene. For both trees, distances were computed using the maximum composite likelihood method. Maximum parsimony, maximum likelihood and neighbor-joining bootstrap replicates (1000 replicates) over 50% are indicated at the nodes. *Synechocystis* sp. PCC 6803 concatenated sequences were used as outgroup. '+' indicates that microcystins (MCs) were over the detection limit and at least 2 *mcy* genes were detected. Distance scale units are the number of base substitutions per site. For both trees, we considered *Microcystis* colony X40 as the same as colony X39 and *Microcystis* colony We3 as the same as colony We2

Those 3 housekeeping genes were successfully amplified and sequenced for 10 *Microcystis* colonies. It seems that each colony is a genetically homogeneous group of cells that share the same sequence type (ST). Indeed, in all chromatograms, neither ambiguities nor mixtures of sequences were observed after careful examination. This also suggests that cells from each colony have a clonal origin, in agreement with the ITS-DGGE results. All housekeeping gene sequences were highly similar (98.2 to 100%) to sequences obtained from the previous studies of *Microcystis* strains (Tanabe et al. 2007, 2009, Tanabe & Watanabe 2011; Table S3 in the Supplement). Description of the sequence types is given in Table 1. Analysis of the alignment of concatenated sequences (1196 bp) revealed 8 ST for 10 colonies. Moreover, *Microcystis* colonies X39 and X40 shared the same ST based on 3 loci. It seems likely that X40
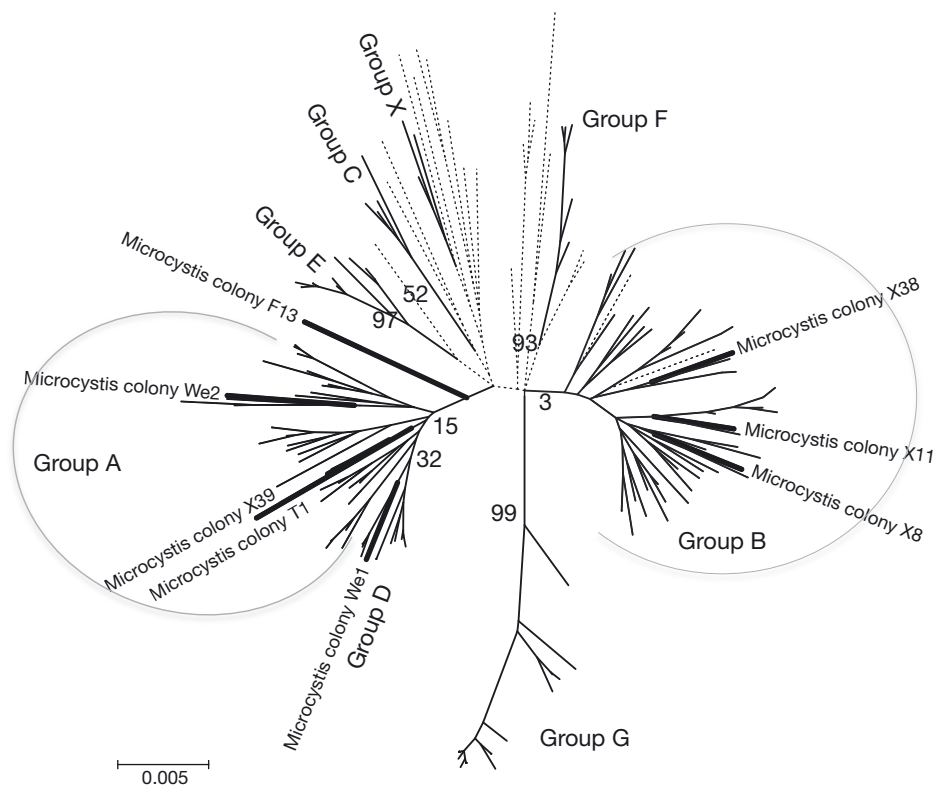
Fig. 3. *Microcystis.* Unrooted distance tree of 179 sequence types (STs) (group names after Tanabe & Watanabe 2011), plus the 8 Belgian STs (bold branches) identified in this study, using the neighbor-joining method implemented in MEGA5 (Tamura et al. 2011). Numbers in the tree indicate the bootstrap values at the groups' basal nodes. There were a total of 1192 nucleotides in the final dataset. The evolutionary distances were computed using the Maximum Composite Likelihood model and are in the units of the number of base substitutions per site. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions containing gaps and missing data were eliminated

was a sub-colony, with the same morphotype and genotype as X39. Colonies We2 and We3 also shared the same ST.

