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Biodiversity and seasonal variation of the cyanobacterial assemblage in a rice paddy field in Fujian, China

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Abstract

Cyanobacteria are one of the main components of the microbiota in rice paddy fields and significantly contribute to its fertilization. The diversity and changes of the cyanobacterial assemblage were investigated during a rice growth season and after harvest in a paddy field located in Fujian Province, China. The cyanobacterial populations were analyzed by a semi-nested PCR, followed by denaturing gradient gel electrophoresis analysis. Twenty-four phylotypes were identified from the denaturing gradient gel electrophoresis profiles. The number of cyanobacterial phylotypes showed a seasonal variation and reached a peak in September, both in the upper (0–5 cm) and the deeper (10–15 cm) soil fractions. Some cyanobacterial sequences were only present during the rice growth season, while others were only found after harvest.

Keywords: Biodiversity; Cyanobacteria; Paddy field soil; PCR-DGGE; 16S rRNA gene

1. Introduction

Biological nitrogen fixation performed by diazotrophs constitutes an important source of nitrogen in many ecosystems. In rice paddy fields, biological nitrogen fixation contributes up to 80 kg N ha^{-1} $year^{-1}$ [1]. Cyanobacteria are one of the main components of the microbiota in rice fields [2] and play an important role in the maintenance and build-up of soil fertility, consequently increasing rice growth and yield [1,3,4]. Experiments using cyanobacteria as biofertilizer have been undertaken, but the inabilities to produce good quality inocula and the difficulties of re-establishing the inoculated strains in the field have made large-scale use difficult [5]. To succeed in introducing cyanobacteria as a biofertilizer in rice paddy fields, an extensive knowledge of the indigenous populations is necessary. Our knowledge of the native cyanobacterial populations in rice fields has so far exclusively been achieved by means of cultivation-based analysis, followed by morphological identification of individual isolates [6–8]. Such indirect approaches have certain limitations for studying diversity in complex natural systems, as only a limited number of bacteria can be recovered from the soil by traditional cultivation techniques [9,10]. However, recent years application of molecular methods, such as 16S rRNA gene analysis for studying natural microbial communities, has increased our understanding of the complexity and dynamics of individual populations under different environmental conditions [9,11]. Molecular studies on methanotrophic

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and anaerobic microbial populations in rice field soil have been published in recent years [12–15]. In spite of the significant importance of cyanobacteria in the rice field ecosystem, no attempts have been made to apply molecular methods to study the structure of indigenous cyanobacterial populations in the paddy field. For investigation of cyanobacterial populations in natural habitats, primers targeting a specific region in the 16S rRNA gene were constructed by Nübel et al. [16]. The primers, which are specific for cyanobacteria and plastids have successfully been used for studying diversity and dynamics of populations in different ecosystems [9,17–19].

The aim of this study was to investigate the diversity of the indigenous cyanobacterial assemblage in the rice field and to follow the changes in the population structures during the rice growth season and after harvest. The cyanobacterial populations in two soil fractions were investigated; the upper 0–5 cm and deeper 10–15 cm. DNA from the soil fractions was analyzed by a semi-nested PCR, followed by separation on denaturing gradient gel electrophoresis (DGGE) and further sequencing of individual bands.

2. Materials and methods

2.1. Soil sampling and field management

Soil samples were collected from a 650 square meter rice field at The Wheat and Rice Research Institute, located outside Fuzhou, Fujian Province, South-East China (25°59′N; 119°22′E). In this field, rice was harvested twice a year with the first growth season from April to July and the second from July to October. In the period between rice crops (November–April), the field was not cultivated. The soil was mixed before transplanting and after harvest each growth season.

The field was fertilized according to local custom; 375 kg ha^{-1} ammonium bicarbonate and 225 kg ha^{-1} calcium perphosphate were spread before transplantation. Seven days after transplantation, 150 kg ha^{-1} urea were added into the field and after 15 and 50 days, 112.5 kg ha^{-1} urea (N:P:K (14:14:14)) were spread. In addition, 3.75 kg ha^{-1} of the herbicide Butachlor was applied seven days after transplantation.

A bulk soil sample was analyzed at the Soil and Fertilizer Institute of Fujian. The soil properties were: total N, 0.338% (w/w); total P, 0.027% (w/w); total K, 2.368% (w/w) and pH 5.9. The amount of available nitrogen (N), phosphorous (P) and potassium (K) were 251, 51 and 120 mg kg^{-1}, respectively. The concentrations of cadmium (Cd), and lead (Pb) were analyzed using atomic absorption spectrometry (AAS) and arsenic (As) and mercury (Hg) with atomic fluorescence spectrometry. The values were 1.718, 78.61, 2.18 and 0.274 mg kg^{-1}, respectively.

In September, the middle of the rice growth season, the field was submerged in water. In October, the soil was drained and stayed dry throughout January. The air temperature dropped from 30–35 °C in September to 4–15 °C in January. Soil samples were collected two times during the rice cultivation period, September 13 and October 15 (2001) and two times after harvest, November 10 (2001) and January 30 (2002).

