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Diversity of microcystin genotypes among populations of the filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*

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Abstract

Microcystins (MCs) are toxic heptapeptides that are produced by filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii via* nonribosomal peptide synthesis. MCs share a common structure cyclo (-D-Alanine[1]-L-X[2]- D-erythro-ß-iso-aspartic acid[3]-L-Z[4]-Adda[5]-D-Glutamate[6]- *N*-methyl-dehydroalanine[7]) where X[2] and Z[2] are variable L-amino acids in positions 2, 4 of the molecule. Part of the *mcy*B gene (1,451 bp) that is involved in the activation of the X[2] amino acid during MC synthesis was sequenced in 49 strains containing different proportions of arginine, homotyrosine, and leucine in position 2 of the MC molecule. Twenty-five genotypes were found that consisted of eight genotype groups (A-H, comprising 2-11 strains) and 17 unique genotypes. *P. rubescens* and *P. agardhii* partly consisted of the same *mcy*B genotypes. The occurrence of numerous putative recombination events that affected all of the genotypes can explain the conflict between taxonomy and *mcy*B genotype distribution. Genotypes B (homotyrosine and leucine in X[2]) and C (arginine in X[2]) showed higher nonsynonymous/synonymous (d_N/d_S) substitution ratios implying a relaxation of selective constraints. In contrast, other genotypes (arginine, leucine, homotyrosine) showed lowest d_N/d_S ratios implying purifying selection. Restriction fragment length polymorphism (RFLP) revealed the unambiguous identification of *mcy*B genotypes, which are indicative of variable X[2] amino acids in eight populations of *P. rubescens* in the Alps (Austria, Germany, and Switzerland). The populations were found to differ significantly in the proportion of specific genotypes and the number of genotypes that occurred over several years. It is concluded that spatial isolation might favour the genetic divergence of microcystin synthesis in *Planktothrix* spp.

Keywords: geographical isolation, microcystin synthesis, microevolution, plankton ecology, population genetics, toxicity

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Introduction

Toxic heptapeptides microcystins are produced by various genera of the phylum of cyanobacteria, i.e. the genera *Microcystis*, *Anabaena*, and *Planktothrix*. The filamentous cyanobacteria *Planktothrix* spp. are one of the most important microcystin producers and can be found in freshwater habitats in the temperate region of the Northern Hemisphere (Fastner *et al*. 1999). *Planktothrix agardhii* co-occurs with *Limnothrix redekei* and other filamentous cyanobacteria, e.g. *Pseudoanabaena limnetica* in high abundance in shallow and eutrophic lakes (Rücker *et al*. 1997). In contrast *Planktothrix rubescens* forms metalimnetic layers in deep stratified and less eutrophic lakes, and often monopolizes resources and dominates phytoplankton completely (Anneville *et al*. 2004).

Microcystins (MCs) are cyclic heptapeptides that are produced by cyanobacteria and share a common structure cyclo (-D-Ala[1] -L-X[2] -D-MeAsp[3] -L-Z[4] -Adda[5] -D-Glu[6] -Mdha[7]), where X and Z are variable L-amino acids (e.g. LR refers to leucine and arginine in the variable positions), D-MeAsp is D-erythro-β-iso-aspartic acid, Adda is (2 *S*, 3 *S*, 8

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S, 9 *S* -3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), and Mdha is N-methyl-dehydroalanine (Carmichael *et al*. 1988). MCs are synthesized by thiotemplate mechanism such as other nonribosomal peptides produced by bacteria and fungi (Marahiel *et al*. 1997). The large enzyme complex encoded by the *mcy* gene cluster is composed of peptide synthetases, polyketide synthases, and tailoring enzymes (Tillett *et al*. 2000; Christiansen *et al*. 2003). It has a modular structure, each module of which contains specific functional domains for activation = aminoacyl adenylation (A) domains, and thioesterification (thiolation domains) of the amino acid substrate and for the elongation (condensation domains) of the growing peptide (Tillett *et al*. 2000). Adenylation domains show a high degree of conservation (core motifs) enabling the definition of general rules for the structural basis of substrate recognition of nonribosomal peptide synthetases (Marahiel *et al*. 1997). The determination of substrate specificity is based on the crystallization of the adenylation domain of gramicidin synthetase (*Grs*A) (Conti *et al*. 1997). This enabled the identification of core motifs constituting a putative binding pocket for the activation of the amino acid substrate during peptide synthesis and the *in silico* identification of critical binding pocket residues that are located between core motifs A3-A6 (Stachelhaus *et al*. 1999; Challis *et al*. 2000).

*Mcy*BA1 is responsible for the activation of amino acids as aminoacyl adenylate followed by peptide bond formation to the growing microcystin molecule by the condensation domain located upstream of *mcy*BA1 (Tillett *et al*. 2000). In *Planktothrix* spp., three different amino acids have been described in position 2 of the microcystin molecule: leucine, arginine, homotyrosine (Luukkainen *et al*. 1993; Henriksen & Moestrup 1997; Fastner *et al*. 1999; Kurmayer *et al*. 2005). In an earlier study, we described the occurrence of novel microcystin variants, which appeared to be unique and dominant for *Planktothrix* and that exclusively occurred in a specific lake, Schwarzensee in the Austrian Alps (Kurmayer *et al*. 2004). It has been speculated that the populations of *P. rubescens* may be geographically isolated; for example Schwarzensee, the lake of origin of the isolates containing those novel microcystin variants is at an altitude of 716 m above sea level (a.s.l.), while many other lakes have a lower altitude, such as Mondsee, which is located only 11.4 km away.

Typically, the distribution of microorganisms is not restricted by geographical barriers (Finlay 2002). Studies have shown that recombination and migration rates in natural populations of soil bacteria (*Bacillus* spp.) and aquatic cyanobacteria (*Microcoleus* sp., *Nodularia* sp.) were high, and were not influenced by geographical factors (Roberts & Cohan 1995; Barker *et al*. 2000; Lodders *et al*. 2005). On the other hand, the occurrence of physical isolation, which is defined as the spatial separation of two or more populations among geographically isolated habitats (Papke & Ward 2004) cannot a priori be excluded. Geographical isolation has been observed among isolated populations of extreme habitats, for example, *Synechococcus* inhabiting hot springs (Papke *et al*. 2003) and hyperthermophilic Archaea (Whitaker *et al*. 2003), but also for free living *Pseudomonas* spp. in pristine soil samples (Cho & Tiedje 2000). The question of isolation is of fundamental importance to understand the evolution of microcystin synthesis, i.e. populations may diverge through neutral processes (i.e. random drift, Kimura 1983), local adaptation processes, or both.