Using the 3 loci, we carried out a haplotype analysis using the dataset of Tanabe et al. (2007, 2009) and Tanabe & Watanabe (2011), and obtained 187 STs in total. The 8 Belgian STs differed from the 179 Asian STs. The phylogenetic analysis of 187 STs using the 3 concatenated loci (Fig. 3) resulted in the identification of the same groups (A to G and X), described by Tanabe et al. (2009) using 7 concatenated loci. Due to the low numbers of discriminating nucleotides in our alignment, rather low bootstrap values for groups A, B, C, D, E, F and X probably resulted, whereas group G was supported by a 99% bootstrap, as in Tanabe et al. (2009). Seven of the Belgian STs belonged to groups A, B, and D. Colonies X39, X40 and T1 were clustered into the complex of MC-producing strains which forms group A. Colonies X8, X11 and X38 were positioned in group B, which is composed of MC-producing and non-producing STs. Colony We1 clustered with the non-producing strains of group D. In Tanabe et al. (2009), groups A and D were only composed by STs from Asian strains. Group B included strains from 3 continents of the northern hemisphere. This finding suggests that groups A and D are also potentially widespread in this region.

The ST of colony F13 was not affiliated with any of the groups previously described (Fig. 3). F13 may thus be a representative of a novel *Microcystis* lineage.

In conclusion, the comparison between the phylogeny based on the ITS and housekeeping gene sequences from the same strains (Fig. 2) suggests that both types of taxonomic markers may have undergone independent evolution.

Woronichinia

Our attempts to cultivate strains of *Woronichinia* from fresh environmental samples were unsuccessful. Six *Woronichinia* colonies with a typical morphology (Komárek & Anagnostidis 1999) were isolated from Tervuren Pond. The colonies consisted of an internal system of radially and more or less parallel oriented, unbranched thick stalks connected to the cells and usually having the same width as the cells. The colonies were dense and embedded in mucilage.

Successful WGA were obtained for the 6 colonies, as shown by the subsequent cyanobacteria-specific PCRs (Table S2 in the Supplement). In order to validate the identification of the colonies on a molecular basis, we partially sequenced the 16S rRNA of T2
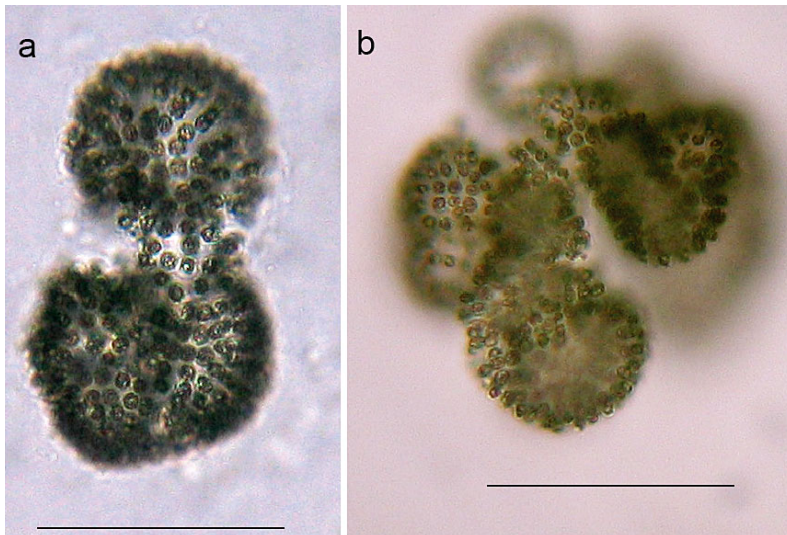
Fig. 4. *Woronichinia*. Colonies taken from water bodies in Belgium: (a) T2; (b) T5. Scale bars = 50 μm

cluster for which the primer sequences used for the PCR are not conserved.