Five sites in the field were marked and used at each sampling occasion. At each site, the upper 5 cm soil and a 10–15 cm deep sample were collected, using a 5 cm diameter plastic cylinder. The samples are referred to as upper soil fraction and deeper soil fraction. The collected samples were transported to the laboratory on ice and stored at −20 °C.

2.2. DNA extraction and purification

Total DNA was extracted from 5 g aliquots of bulk soil using the sodium dodecyl sulfate (SDS)/hexadecylmethylammonium bromide (CTAB) method described by Zhou et al. [20]. To avoid interference from humic acids during PCR amplification, DNA was further purified by gel electrophoresis in 1% (w/v) low-melting (LM) agarose at 85 V for 45 min, at 4 °C. The DNA band was excised from the gel and further extracted by incubation for 5 min at 65 °C in Tris–EDTA (TE) buffer, followed by phenol–chloroform purification. The precipitated DNA was resuspended in 20 μl of TE buffer and stored at −20 °C.

2.3. PCR amplification

Different PCR protocols for the amplification of the 16S rRNA gene fragment were tested for optimization. A semi-nested PCR set-up was found to be the most efficient method for amplification of the variable V3–V4 region of the 16S rRNA gene. The approach was chosen in order to get sufficient PCR products from all of the samples. In order to eliminate the accumulation of primer-dimers during the PCR, due to a six bp overlap between the cyanobacteria-specific PCR primers CYA359F and CYA781R used in the second PCR (Table 1), different primer concentrations ranging from 0.125 to 2 pmol reaction^{-1} were tested (data not shown). The optimal primer concentration was found at 0.16 pmol reaction^{-1} for each primer. The first PCR was performed using primers CYA359F and 16S-1051R (Table 1), producing the expected 711 bp fragment. The PCR was carried out in 25-μl volumes, containing 0.1 μl purified DNA template, 1× GeneAmp PCR Buffer II (Applied Biosystems, Warrington, UK), 2 mM $MgCl_2$, 200 μM dNTP, 2 μM of each primer and 0.025 U μl^{-1} AmpliTaq Gold (Applied Biosystems,

Table 1
Primers used during amplifying and sequencing of the 16S rRNA gene

Primer	Target site[a]	Sequence (5′–3′)	Reference/designed by
CYA359F[b]	359–378	GGG GAA TYT TCC GCA ATG GG	Nübel et al. [16]
16S-1051R	1051–1070	GCT GGC AAC TAA AAA CGA GG	This study
CYA781R(a)[c]	781–805	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel et al. [16]
CYA781R(b)[c]	781–805	GAC TAC AGG GGT ATC TAA TCC CTT T	Nübel et al. [16]

[a] *E. coli* numbering of 16S rRNA nucleotides.
[b] In PCR for DGGE analysis, a 40-nucleotide GC-rich sequence (5′-CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG CCC G-3′) was attached to the 5′end.
[c] Reverse primer CYA781R was an equimolar mixture of CYA781R(a) and CYA781R(b).

Warrington, UK). The thermal PCR profile was as follows: initial denaturation for 10 min at 95 °C, followed by 35 incubation cycles each consisting of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a final elongation step at 72 °C for 10 min. 0.1 μl of the PCR product was used as template in a subsequent PCR, performed in 50 μl volume under the same conditions as above, except using different primers: CYA359F (with a 40-nucleotide GC rich sequence attached to the 5′ end) and CYA781R (0.16 pmol reaction^{-1} each). The annealing temperature in the PCR profile was increased to 60 °C.

2.4. Analysis of PCR products by DGGE

DGGE was performed using the Dcode universal mutation detection system as described in the manufacturer's manual (Bio-Rad, Hercules, CA, USA). Aliquots (40 μl) of the 446 bp PCR products were combined with 10 μl of 5× DGGE loading buffer (12.5% Ficoll, 25 mM Tris, 5 mM EDTA, 0.5% SDS, 0.1% bromphenol blue, and 0.1% xylene cyanol) and applied directly onto a 6% (w/v) polyacrylamide gel in 0.5× Tris–acetate–EDTA (TAE) buffer (20 mM Tris-base, 10 mM acetic acid and 5 mM EDTA [pH 8.0]) with a linear 20–60% denaturant gradient (100% denaturation was defined as 7 M urea and 40% [v/v] formamide). DGGE was carried out at 60 °C (constant temperature) for 16 h at 75 V. Two parallel gels were run; one was stained in Vistra Green Nucleic Acid Stain (Amersham Biosciences, Freiburg, Germany) (1000 times diluted in 0.5× TAE buffer) for 10 min, in accordance to the instruction by the manufacturer, and scanned with Typhoon 8600 (Amersham Biosciences, Freiburg, Germany) (excitation 490 nm, emission 520 nm, PMT voltage 700–800 V) for image analysis. The second gel was stained for 8 min with ethidium bromide (1 μg ml^{-1} in 0.5× TAE) and visualized by UV illumination for further excision of the bands from the gel. A genetic marker containing PCR products of three cultured cyanobacteria (*Nostoc* strains PCC 9229, PCC 73102 and PCC 7422) was included as genetic marker on each gel alongside the environmental samples (not shown).