The ability to detect and interpret genotypic variation depends on the type of the gene under investigation and the resolving power of the genetic markers. Genes showing high variability with potential functional consequences are considered highly suitable to interpret genotypic variation in potentially isolated populations (Palys *et al*. 1997). The species *P. rubescens* (*sensu* Suda *et al*. 2002) only consists of microcystin genotypes while — similar to *Microcystis* sp. — populations assigned to *P. agardhii* consisted of microcystin genotypes and genotypes without the *mcy* genes (Kurmayer *et al*. 2004). It has been shown that *Microcystis* sp. colonies that were isolated from field samples of a eutrophic lake show a high genetic variability within *mcy*BA1 (Kurmayer *et al*. 2002), for example, due to domain duplication and recombination within the *mcy*B gene (Mikalsen *et al*. 2003).

It was the aim of the present study to: (i) analyse the genetic variation within strains, (ii) identify genotypes that are indicative of the production of specific MC variants, (iii) and directly detect and quantify those genotypes in various lakes in the Alps. This knowledge is necessary to understand MC net production in phytoplankton as well as the microevolution of microcystin synthesis in cyanobacteria. For this purpose, 49 *Planktothrix* strains isolated in Europe were sequenced for the first adenylation domain (A1) of the *mcy*B gene. In order to characterize populations directly (and to overcome a possible isolation bias), restriction fragment length polymorphism (RFLP) profiles were assigned to each *mcy*BA1 genotype and subsequently used to distinguish the gene pools of spatially separated populations.

Materials and methods

Cultivation and sampling of cyanobacteria

The 49 strains that were used in this study were either isolated from several European freshwater habitats, as described in Kurmayer *et al*. (2004), or acquired from international culture collections (Table 1). The isolates were assigned to the genus *Planktothrix* (*P. agardhii* and *P. rubescens*, Suda *et al*. 2002; minimum 99.2% similarity in 16S rDNA) according to the morphological criteria provided by Komarek (2003). In addition, all of the strains were genetically

Table 1 Variation of amino acids in position 2 of microcystin molecules (Arg, arginine; Hty, homotyrosine; Leu, leucine) produced among 49 strains of *Planktothrix rubescens* (rub) and *Planktothrix agardhii* (aga). Strains are ordered according to the proportion of each of the structural variants produced (mean ± 1 SE). The number of MC measurements using HPLC analysis is indicated for each strain. Strains marked by asterisks have been shown to be inactive in MC production (Christiansen *et al*. 2006). The corresponding *Alu*I (I–X) and *Tse*I (I–IV) restriction types, and the sequence accession numbers of *mcy*BA1 are indicated. Supercripts A–H identify identical genotypes. Strains have been isolated during the study by Kurmayer *et al*. (2004). Country codes (ISO format). SAG Culture Collection of Algae (Göttingen, Germany), PCC Pasteur Culture Collection (Institute of Pasteur, Paris, France), CCAP Culture Collection of Algae and Protozoa (Windermere, UK)

	Microcystin data					Restr. type		Genotype		Strain data		
Amino acid in pos. 2	Arginine	Homotyrosine	Leucine	Undetermined	No. of MC measurements	*Alu*I	*Tse*I	PC-IGS Acc. no.	*mcy*BA1 Acc. no.‡	Species	Strain no.	Origin
Arg	99 ± 1			1 ± 1	5	I	I		AJ749276[A]	rub	3	Mondsee (AT)
	100				2	I	I		AJ890255[A]	rub	10	Mondsee (AT)
	—				2	I	I		AJ890263[H]	rub	40*	Mondsee (AT)
	—				4	I	I		AJ890264	rub	91/1*	Mondsee (AT)
	99 ± 1			1 ± 1	2	I	I		AJ890256[A]	rub	99	Mondsee (AT)
	97			3	2	I	I		AJ890257[A]	rub	109	Mondsee (AT)
	97 ± 1			3 ± 2	4	I	I		AJ749282[A]	rub	111	Mondsee (AT)
	95 ± 2			5 ± 2	2	I	I		AJ890258[A]	rub	113	Mondsee (AT)
	—				2	I	I		AJ890259[A]	rub	119*	Mondsee (AT)
	97 ± 1			3 ± 1	4	I	I	AJ558156	AJ890260	rub	34	Ammersee (DE)
	99 ± 1			1 ± 1	2	I	I		AJ890261[A]	rub	81	Ammersee (DE)
	97 ± 1			3 ± 1	2	I	I		AJ890262[H]	rub	86	Ammersee (DE)
	—				2	II	I	AJ558153	AJ890265[D]	rub	62*	Irrsee (AT)
	—				3	II	I		AJ890266[D]	rub	65*	Irrsee (AT)
	100 ± 1			1 ± 1	4	III	I		AJ749277	rub	64	Wörthersee (AT)
	100				3	V	IV	AJ558160	AJ749272[C]	aga	CCAP1459/11A	L. Windermere (UK)
	100				3	V	IV	AJ558159	AJ749274[C]	aga	CCAP1459/21	Esth. Water (UK)
	100				2	IV	I		AJ890267	rub	72	Zürichsee (CH)
Hty/ Leu		42 ± 5	56 ± 6	2	2	VI	II		AJ863131[B]	rub	21-	Figur (AT)
		54 ± 3	44 ± 1	1 ± 1	2	VI	II		AJ890275[B]	rub	21/2	Figur (AT)
		93 ± 7†		3 ± 3	2	VI	II		AJ890276[B]	rub	13	Schwarzensee (AT)
		95 ± 5†		3 ± 4	2	VI	II		AJ890277[B]	rub	18	Schwarzensee (AT)
		94 ± 6†		3 ± 2	2	VI	II		AJ890278[B]	rub	59	Schwarzensee (AT)
		88†		13	2	VI	II		AJ890279[B]	rub	60	Schwarzensee (AT)
		86 ± 2†		14 ± 2	5	VI	II	AJ558152	AJ749278[B]	rub	80	Schwarzensee (AT)
		69 ± 3	32 ± 4		3	VI	II	AJ558158	AJ749273[B]	aga	CCAP1459/16	Blelham Tarn (UK)
		75 ± 9	23 ± 7	2 ± 2	2	VI	II		AJ863133[B]	aga	CCAP1459/17	Blelham Tarn (UK)
		90	10		2	VI	II		AJ863134[B]	aga	CCAP1459/31	White Lough (UK)
		75 ± 3	27 ± 2		3	VI	II		AJ749275[B]	aga	CCAP1460/5	L. Kasumigaura (JP)