For colony T2, a PCR product was detected with one of the 2 primer sets for *mcyE* (Rantala et al. 2004). The 689 bp amplicon was cloned and 2 inserts were sequenced, giving an identical sequence. Alignment and phylogenetic analysis of the sequenced fragments with all published *mcyE* sequences (Rantala et al. 2004) showed no relation to known *mcy* gene sequences (data not shown). BLAST (BLASTP) analysis showed no relatedness to *mcyE* but a 50% identity to a ketoacyl carrier protein synthase in *Microcystis* NIES843 (Kaneko et al. 2007). Beta-ketoacyl-ACP synthases are important enzymes involved in polyketide and fatty acid synthesis. This suggests the presence of a ketoacyl synthase (KS) region. This KS region may be part of a NRPS-PKS or PKS complex that may be involved in the formation of one or more unknown secondary metabolites.

and T5 colonies (Fig. 4). Both formed a subcluster with *Woronichinia naegeliana* 0LE35S01 (AJ781043) with a high bootstrap value in the 3 phylogenetic trees built with 3 methods (Fig. 5). Moreover, the sequences of colonies T2 and T5 were 99.8 and 100% similar to *Woronichinia* 0LE35S01, respectively. This cluster is part of the sub-family of Gomphosphaerioideae with another cluster formed by *Snowella* sequences, in agreement with the taxonomic system of Komárek & Anagnostidis (1999).

We performed the ELISA anti MC-LR for the colonies T2, T5, T8, T9, and T11 but not for T4, as the resuspended volume evaporated. MCs were not detected in colonies T2 and T11. In colonies T5, T8, and T9, 0.021 to 0.051 ng of MC-LR equivalents per colony was measured, but the *mcyA* and *mcyE* PCR reactions were negative (Table S2). Thus, it is not possible to infer whether the MC(s) detected was (were) a product of the colony or whether it/they had been acquired from the environment. An alternative explanation could be the presence of a *mcy* gene

Fig. 5. *Woronichinia*. 16S rRNA-based distance tree constructed using the neighbor-joining method implemented in MEGA5 (Tamura et al. 2011). There were a total of 994 nucleotides in the final dataset. Distances were computed using the Jukes and Cantor correction. Maximum parsimony, maximum likelihood and neighbor-joining bootstrap replicates (1000 replicates) over 50% are indicated at the nodes as percentages. The *Anabaena* sp. 90 16S rRNA sequence was used as outgroup. Sequences obtained during this study are in bold. Distance scale is expressed as the number of base substitutions

CONCLUSIONS

Two accurate methods—WGA and ELISA for MC immunoassay—were successfully applied for the concurrent detection of MC synthase genes and the quantification of MCs in single colonies of 2 cyanobacterial bloom-forming genera, *Microcystis* and *Woronichinia*. This approach allowed the detection and quantification of MCs in single environmental colonies of *Microcystis*, plus the characterization of their genotypes on the basis of the presence or absence of 3 *mcy* genes (*mcyA, mcyB*, and *mcyE*) and the sequences of 3 gene loci (*ftsZ, gltX*, and *recA*) in the same colonies. The STs of the colonies from Belgian water bodies were easily compared to the large analysis of strains from Asian waters obtained by Tanabe & Watanabe (2011). It resulted in the discovery of a potential cryptic lineage, represented by colony F13.

In addition, this approach yielded gene sequences from *Woronichinia*, a cyanobacterium that is difficult to isolate and maintain in laboratory culture. The 16S rRNA analysis confirmed colony identification. The first discovery of a polyketide synthase-like DNA sequence in one *Woronichinia* colony highlights the need for further study of this widely occurring genus for its ability to produce MCs and/or related metabolites.

Advances in microbial ecology can be hindered by problems with strain isolation and cultivation. In the last 10 yr, this problem has been solved to some extent by metagenomic approaches (e.g. Venter et al. 2004). However, the need to identify and characterize microbes, which are responsible for the production of antibiotics, toxins and other bioactive compounds in natural and anthropogenic environments, remains high. It is also mandatory to define the role that uncultivated organisms play in their environment. The approach presented here provides a high-resolution analysis from individual colonies of cyanobacteria taken directly from the environment.

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