2.5. Re-amplification and sequencing of 16S rRNA gene fragments

From the gels, 140 DGGE bands were carefully excised using a sterile surgical scalpel. DNA from the excised bands was extracted by incubation overnight at 37 °C in 0.3 M NaCl, 3 mM EDTA and 30 mM Tris (pH 7.6), according to Rölleke et al. [21]. The purity of the excised band was confirmed by re-amplification of the eluted DNA with the primer pair CYA359F (with GC clamp) and CYA781R, subsequently followed by DGGE as described above. Only products that resulted in a single band, with the predicted mobility, were further processed. Those samples were separated from the primer-dimer band by 1.5% agarose gel electrophoresis. The specific DNA product was excised and purified with the QIAquick gel extraction Kit (Qiagen, Hilden, Germany) and cloned using the Topo TA Cloning Kit (Invitrogen, Paisley, UK) in accordance with the manufacturers' instructions. The cloning was performed to insure high quality sequencing from a single product. Extracted plasmid DNA was purified using the QIA prep Spin Miniprep Kit (Qiagen, Hilden, Germany), following the manufacturer's instruction. 200–400 ng of purified plasmid was used as template in sequencing reactions by applying the ABI prism BigDye Terminatior Cycle Sequencing Ready reaction Kit (Applied Biosystems, Warrington, UK). Sequences of the plasmid insert were determined by using the CYA359F and CYA781R primers. Products were analyzed using an Applied Biosystems 377 automated DNA Sequencer.

2.6. Determination of relationships

The sequences obtained were manually corrected by comparing forward and reverse sequences. The length of the corrected sequences varied between 352 and 387 bp. The sequences were initially aligned in the SDSC Biology Workbench (http://workbench.sdsc.edu) using the Clustal W function [22]. Identical sequences with the same migration on DGGE were treated as one. Further manual amendments to the alignment were performed using Se–Al (http://evolve.zoo.ox.ac.uk).

The 36 different sequences (phylotypes) considered in the alignment were subjected to a NCBI BLASTN search (http://ncbi.nlm.nih.gov/blast/) to get an initial assessment of sequence similarity.

Additionally, 53 full-length 16S rRNA sequences were obtained from Genbank and added to the alignment. Of these, 5 were non-cyanobacterial bacteria; 7 were chloroplasts-covering diatoms (*Bacillaria*, *Lauderia*), green algae (*Chlamydomonas*, *Chlorella*), a land plant relative (*Coleochaete*), a fern (*Adiantum*) and a flowering plant (*Oryza*); and 41 were cyanobacteria selected from the five main cyanobacterial sections [23].

The general time reversible model [24,25] with gamma distribution of rates [26] (GTR + G) was selected for the data set by using MrAIC (http://www.ebc.uu.se/systzoo/staff/nylander.html) and PHYML [27] with the AICc criterion. A phylogeny was estimated in MrBayes [28] ver. 3 under Linux. Two separate analyses were performed starting from flat priors and a random tree, each running 2,000,000 generations of the Markov Chain (Monte Carlo) with six separate chains and a chain temperature setting of 0.1. Sampling frequency was set to once every 100 generations and the initial 7500 samples (the chain "burnin") were discarded before summarizing the results. The tree presented was based on graphical output from TreeView [29].

2.7. Nucleotide sequence accession numbers

The sequences obtained in this study are available in EMBL under accession numbers AJ889085–AJ889108 (bands 1–24), AJ889109–AJ889113 (bands I–V) and AJ889114–AJ889120 (bands A–G).

3. Results and discussion

In the present study, a seasonal variation in the cyanobacterial populations could be demonstrated considering both soil depth and time. Since the rice field soil is mixed several times during the year (after harvest and before transplanting each growth season), samples from the upper (0–5 cm) as well as the deeper (10–15 cm) soil fractions were analyzed in order to obtain information on the total available cyanobacterial populations in the soil. The DGGE fingerprint profiles presented in Figs. 1 and 2 reveal higher cyanobacterial diversity in the deeper soil fraction compared to the upper soil frac-