Table 1 *Continued*

Amino acid in pos. 2	Microcystin data					Restr. type		Genotype		Strain data		
	Arginine	Homotyrosine	Leucine	Undetermined	No. of MC measurements	*Alu*I	*Tse*I	PC-IGS Acc. no.	*mcy*BA1 Acc. no.‡	Species	Strain no.	Origin
Arg/Leu	90		9	1 ± 1	5	IV	I		AJ749269	rub	39	Wannsee (DE)
	89		8 ± 3		2	IV	I		AJ890268	rub	42	Wörthersee (AT)
	91 ± 3		9 ± 3		2	IV	I		AJ890269	rub	101	Mondsee (AT)
	88 ± 4		12 ± 4		2	IV	I		AJ890270	rub	118	Mondsee (AT)
	70 ± 2		30 ± 2		5	VI	I	AJ558147	AJ749284	rub	CCAP1459/30	Plöner See (DE)
	87 ± 4		13 ± 4		2	VI	I	AJ558150	AJ890271	rub	CCAP1459/24	L. Ören (SE)
	80 ± 3		19 ± 2	1	3	VII	III	AJ558137	AJ749271	rub	SAG6.89	Plußsee, Plön (DE)
	95 ± 1		5 ± 1		4	VII	III	AJ558154	AJ749283	rub	PCC7821	L. Gjersjøen (NO)
	89 ± 1		11	1 ± 1	4	VIII	III		AJ749268	aga	32	Wannsee (DE)
	94		2	4	2	IX	I		AJ890280[F]	rub	77	Wörthersee (AT)
	93 ± 2		3 ± 1	5 ± 1	2	IX	I		AJ890281[F]	rub	89	Wörthersee (AT)
	—				2	IX	I		AJ890282[F]	rub	120*	Mondsee (AT)
	95 ± 1		4 ± 1		5	X	I		AJ749270[E]	aga	79	L. Arresø (DK)
	99		1		4	X	I		AJ749281	rub	108	Irrsee (AT)
	90 ± 2		9 ± 2	1 ± 1	7	X	I	AJ558135	AJ441056[E]	aga	CYA126/8	L. Langsjön (FI)
Arg/Hty/Leu	94 ± 2	1 ± 1	5 ± 1		2	VI	I	AJ558151	AJ890272[G]	rub	46	Zürichsee (CH)
	—				2	VI	I		AJ890273[G]	rub	67*	Wörthersee (AT)
	91 ± 3	2	7 ± 3		2	VI	I		AJ890274	rub	75	Zürichsee (CH)
	83 ± 2	1 ± 1	14 ± 3	2 ± 1	4	VII	III		AJ749267	aga	31/1	Wannsee (DE)
	95 ± 1	1 ± 0	5 ± 1		6	X	I		AJ749279	rub	82	Ammersee (DE)

†New microcystins with Hty/Tyr in position 2 of the microcystin molecule (R. Kurmayer, W. Yoshida, K. Ishida, T. Hemscheidt, unpublished).
‡Accession nos AJ749267–AJ749284, AJ863131–AJ863134 have been published by Kurmayer *et al*. (2005).

Table 2 Morphometric and limnological characteristics of lakes sampled for *Planktothrix rubescens*. Trophic data (M, mesotrophic; O, oligotrophic) are yearly minimum–mean–maximum value (sample size) of the year 2001 from the Kärntner Institut für Seenforschung, Carinthia (Afritzersee, Wörthersee), Bayrisches Landesamt, Wielenbach, Germany (Ammersee), Department for Environment, Kanton Aargau (Hallwilersee), Federal Agency for Water Management, Scharfling, Upper Austria (Irrsee, Schwarzensee) and Zurich Water Supply (Zürichsee). Chlorophyll *a* values were integrated over the water column. As inferred from microscopical inspection of net samples (30 μm mesh size) the abundance of *Planktothrix rubescens* is given: D, dominant; A, abundant; and R, rare; m a.s.l. is metres above sea level

Lake	N(°)	E(°)	m a.s.l.	Area (km^2)	Tropic status	Secchi depth (m)	Chlorophyll a (μg/L)	Abundance *Planktothrix*
Afritzer See, AT	46°44	13°46	750	0.5	M	3.3–4.0–5.2 (4)	2.4–4.4–6.8 (4)	A
Ammersee, DE	47°16	11°4	533	47	M	1.6–4.4–6.5 (11)	1.5–5.1–9 (11)	A
Hallwilersee, CH	47°20	8°10	449	10	M	1–2.7–5 (12)	8.8–18.9–30 (12)	D
Irrsee, AT	47°56	13°19	553	3.6	M	2.2–5.1–8.2 (12)	1.4–2.8–4.3 (7)	R
Mondsee, AT	47°48	13°22	481	14.2	M	2.4–3.8–5 (24)	1.8–4.3–9.6 (24)	A
Schwarzensee, AT	47°45	13°30	716	0.5	O	5.5–6.5–7.5 (3)	0.5–0.9–1.5 (3)	R
Wörthersee, AT	46°36	14°3	440	19.4	M	3.5–4.9–7.5 (7)	5.4–9–13.3 (7)	D
Zürichsee, CH	47°22	8°32	406	65	M	2.3–3.9–5.9 (18)	5–9.5–15.7 (12)	D

tested using *Planktothrix* specific primers binding to the intergenic spacer region within the phycocyanin operon (PC-IGS) as described in Kurmayer *et al*. (2004). Clonal strains were grown in BG-11 medium (Rippka 1988) modified to contain 2 mM $NaNO_3$ + 10 mM $NaHCO_3$ and kept in batch culture at 15 °C under continuous light conditions (5–10 μmol m^{-2} s^{-1}, Osram Type L30W/77 Fluora).

Planktothrix rubescens was sampled by pulling a plankton net (30 μm in mesh size) from a depth of 20 m to the surface at the deepest part of eight lakes in the Alps (Table 2). Aliquots (a few mL from net samples) were filtered onto glass fibre filters (GF/C, Whatman) under vacuum pressure and stored frozen (–20 °C) until DNA extraction. In this study, each lake was sampled a total of three times from 2001 to 2005.