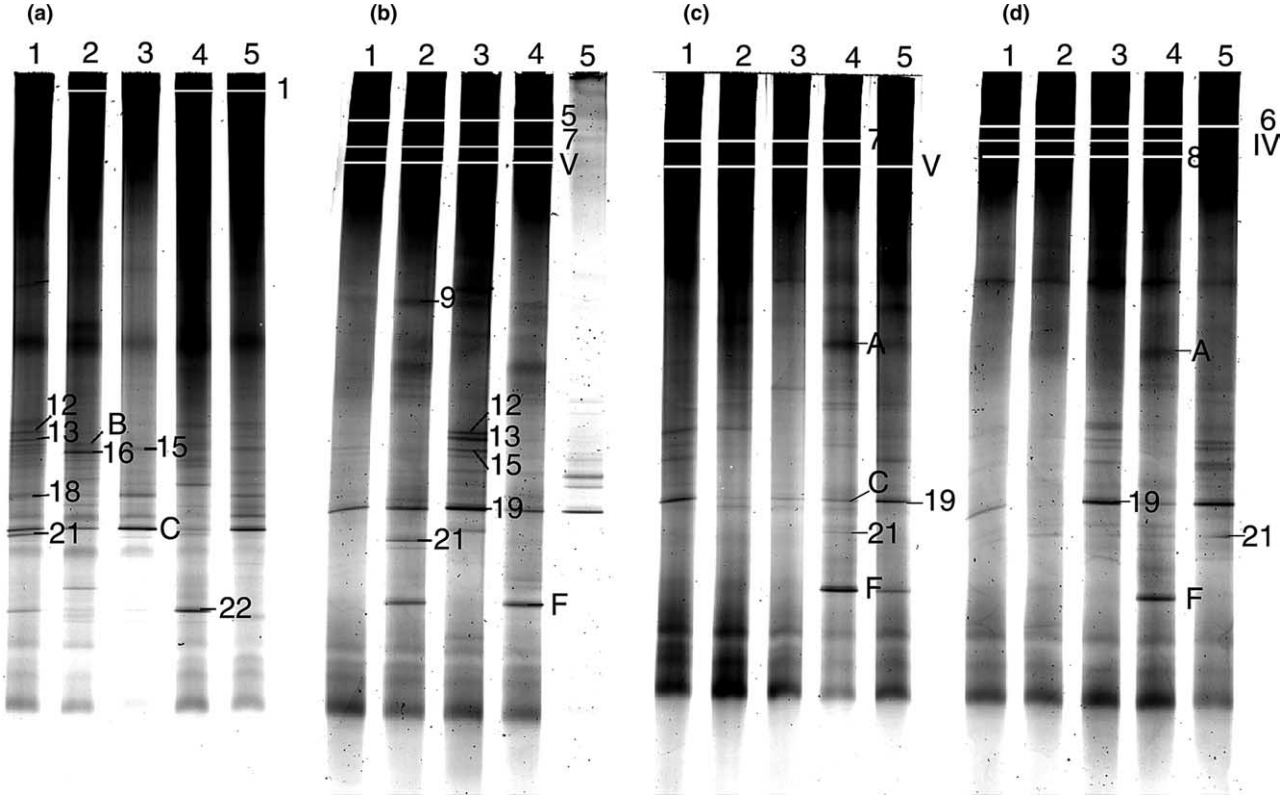

Fig. 1. DGGE fingerprint obtained with cyanobacterial specific primers targeting the 16S rRNA region of DNA extracted from rice field soil at 0–5 cm depth. (a) September 13, 2001, (b) October 15, 2001, (c) November 10, 2001 and (d) January 30, 2002. Numbers 1–24 indicate cyanobacteria, I–V diatom chloroplasts and A–H plant chloroplasts. Lanes 1–5 represent the five different sampling points in the paddy field.

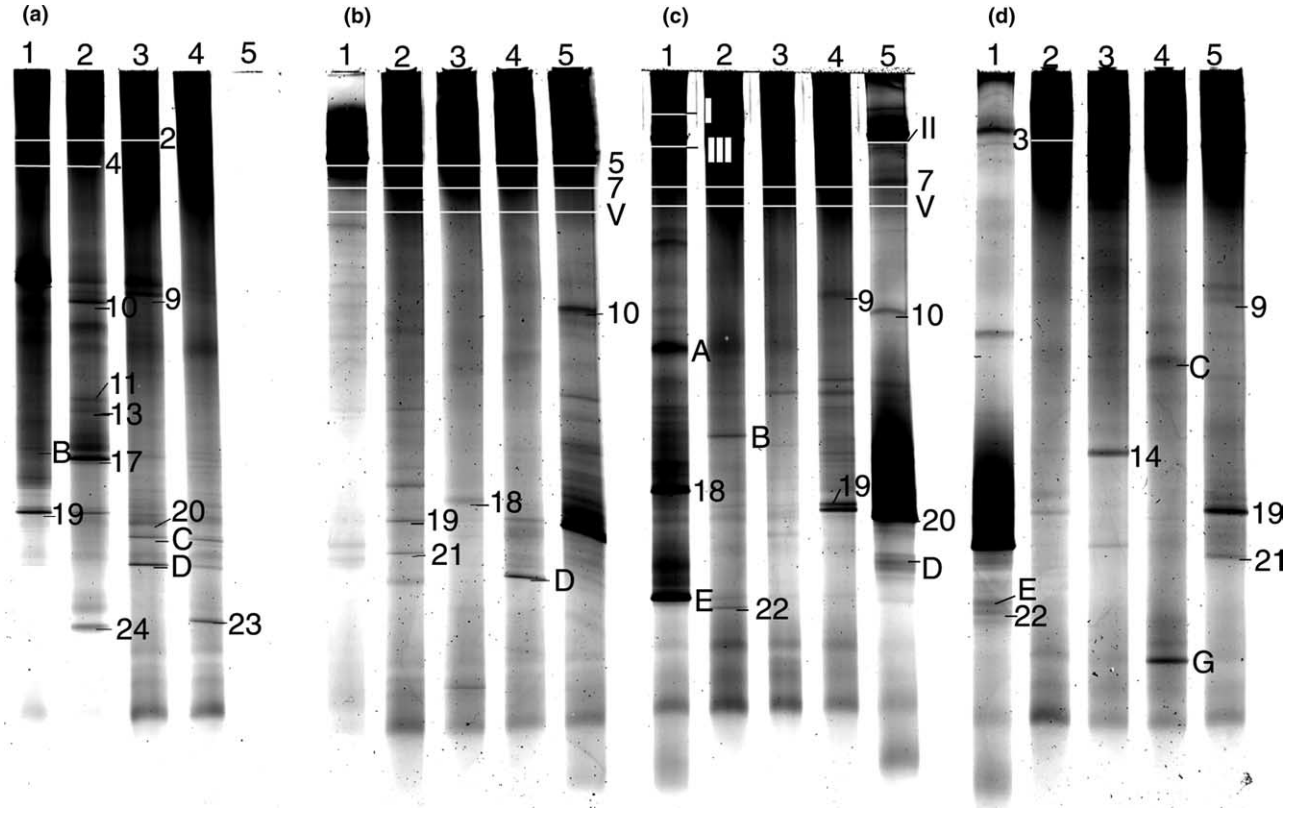

Fig. 2. DGGE fingerprint obtained with cyanobacterial specific primers targeting the 16S rRNA region of DNA extracted from rice field soil at 10–15 cm depth. (a) September 13, 2001, (b) October 15, 2001, (c) November 10, 2001 and (d) January 30, 2002. Numbers 1–24 indicate cyanobacteria, I–V diatom chloroplasts and A–H plant chloroplasts. Lanes 1–5 represent the five different sampling points in the paddy field.

tion. In addition, the highest diversity was found in the middle of the growth season and the lowest after harvest.

3.1. DGGE sequence identity

The closest sequence similarity of the excised and sequenced DGGE bands, shown in Figs. 1 and 2, revealed 36 different phylotypes using NCBI BLASTN (Table 2). All of the sequences obtained were of cyanobacterial or plastid origin, verifying the specificity of the primers described by Nübel et al. [16]. Five of the phylotypes had closest identity to the 16S rRNA gene from diatom chloroplasts (I–V), seven to chloroplasts from plants (A–G) and 24 to cyanobacteria (1–24).

According to the closest sequence similarity to cultured strains using BLASTN (Table 2) the cyanobacterial phylotypes were represented by 11 genera, two filamentous heterocystous (*Nostoc*, *Scytonema*), five filamentous non-heterocystous (*Leptolyngbya*, *Phormidium*, *Microcoleus*, *Spirulina*, *Chroococcidiopsis*) and four unicellular (*Synechococcus*, *Cyanothece*, *Chamaesiphon*, *Synechosystis*). Nine of the sequences had closest identity to the genus *Leptolyngbya*, four to the genus *Nostoc*, three to the genus *Synechococcus* and one to each of the genera *Chamaesiphon*, *Chroococcidiopsis*, *Cyanothece*, *Microcoleus*, *Phormidium*, *Scytonema*, *Spirulina* and *Synechosystis*.

In the phylogenetic analysis using MrBayes (Fig. 4) most of the cyanobacterial phylotypes were found within six distinct clades: I (*Synechococcus*), II (*Synechosystis*), III (*Nostoc*/*Anabaena*), IV (*Phormidium*), V and VI (*Leptolyngbya*). The credibility values were 1.0 for clade III, IV, V and VI, and 0.92 and 0.97 for clade I and II, respectively. In addition, the green plant chloroplasts (clade VII) and the diatom chloroplasts (clade VIII) show a clade credibility value of 1.0. This result strongly indicates the accuracy of the BLASTN results.

However, the identity of some bands (band 1, 7, 11, 12, 13, and 15), showing a low sequence similarity (90–93%) using BLASTN search, could not be verified with a strong credibility value to any defined genera in the phylogenetic analysis. However, all of them are well-nested within the cyanobacterial group. Moreover, bands 3, 8, and IV are all found in clade VI (*Leptolyngbya*) and not with the closest cultured match, according to sequence similarity (Table 2).