Microcystin analysis

All strains were analysed for their composition of microcystin variants, i.e. amino acid composition in position 2 of the MC molecule by HPLC-DAD (high performance liquid chromatography with diode array detection) as described (Kurmayer *et al*. 2004, 2005). Briefly, the cells were filtered on glass fibre filters (GF/C, Comesa) and MCs were extracted using 75% (w/v) aqueous methanol. MC variants were quantified at 240 nm by their characteristic absorption spectra (original spectrum and first order derivative) and retention times (Fastner *et al*. 1999) using a linear gradient of acetonitrile (0.05% TFA, Trifluoroacetic acid) against water (0.05% TFA) according to Lawton *et al*. (1994).

Demethylated variants containing arginine in position 2 of the molecule, i.e. [D-Asp^3, Mdha^7]-RR and [D-Asp^3, Dhb^7]-RR eluted from 13.4 to 13.9 min and 14.3 to 14.8 min, each, those containing homotyrosine (Hty) in position 2, [Asp^3]-MC-HtyR eluted from 18.3 to 18.5 min, and those containing leucine in position 2, [Asp^3]-MC-LR eluted 19.3–19.6 min (Kurmayer *et al*. 2005). All strains from Lake Schwarzensee were found to produce new microcystin variants eluting from 23.5 to 24.0 min (Kurmayer *et al*. 2004). Amino acid analysis, one-dimensional, and two-dimensional NMR revealed that the two novel variants contained homotyrosine/tyrosine in position 2 of the molecule: [D-Asp^3 (*E*)-Dhb^7]-MC-HtyY and [D-Asp^3 (*E*)-Dhb^7]-MC-HtyHty (R. Kurmayer, W. Yoshida, K. Ishida, T. Hemscheidt, unpublished).

Genetic analysis

Two millilitres of each strain culture were incubated 1 h on ice and centrifuged at 16 000 g for 10 min. The pellet was lyophilized in a vacuum centrifuge at 30 °C. DNA extraction from the strains or field samples was performed as described in Kurmayer *et al*. (2003).

Polymerase chain reaction (PCR) amplifications were performed in a vol. of 20 μL, containing 2 μL of QIAGEN PCR buffer (QIAGEN), 1.2 μL $MgCl_2$ (25 mM, QIAGEN), 0.6 μL deoxynucleotide triphosphates (10 μM each, MBI Fermentas), 1 μL of each primer (10 pmol/μL), 0.1 μL *Taq* DNA polymerase (5 U/μL^{-1}, QIAGEN), 13.1 μL sterile Millipore water and 1.0 μL of the diluted DNA extract (1:100). The PCR thermal cycling protocol included an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, with an annealing temperature of 52 °C for 30 s, and elongation at 72 °C for 2 min, followed by 72 °C for 5 min.

Oligonucleotide primers for *mcy*BA1 amplification and sequencing are published in Kurmayer *et al*. (2005). *Mcy*BA1totfwd and *Mcy*BA1totrev were used to amplify the total fragment of the first adenylation domain (A1) of *mcy*B in strains and field samples (1693 bp, including the core motifs A1-A10; Marahiel *et al*. 1997). The primers were

Table 3 Schematic representation of *Alu*I and *Tse*I restriction profiles within *mcy*BA1 (1693 bp) of *Planktothrix* spp. The size of the restriction fragments in bp was calculated from the sequences

	*Alu*I										*Tse*I			
Fragments (bp)	I	II	III	IV	V	VI	VII	VIII	IX	X	I	II	III	IV
1693														—
865											—		—	
828												—	—	
798												—		
735											—			
328	—	—	—	—	—	—	—	—	—	—				
289	—	—	—	—	—	—	—	—	—	—				
272	—	—	—	—	—	—								
263	—	—	—						—	—				
243				—	—	—	—	—						
225									—	—				
208		—			—		—							
201	—	—	—	—	—	—	—	—	—	—				
179							—	—						
132	—	—	—	—	—	—	—	—	—	—				
114			—			—		—		—				
106	—			—					—					
102	—			—					—					
94			—			—		—		—				
93											—			
67												—		
47							—	—	—	—				
46							—	—						
20				—	—	—	—	—						

specific for *Planktothrix*. Amplification products for *mcy*B were purified using the QIAGEN QIAquick PCR Purification Kit (QIAGEN,) and sequenced directly by standard automated fluorescence techniques (Applied Biosystems, Weiterstadt, Germany). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under Accession nos AJ890255–AJ890282 (Table 1).

In addition, *mcy*BA1 PCR products were digested using *Alu*I and *Tse*I. The restriction profiles were compared with the *mcy*BA1 sequences. Restriction analysis was performed directly from the PCR amplification product using *Alu*I (MBI Fermentas) and *Tse*I (New England Biolabs) according to the manufacturer's instructions. Digestions were performed in a vol. of 20 μL, containing 0.2 μL of the restriction enzyme (5 $U/\mu L^{-1}$) and 7 μL of the PCR products and incubated at 37 °C (*Alu*I) and 65 °C (*Tse*I) for 3 h. The PCR products for *mcyB* were visualized in 1% agarose in 0.5 × TBE (Tris-borate-EDTA buffer) and ethidium-bromide staining performed *via* agarose gel electrophoresis according to standard procedures (Sambrook *et al*. 1989). For the electrophoresis of restriction fragments, 2% agarose was used.

PCR products for *mcy*BA1 obtained from field samples were cloned using the pDrive Cloning Vector system (QIAGEN,) according to the manufacturer's instructions. The transformation efficiency was 2.1×10^4–7.0×10^4 colonies per μg of DNA. After the transformed colonies were grown on an agar plate overnight, white colonies were randomly picked using the tip of a pipette, and redissolved in 10 μL of Millipore water each. Re-PCR amplification from the white clones was always successful and the subsequent restriction analysis of colony specific PCR products revealed the unambiguous identification of a specific restriction type. The sequencing of m*cy*BA1 primer binding sites performed for all of the different *Alu*I restriction types observed among strains (Table 3) revealed high similarity, i.e. identical nucleotides for the binding site of the m*cy*BA1totrev primer (5′-AGA CTT GTT TAA TAG CAA AGG C-3′). One substitution of adenine by cytosine in position 6 of the *mcy*BA1totfwd primer for restriction types I, IV (5′-CAC CTA GTT GAA GAA CAA GTT CT-3′) was found. Consequently, the PCR amplification of the restriction types from the field samples was considered unbiased.