Three of the genera (*Nostoc*, *Phormidium*, and *Synechosystis*) have previously been described as common cyanobacteria in rice field soil, and have been isolated from various rice fields located in India, Bangladesh,

Table 2
Amplified 16S rRNA gene fragments were excised from DGGE, cloned and sequenced

Band	No. of bases	Closest cultured match		
		Description	GeneBank accession number	Sequence similarity % (no. of bases)
1	377	*Microcoleus steenstrupii*	AF355395	91 (375)
2	383	*Synechococcus* sp.	AF448075	95 (382)
3	373	*Cyanothece* sp.	AB067581	92 (335)
4	384	*Phormidium autumnale*	AF218371	92 (381)
5	378	*Leptolyngbya* sp.	AY239603	93 (376)
6	352	*Nostoc sp.*	AF506243	96 (352)
7	373	*Chamaesiphon subglobosus*	AY170472	90 (360)
8	373	*Spirulina* sp.	X75045	92 (321)
9	369	*Leptolyngbya* sp.	AY269600	90 (367)
10	378	*Nostoc* sp.	AF506238	100 (378)
11	387	*Scytonema hofmanni*	AF132781	91 (383)
12	387	*Synechococcus* sp.	AF448074	93 (387)
13	372	*Synechococcus* sp.	AF448074	91 (373)
14	383	*Leptolyngbya* sp.	AF317507	91 (385)
15	374	*Chroococcidiopsis* sp.	AJ344557	92 (375)
16	371	*Leptolyngbya* sp.	AY239602	94 (371)
17	385	*Synechosystis* sp.	AY224195	94 (385)
18	385	*Leptolyngbya* sp.	AY239600	92 (381)
19	380	*Leptolyngbya* sp.	AF317507	92 (382)
20	382	*Leptolyngbya* sp.	AY239602	95 (381)
21	384	*Leptolyngbya* sp.	AY239603	94 (375)
22	381	*Nostoc* sp.	AF506238	100 (381)
23	377	*Leptolyngbya* sp.	AY239603	95 (377)
24	381	*Nostoc* sp.	AF506238	99 (381)
I	380	*Haslea wawrikae*	AF514855	98 (380)
II	386	*Lauderia borealis*	AJ536459	92 (314)
III	379	*Gyrosigma fasciola*	AF514847	98 (379)
IV	381	*Lauderia borealis*	AJ536459	91 (305)
V	380	*Bacillaria paxillifer*	AJ536452	97 (379)
A	386	*Tobacco* chloroplast	V00165	95 (382)
B	386	*Magnolia* chloroplast	AF244557	95 (386)
C	370	*Calycanthus* chloroplast	AJ428413	97 (348)
D	384	*Calycanthus* chloroplast	AJ428413	99 (385)
E	381	*Tobacco* chloroplast	V00165	99 (382)
F	385	*Calycanthus* chloroplast	AJ428413	99 (386)
G	386	*Physcomitrella* chloroplast	AP005672	100 (386)

The identity of the sequences was determined using NCBI BLASTN search.
Numbers 1–24 indicate cyanobacteria, I–V diatom chloroplasts and A–G plant chloroplasts.

Spain and Uruguay [6–8,30]. In those studies, which are based on culture-dependent methods, the heterocystous cyanobacteria were the most abundant genera, comprising 50% from rice fields in Bangladesh and 95% from rice fields in Spain [6,8]. In the present study, a much lower frequency of heterocystous cyanobacteria was observed. According to the BLASTN results and the phylogenetic analysis, only 18% of the cyanobacterial phylotypes had closest sequence similarity to heterocystous cyanobacteria. Even though a direct comparison is difficult to do since different methods have been used, it cannot be excluded that the dominance of heterocystous cyanobacteria has been overestimated in the previous studies, based on a selection during culturing. Thus, as indicated from our results, the cyanobacterial assemblage in the paddy field might constitute highly complex populations.

However, the use of molecular approaches for studying soil microbial communities has limits and drawbacks [31]. Differences in the relative abundance of the target microorganisms may result in the inability to detect certain organisms due to competition during the PCR, and the generation of bias is inevitable in PCR reactions. In this study, a non-quantitative PCR approach was used and therefore we can not make any conclusions on the abundance of particular species. Nevertheless, this approach gives very valuable information on the composition and diversity of the cyanobacterial populations.

3.2. Cyanobacterial diversity and dynamics

The distribution and abundance of cyanobacteria in the paddy field are known to be influenced by numerous environmental factors such as soil properties, water

availability, light intensity, chemical fertilizers and pesticides [6–8,30,32,33]. In this study, a seasonal variation in the cyanobacterial populations could be demonstrated. The cyanobacterial diversity (total number of phylotypes) was highest in the deeper soil samples where a total of 18 different sequence types were observed, compared to 14 from the upper soil samples (Fig. 3). Moreover, most cyanobacterial phylotypes were found in September, both in the upper (eight different types) and the deeper soil fraction (11 different types). At this time, the field was submerged with approximately 10 cm of water and the light intensity at the soil surface was low, due to the rice canopy. In contrast, in November and January (after harvest) when the soil was dry and the surface exposed to full sunlight, the number of different phylotypes declined to three and four in the upper and seven and six in the deeper soil fraction, respectively (Fig. 3). This change indicates that water availability, light intensity and temperature are important and might be the main factors regulating the cyanobacterial populations. Changes in the natural cyanobacterial populations in rice fields have previously shown to be influenced by light intensity [33,34].

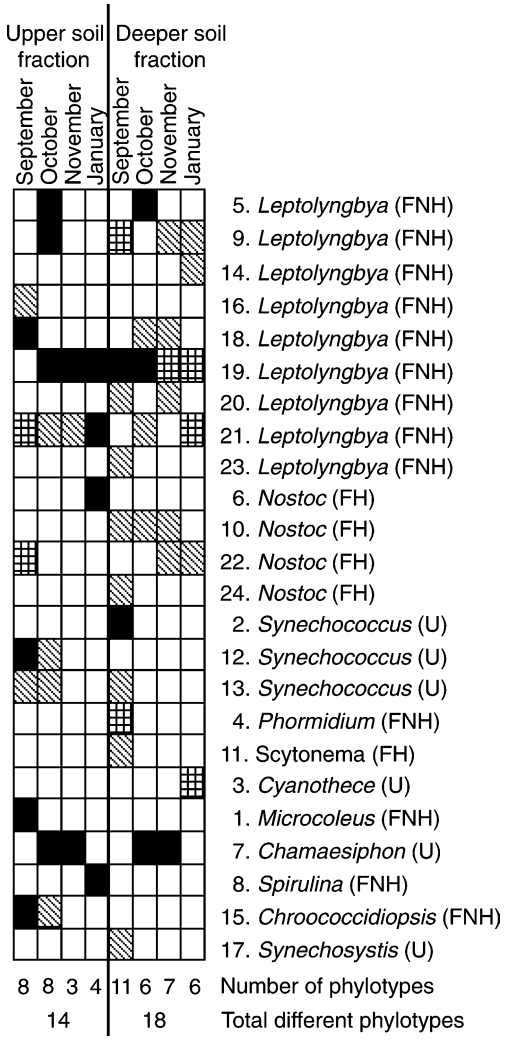

Fig. 3. Distribution of different cyanobacterial phylotypes with regard to sampling time and soil depth. The numbers indicate the different bands on the DGGE gel (Figs. 1 and 2), and the names refer to the closest cultured match using BLASTN search (Table 2). Abbreviations: Filamentous non-heterocystous (FNH), filamentous heterocystous (FH), unicellular (U). Different patterns indicate the number of samples where the strains have been identified: 3–5 samples (black), 2 samples (checked) and 1 sample (striped).

Among the different genera found, phylotypes of *Leptolyngbya* and *Nostoc* were found throughout the whole sampling period, either in the upper or deeper soil fractions, or in both (Fig. 3). In contrast, *Synechococcus*, *Phormidium* and *Synechosystis* showed a restricted appearance, both temporally and spatially. Most of the cyanobacterial phylotypes, which show a restricted time appearance, were commonly found in more than three sampling points in the field. Moreover, the individual phylotypes within the genera *Nostoc* and *Leptolyngbya* show a specific appearance, either temporal or spatial (Fig. 3).

As seen in Table 2, nine of the phylotypes show closest sequence similarity to *Leptolyngbya*, and in the phylogenetic analysis 12 phylotypes grouped with *Leptolyngbya* (Fig. 4). To our knowledge, the genus *Leptolyngbya* has never been reported in rice fields before, but is common in freshwater environments. *Leptolyngbya* is morphologically very similar to *Lyngbya* [23], which has been reported and isolated from rice fields in Bangladesh, India and Uruguay [6,7,30]. However, none of the sequenced bands from this study show any match to sequences of *Lyngbya*, either in the BLASTN search (Table 2) or in the phylogenetic analysis (Fig. 4). Consequently, the possibility exists that some previous isolates might have been identified as *Lyngbya* instead of *Leptolyngbya*. Additionally, *Leptolyngbya* has been observed abundantly in polluted freshwater [35]. The soil had an elevated value of cadmium, 1.718 mg kg^{-1}, compared to average soil concentration of 0.5 mg kg^{-1} [36]. This might have influenced the cyanobacterial population and in particular the presence of *Leptolyngbya*, as studies have indicated a genera/species dependent effect of cadmium and other heavy metals on cyanobacteria. For instance, *Anabaena*, *Microcystis* and *Nostoc* were inhibited in growth and nitrogenase activity by cadmium [37,38], whereas other cyanobacteria such as *Gleoethece* and *Synechococcus* have a high tolerance and therefore might be potential bioremediators of contaminated soils [39,40]. Furthermore, the unicellular genus *Synecococcus* has not been observed previously in rice fields. However, the absence of this genus in earlier studies could be due to a local diversity, as well as to the time of sampling in respect to the rice growth season, since it shows a rather restricted period of appearance.