Phylogeny and statistical analysis

Sequences (1451 bp) were aligned using multiple sequence alignment (CLUSTAL W 1.8) and similarities between

nucleotide sequences were calculated using DNADIST in the PHYLIP package (version 3.6 alpha; Felsenstein 1989). Maximum likelihood analysis was used to estimate nucleotide substitution parameters under a general time-reversible nucleotide substitution model by estimating the gamma distribution for variable rates among the sites. Ambiguous sites at which at least one sequence showed an undetermined nucleotide were removed (two sites) and the discrete gamma algorithm was used to approximate a continuous gamma distribution using five categories of rates (ncatG = 5) in the programme BASEML of the PAML package (version 3.14, Yang 1997). The best fit of the model showed (log-) likelihood ln L = –2871.96 with parameter estimates for the transition/transversion ratio $\kappa = 2.07$ and the shape parameter $\alpha = 0.006$. Statistical significance of the branches was estimated by bootstrap analysis generating 100 replicates of the original data set using the PHYLIP package. Finally consensus trees following the 50% majority rule were computed.

The ratio of nonsynonymous (d_N) and synonymous (d_S) substitution rates per site was determined using the likelihood approach, which was implemented in the program CODEML of the PAML package (Yang 1998). The 'one ratio' model, assuming the same ratio for all of the branches in the phylogeny and the 'free ratio' model, assuming as many ratios as the number of branches in the phylogenetic tree were used. Branch-site models (Zhang *et al*. 2005) were employed to test for positive selection acting on specific branches in the phylogenetic tree showing increased d_N/d_S ratios. Branches of the tree were divided a priori into foreground and background lineages, and a likelihood ratio test was constructed by comparing a model that allows positive selection on the foreground lineages (the alternative model) with a model that does not allow such positive selection (the null model). The improved branch-site likelihood method for detecting positive selection implemented in the PAML package was used (version 3.15, Anisimova *et al*. 2003; Zhang *et al*. 2005).

Following Tanabe *et al*. (2004) the runs test implemented in the program GENECONV (version 1.81, Sawyer 1999) was used to investigate whether substitutions were significantly clustered, and whether gene conversion (recombination) events occurred within *mcy*BA1. The settings used were the default (/g0 = mismatches within fragments were not allowed). The global *P*-value calculated from 10 000 random permutations of the alignment was used to assess the significance of any unusually long fragments that were sufficiently similar to be suggestive of past gene conversion. Recombination events were independently quantified using a recombination detection program (RDP) developed by Martin & Rybicki (2000) using the default settings (window size 10, highest acceptable *P*-value 0.05).

The frequency of the occurrence of genotypes and restriction types between the *Planktothrix* species was compared using the Yates-corrected chi-square statistic in the analyses of 2×2 contingency tables (Zöfel 1992). The occurrence of restriction types between lakes was statistically compared using chi-square statistics for 8×2 contingency tables. Because the test can produce false results when the expected frequencies are small, proportions with an expected frequency < 1 were not tested (Jongman *et al*. 1995). The differences in the total number of restriction types found in a population were tested using one-way ANOVA followed by the Tukey test for pairwise comparison (overall significance $P = 0.01$).

Results

Genetic variation within the microcystin mcyB *gene*

Among the *mcy*BA1 sequences obtained from the 49 *Planktothrix* strains, 25 different genotypes were detected, which differed in at least one base pair. We found eight *mcy*BA1 genotypes (A-H) consisting of 2–11 strains each and 17 unique genotypes (Table 1). The genetic variability was between zero and 3.6%. Genotype B (11 strains) consisted of both species *Planktothrix agardhii* and *Planktothrix rubescens* while A (8), H (2), G (2), D (2), F (3) consisted of *P. rubescens* only. In contrast, C (2) and E (2) occurred significantly in *P. agardhii* only (chi-square test, χ^2 (d.f. = 1), $P < 0.05$).

The ratio of nonsynonymous (d_N) and synonymous (d_S) substitution rates for the entire phylogenetic tree was $d_N/d_S = 0.19$. Genotypes B, C, and the clade consisting of genotypes A (D, E, H, strains No34, 64, 91/1) showed a relatively high frequency of nonsynonymous substitutions resulting in $d_N/d_S = 0.73, 0.87, 0.23$, respectively. The branch-site model was used to detect a possible positive selection acting on genotypes B, C and the clade consisting of genotypes A, D, E, H, No34, 64, 91/1. Although the log-likelihood value of the alternative model was found improved when compared with the log-likelihood value of the null model, the difference between the two models was not found to be significant ($2\Delta l < 3.84$, d.f. = 1, $P > 0.05$).

The runs test implemented in GENECONV revealed 146 possible recombination events, i.e. gene fragments that were sufficiently similar to be suggestive of past gene conversion within *mcy*BA1 (123–1379 bp, 12–68 polymorphic sites). All of the genotypes showed at least one putative recombination event with another genotype, in total 8, 85, and 53 events occurred within *P. agardhii*, *P. rubescens*, and between both species, respectively. The most frequent breaking points were from Pos. 526–883 (27), 707–890 (28) and 707–883 (10) of strain No10 (AJ890255). A high number of recombinations (250) was also detected using RDP.

Microcystin genotypes and microcystin variants

Genotypes A, C, D, H, No34, 64, 72 were found to contain exclusively arginine in position 2 of the molecule (Table 1,

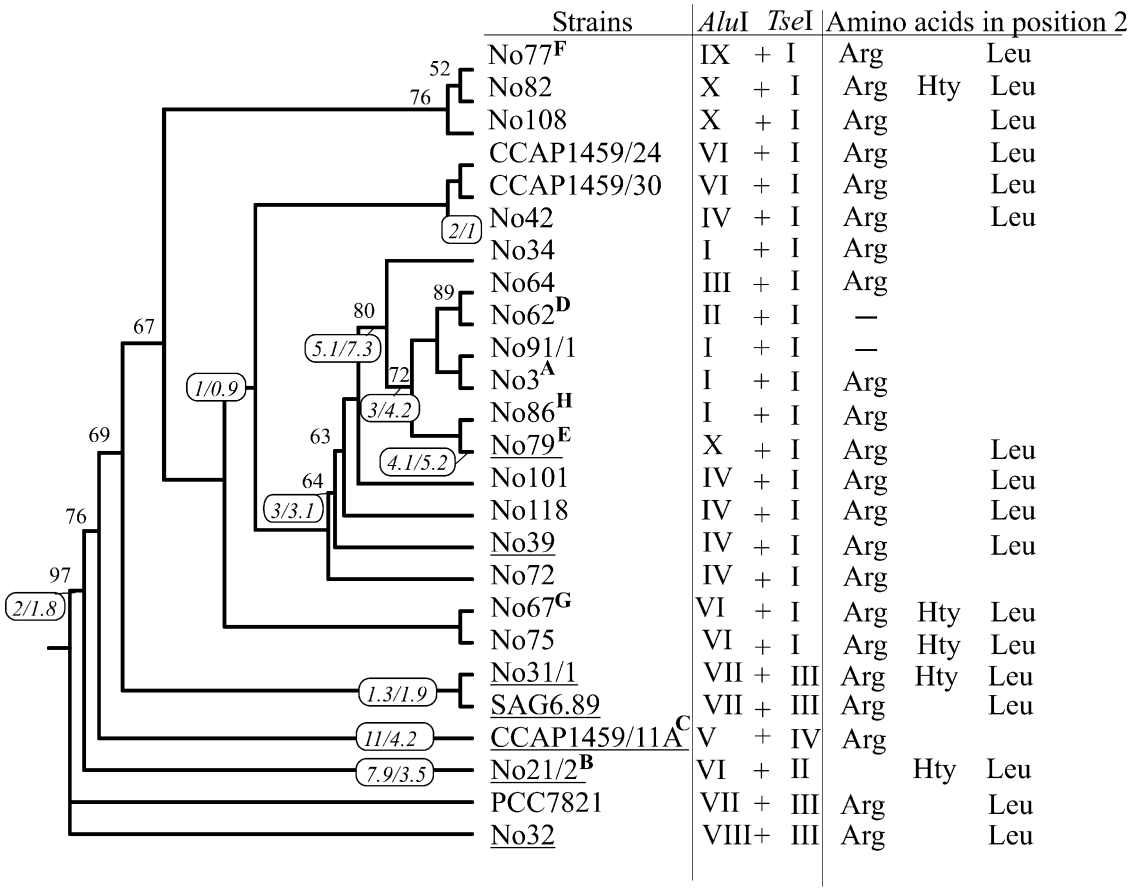

Fig. 1 Phylogenetic tree based on ML analysis from *mcy*BA1 sequences (1.451 bp) from 49 *Planktothrix* spp. strains. Strains assigned to *Planktothrix agardhii* are underlined. Capitals A–H in the superscripts indicate the genotype groups (consisting of 2–11 strains) as indicated in Table 1. Genotype B consisted of both *Planktothrix rubescens* and *P. agardhii*. For each strain the corresponding restriction profile (*Alu*I, *Tse*I) and the occurrence of amino acids in position 2 of the microcystin molecule is indicated. The significant bootstrap percentages were obtained from 100 pseudo replicates. The two numbers shown in boxes are the maximum-likelihood estimates of the numbers of nonsynonymous/synonymous substitutions for the entire *mcy*BA1 along that branch (only ratios higher than the background ratio of the 'one-ratio' model ($d_N/d_S = 0.19$) are shown, see text).

Fig. 1). Notably, the gene region coding for the putative binding pocket, which is responsible for substrate activation during microcystin synthesis (A4-A5, Stachelhaus *et al*. 1999, position 424–735 of strain No10, AJ890255, 312 bp), showed two binding pockets only: the first arginine binding pocket genotype comprised genotype C, while all other strains (A, D, H, No34, 64, 72) containing arginine only formed another binding pocket genotype differing by 4.1% from C, $d_N/d_S = 0.704$, estimated through pairwise comparison by the method of Yang & Nielsen (2000). Strains of genotype B contained microcystin with homotyrosine (tyrosine) and leucine, but never arginine in position 2. Its putative binding pocket differed from the arginine binding pocket of genotype C by 3.2% ($d_N/d_S = 3.34$) and from the other arginine binding pocket of genotype A (D, H, strains No34, 64, 72) by 2.9% ($d_N/d_S = 0.61$). All other genotypes contained arginine and leucine, and in some cases additional low levels of homotyrosine (G, No31/1, 75, 82). Strains No40, 62, 65, 67, 91/1, 119, 120 were found inactive in MC production, which was partly caused by transposases or gene deletions (Christiansen *et al*. 2006); however, those strains did not differ in sequence compared with active *mcy* genotypes (Table 1).

Distribution of microcystin gene restriction types among strains

Out of the 25 genotypes, 10 restriction types were obtained with *Alu*I and four restriction types were obtained with *Tse*I (Table 3, Fig. 1). In total, 11 *Alu*I+*Tse*I combinations were observed. While some restriction types were found in both strains of *P. rubescens* and *P. agardhii* (VI+II) others were found only in *P. agardhii* (V+IV, VIII+III) or in *P. rubescens* (I+I, II+I, III+I, IX+I). The occurrence of I+I in *P. rubescens* and V + IV in *P. agardhii* was statistically significant (chi-square test, χ^2 (d.f. = 1), $P < 0.05$). The

restriction types I+I, II+I, III+I comprised a group of most closely related genotypes of *P. rubescens*, which differed in 0.5% of the base pairs only (15 strains, A, D, H, No34, 64, 91/1).

Restriction types I+I, II+I, III+I, V+IV identified those genotypes with arginine only in position 2 of the molecule. Genotype B lacking arginine was identified by restriction type VI + II. Those genotypes containing arginine/leucine (and homotyrosine) were identified by VI+I, VII+III, VIII+III, IX+I, X+I.

Distribution of microcystin gene restriction types among populations

In order to test the reproducibility of the results on the restriction type proportions from cloning libraries, *mcy*BA1 genes from field samples (Mondsee, 9 December 2003, Wörthersee, 28 August 2003) were independently amplified by PCR threefold, cloned, and subsequently digested using *Alu*I after the re-amplification of 40 clones. The results were reproducible for the dominant restriction types (I, II, IV, VI) as well as the absent restriction types (VII, VIII). The results for the subdominant genotypes were more variable (Table 4).

In total, 24 *Alu*I+*Tse*I combinations were observed. Out of a total of 985 clones, the five most frequently detected restriction types were IV+I (23.8%), I+I (20%), VI+II (13%), X+I (10%), and VI+I (5.1%). All of the other restriction types occurred < 5% (Fig. 2). Nine restriction types occurred only once (I+III, I+IV, II+III, III+II, III+III, IX+III, IV+III) or twice (VII+III, X+III). Other restriction types showed new *Alu*I or *Tse*I profiles that were derived from unknown organisms and that occurred at all of the sampling sites (11.9%). Two restriction types that were observed among the strains of *P. agardhii* (V+IV, VIII+III) were not detected in the field samples.