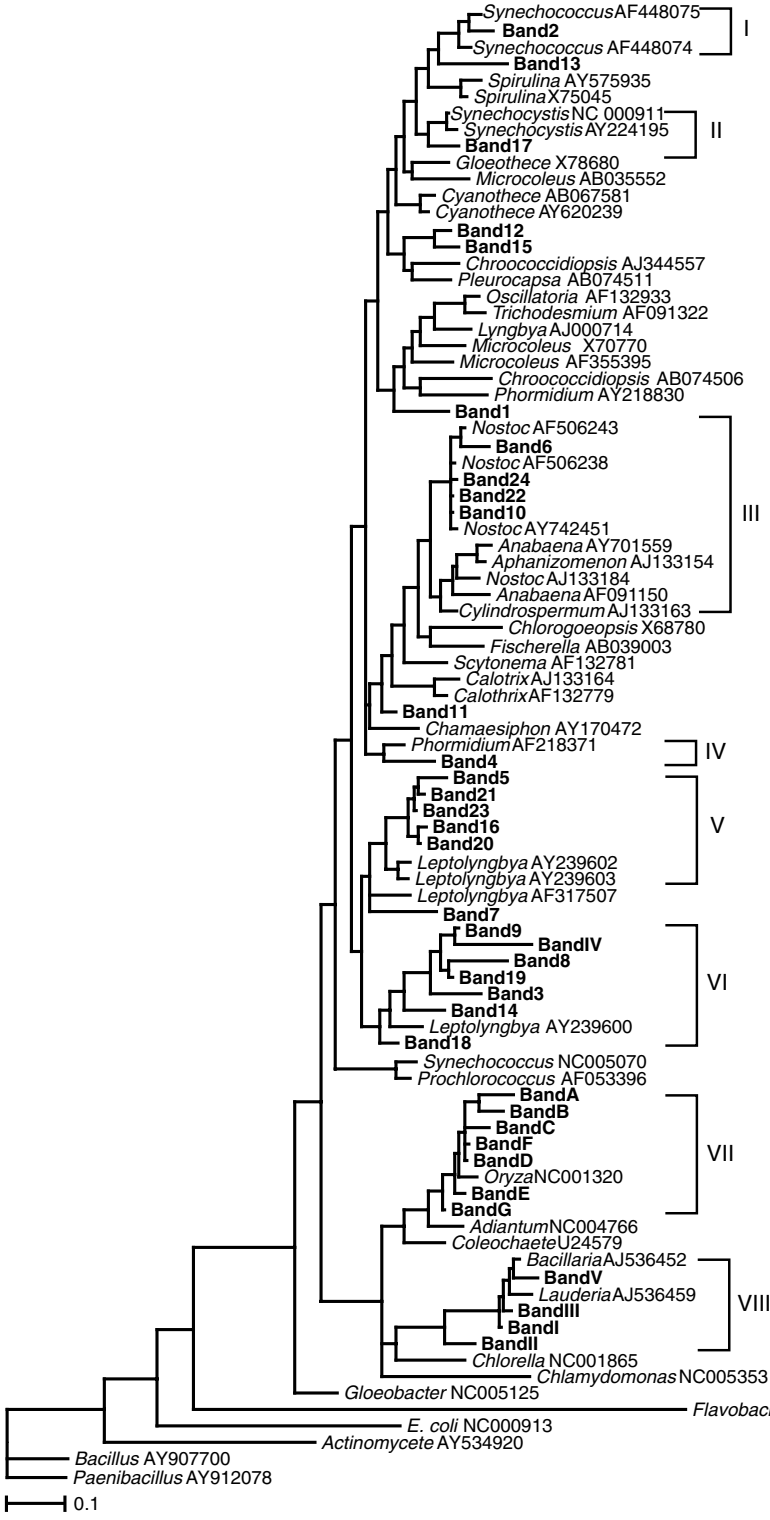

Fig. 4. Estimated phylogeny from the MrBayes analysis. Majority-rule consensus tree (50%) based on the 12,500 trees sampled after initial "burnin" of the Markov Chain. Branch lengths are drawn proportional to the mean estimated change (scale bar is 0.1 substitutions per site). All sequences generated in this study are indicated in bold face. Band numbers 1–24 indicate cyanobacteria, I–V diatom chloroplasts and A–H plant chloroplasts, according to the sequence similarity using BLASTN (Table 2). The tree was rooted on the branch to *Bacillus subtilis*.

In conclusion, we have shown that great phylotype diversity exists in the cyanobacterial populations in the rice paddy field, considering both sampling time and soil depth. Molecular methods have been used to identify 24 cyanobacterial phylotypes. This is, to our knowledge, the first time that a direct molecular approach has been

used to investigate the indigenous cyanobacterial populations in a rice paddy field. Our results show that such an approach is appropriate for studying the dynamics of cyanobacteria in a complex microbial system.

Acknowledgments

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