The distribution of restriction types differed significantly between sites; for example, I+I and IV+I occurred frequently in many lakes and were not found in Schwarzensee. In contrast, Schwarzensee was dominated by VI+II or X+I. Restriction type VI+II occurred in all of the lakes except for Irrsee and X+I was not found in Afritzersee but showed high frequency in Irrsee and Schwarzensee. In addition, Schwarzensee had a significantly lower total number of restriction types when compared to the other lakes (one-way ANOVA, d.f. = 23, $P = 0.01$).

In summary, many populations were found to be genetically heterogeneous. Closely located and spatially separated populations were found to differ significantly in *mcy*BA1 restriction type composition. Most populations contained *mcy*BA1 genotypes indicative of the production of arginine, homotyrosine, and leucine. In contrast, genotypes indicative of arginine were absent in Schwarzensee, and that of homotyrosine were absent in Irrsee during the study period.

Discussion

Taxonomic distribution of microcystin genes in Planktothrix *spp.*

In this study, certain genotypes (C, E) and restriction types (V+IV, I+I) were found to occur exclusively either in one or the other species. On the other hand, genotype B occurred in both species and genotypes E, No32, and No39 of *Planktothrix agardhii* were found to be the most closely related to the genotypes of *Planktothrix rubescens*. This conflict between taxonomy and the phylogenetic assignment of *mcy*BA1 genotypes can be best explained by relatively recent recombination events within *mcy*BA1. Homologous recombination has been increasingly recognized as an important force in prokaryotic evolution (Feil 2004). For example, in pathogenic bacteria, recombination has been estimated to change alleles fivefold to 15-fold more frequently than mutation (e.g. Feil *et al*. 2003). For cyanobacteria, Rudi *et al*. (1998) demonstrated the occurrence of recombination

Table 4 Proportion of restriction types (I–X; New, new restriction types) of *mcy*BA1 obtained through *Alu*I digestion after three independent PCR amplifications from field samples, cloning of the PCR product, and PCR-re-amplification of 40 randomly selected white clones from the respective clone library (1–3)

Clone library	I	II	III	IV	V	VI	VII	VIII	IX	X	New
Mondsee, 9 December 2003											
1	35	8	8	10	3	18	0	0	10	3	8
2	16	22	8	22	3	5	0	0	3	3	19
3	5	33	0	23	5	10	0	0	8	3	13
Mean ± 1 SE	19 ± 9	21 ± 7	5 ± 3	18 ± 4	3 ± 1	11 ± 4	0	0	7 ± 2	3 ± 0	13 ± 3
Wörthersee, 28 August 2003											
1	30	5	3	20	5	13	0	0	3	8	15
2	26	31	10	7	0	17	0	0	0	0	10
3	26	5	5	14	0	19	0	0	12	2	19
Mean ± 1 SE	27 ± 1	14 ± 9	6 ± 2	14 ± 4	2 ± 2	16 ± 2	0	0	5 ± 4	3 ± 2	14 ± 3

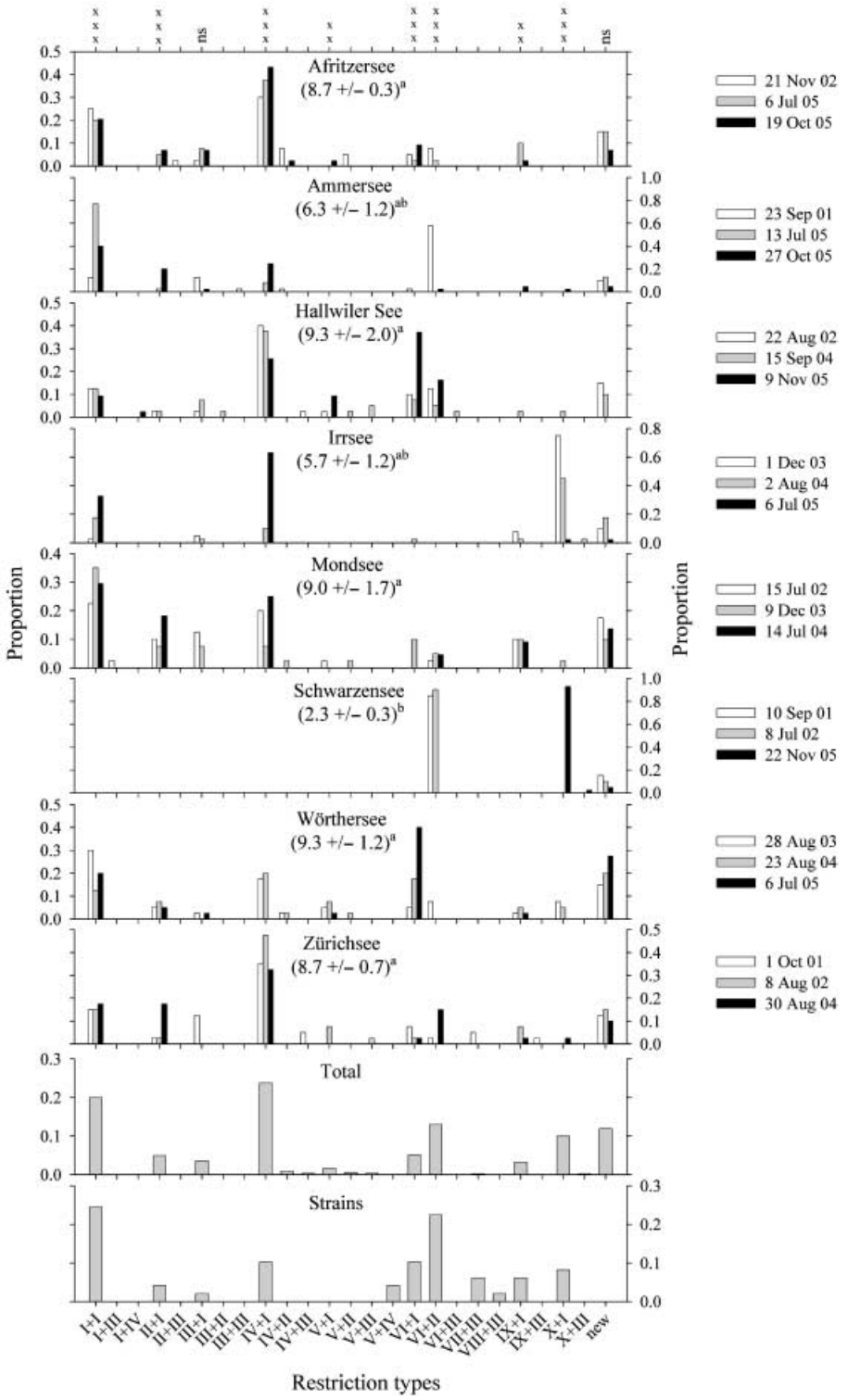

Fig. 2 Proportion of *Alu*I+*Tse*I restriction types obtained from amplified and cloned *mcy*BA1 PCR products from eight different *Planktothrix* spp. populations for three sampling dates. For each sample, 40 clones of *mcy*BA1 PCR products were analysed and the percentage of a specific restriction type was calculated. The proportion for the total population and for the 49 sequenced strains is provided in the lower two graphs. Note that the scales of the y-axes are different. Significant differences of the proportions of restriction profiles between the eight lakes are marked by crosses at the top (xxx, $P < 0.001$, xx, $P < 0.01$, ns, not significant). The mean ± 1 SE number of the total number of restriction types found in a population is given in parentheses. Superscripts a, ab, b indicate homogeneous subsets whose highest and lowest means in the number of different restriction types are not significantly different ($P > 0.05$).

for *rbc*L (D-ribulose 1,5-bisphosphate carboxylase-oxygenase) and the less conserved *rbc*X gene with a possible chaperonin-like function. Based on DNA-DNA hybridization studies, Suda *et al*. (2002) concluded that *P. rubescens* and *P. agardhii* differentiated relatively recently and the relatively high genetic similarity may favour the occurrence of recombination (Roberts & Cohan 1993). Typically, both species do not occur in the same freshwater system; however, in some lakes both species have been found to co-occur over the course of years (e.g. Blelham Tarn, Davis *et al*. 2003). It would be interesting to know whether this co-occurence favours the occurrence of interspecific recombination events.

Microevolution of microcystin genes

In recent years, a number of recombination events within microcystin genes were reported (Mikalsen *et al*. 2003; Tanabe *et al*. 2004; Kurmayer *et al*. 2005), suggesting that microcystin genes are under continuous modification and re-organization. While Tanabe *et al*. (2004) observed the recombination of shorter DNA fragments (< 1000 bp) within *mcy*A (but not within *mcy*D, *mcy*G, *mcy*J), Kurmayer *et al*. (2005) reported the replacement of whole domains, i.e. the typical N-methyl-dehydroalanine adenylation domain (2854 bp) of *mcy*AA1 was replaced by an adenylation domain without the N-methyl transferase (1692 bp) resulting in dehydrobutyrine in position 7 instead of the common N-methyl-dehydroalanine. The flanking regions as well as the site of recombination in *mcy*AA1 were found to be identical within 12 strains containing the replaced domain implying that this recombination event happened only once (R. Kurmayer, C. Molitor, unpublished). In this study, a larger number of short fragments that are indicative of recombination were observed implying that parts of the *mcy*B gene were frequently transferred between lineages of *Planktothrix* spp. It is concluded that the *mcy*A and *mcy*B genes generally show a mosaic structure rather than a bifurcating phylogenetic tree.

The 'one ratio' maximum likelihood model estimated $d_N/d_S = 0.19$, which is similar to the ratios calculated by Tanabe *et al*. (2004), i.e. $d_N/d_S = 0.2$ (*mcy*A), 0.14 (*mcy*D), 0.17 (*mcy*G), 0.11 (*mcy*J). Rantala *et al*. (2004) reported $d_N/d_S < 1$ for *mcy*A, *mcy*D, *mcy*E. An excess of synonymous substitutions over nonsynonymous ones indicates that *mcy* genes are subject to purifying selection and mutations affecting the protein sequence are in general deleterious. Notably, the genotypes B (indicative of homotyrosine, leucine but no arginine) and C (indicative of arginine only) showed higher d_N/d_S ratios implying either relaxation of purifying selection or positive selection. Averaging d_N/d_S rates over all of the sites of the protein typically underestimates positive selection because the ratio is overwhelmed by the ubiquitous purifying selection. To increase the power of detection branch-site models have been invented to test for positive selection acting on specific sites of specific genotypes (Yang & Nielsen 2002; Zhang *et al*. 2005) or on a subset of sites in the whole phylogenetic tree (Yang *et al*. 2000). Indeed employing site models (Yang *et al*. 2000) 3% of the sites within the whole *mcy*BA1 phylogeny were found positively selected ($d_N/d_S \sim 5.0$, unpublished data). However, no statistically significant support for positive selection in specific branches was found, implying that some sites in genotypes B, C rather experienced a relaxation of selective constraints. In the future, quantifying the fitness of specific microcystin ecotypes (i.e. Kurmayer *et al*. 2005) under various environmental conditions will help to elucidate whether the observed differences in d_N/d_S ratios resulted from possible adaptive speciation events or from a relaxation of selective constraints.

Divergence of microcystin genotype composition between populations

In this study, genotype C (two strains isolated from the English Lake District, UK) was never detected in field samples in the Alpine lakes. In addition, populations were found to differ with regard to the occurrence of specific genotypes as well as to the number of genotypes occurring in total in a specific population. This study is the first that documents homogeneous and more heterogeneous populations of aquatic cyanobacteria existing in closely located yet spatially isolated habitats over the course of several years. According to Horner-Devine (2004) three fundamental processes, the rates of dispersal, speciation, and extinction all contribute to the geographical patterns. There is no reason to assume that *P. rubescens* has fewer dispersal capabilities than other aquatic prokaryotes. Typically *P. rubescens* occurs in depths of 9–12 m (in Mondsee) with low light intensity (i.e. neutral buoyancy in Lake Zürich occurred between 4 and 8 μmol m^{-2} s^{-1}, Walsby *et al*. 2004). Notably, the populations in Schwarzensee and Irrsee showing the lowest genetic diversity observed in this study, also had the lowest numbers of individuals when compared with the populations of high genetic diversity (Mondsee, Wörthersee) as reported previously (Christiansen *et al*. 2006; Table 4). Recolonization of those habitats by single genotypes after the extinction of the population during winter or other catastrophic events may contribute to the low homogeneity and the high degree of clonality observed in sparse populations. Consequently isolation combined with low numbers of individuals may lead to divergence in microcystin synthesis through genetic drift.

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Our research aims towards understanding the evolution and maintenance of the impressive diversity of secondary metabolites in cyanobacteria. Apart from the problems involved in using water bodies affected by toxic cyanobacteria, the evolution of genes involved in secondary metabolite synthesis is also interesting from a biological perspective. By linking molecular insights with ecological research, we hope to identify the evolutionary mechanisms affecting the synthesis of natural products on a molecular and ecosystem level.
