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Paralytic Shellfish Poisoning Toxin-Producing Cyanobacterium *Aphanizomenon gracile* in Northeast Germany▽†

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Neurotoxic paralytic shellfish poisoning (PSP) toxins, anatoxin-a (ATX), and hepatotoxic cylindrospermopsin (CYN) have been detected in several lakes in northeast Germany during the last 2 decades. They are produced worldwide by members of the nostocalean genera *Anabaena*, *Cylindrospermopsis*, and *Aphanizomenon*. Although no additional sources of PSP toxins and ATX have been identified in German water bodies to date, the observed CYN concentrations cannot be produced solely by *Aphanizomenon flos-aquae*, the only known CYN producer in Germany. Therefore, we attempted to identify PSP toxin, ATX, and CYN producers by isolating and characterizing 92 *Anabaena, Aphanizomenon*, and *Anabaenopsis* strains from five lakes in northeast Germany. In a polyphasic approach, all strains were morphologically and phylogenetically classified and then tested for PSP toxins, ATX, and CYN by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and enzyme-linked immunosorbent assay (ELISA) and screened for the presence of PSP toxin- and CYN-encoding gene fragments. As demonstrated by ELISA and LC-MS, 14 *Aphanizomenon gracile* strains from Lakes Melang and Scharmützel produced four PSP toxin variants (gonyautoxin 5 [GTX5], decarbamoylsaxitoxin [dcSTX], saxitoxin [STX], and neosaxitoxin [NEO]). GTX5 was the most prevalent PSP toxin variant among the seven strains from Lake Scharmützel, and NEO was the most prevalent among the seven strains from Lake Melang. The *sxtA* gene, which is part of the saxitoxin gene cluster, was found in the 14 PSP toxin-producing *A. gracile* strains and in 11 non-PSP toxin-producing *Aphanizomenon issatschenkoi*, *A. flos-aquae*, *Anabaena planktonica*, and *Anabaenopsis elenkinii* strains. ATX and CYN were not detected in any of the isolated strains. This study is the first confirming the role of *A. gracile* as a PSP toxin producer in German water bodies.

Neurotoxic saxitoxins, also known as paralytic shellfish poisoning (PSP) toxins, as well as neurotoxic anatoxin-a (ATX) and hepatotoxic cylindrospermopsin (CYN), have been detected in several northeast German lakes in the last 2 decades (3, 35). In a survey conducted in 1995 and 1996, ATX was present in 26% of 78 German lakes and PSP toxins were present in 34% of 29 lakes (3). In 2004, a qualitative survey showed that CYN was present in 50% of 127 German lakes investigated (8). *Aphanizomenon flos-aquae* Ralfs ex Born. et Flah. has been identified as producer of CYN in these lakes (33), but sources of PSP toxins and ATX have yet to be identified in German water bodies.

PSP toxins are potent neurotoxic alkaloids produced by marine dinoflagellates and filamentous freshwater cyanobacteria (1, 2, 42). The 21 currently known PSP toxin variants belong to four groups: carbamoyl toxins, decarbamoyl toxins, *N*-sulfocarbamoyl toxins, and deoxydecarbamoyl toxins (15). Carbamoyl toxins are the most potent PSP toxins, including saxitoxin (STX) and neosaxitoxin (NEO), while deoxydecarbamoyl toxins comprise the least potent PSP toxins (38). PSP toxins block neural sodium ion channels, leading to death through respiratory failure (1).

Cyanobacteria belonging to the orders *Oscillatoriales* and *Nostocales*, including members of the genera *Cylindrospermopsis*, *Anabaena*, and *Aphanizomenon*, have been identified as PSP toxin producers in freshwater habitats (4). *Aphanizomenon gracile* Lemmermann and *Aphanizomenon flos-aquae* strains from China, Portugal, and the United States have been described as PSP toxin producers (9, 23, 31). Both species are abundant members of the *Nostocales* and are widely distributed in phytoplankton communities in oligotrophic, mesotrophic, and eutrophic water bodies throughout northeast Germany (35).

Regarding saxitoxins, *Cylindrospermopsis raciborskii* (Woloszyńska) Seenayya et Subba Raju strain T3 was recently found to contain a new candidate saxitoxin gene cluster containing around 35 kb of DNA and comprising more than 26 genes (16). This saxitoxin gene cluster was also found in *Anabaena circinalis* Rabenhorst ex Bornet & Flahault strains from Australia, in *Aphanizomenon* sp. strain NH5, and in *Lyngbya wollei* (Farlow ex Gomont) comb. nov. (16).

Anatoxin-a, a neurotoxic bicyclic alkaloid, has been detected in freshwater bodies worldwide (4). Anatoxin-a production has been found in *Anabaena*, *Aphanizomenon*, *Cylindrospermum*, *Oscillatoria* sp., and *Phormidium* strains (4). Anatoxin-a is a potent agonist for the nicotinic acetylcholine receptor. Its toxic effects include muscle fasciculation, gasping, convulsions, and death by respiratory arrest in vertebrates (2).

Cylindrospermopsin is a potent alkaloid hepatotoxin produced by planktonic cyanobacteria of the order *Nostocales*. It was first detected in Australian *Cylindrospermopsis raciborskii* strains (12) and is additionally produced by *Anabaena bergii* Ostenfeld (36), *Umezakia natans* M. Watanabe (11), *Apha-*

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TABLE 1. Strains of the order *Nostocales* isolated from different German lakes

Species	No. of strains	Lake
Aphanizomenon gracile	34	Langer, Scharmützel, Melang
Aphanizomenon flos-aquae	18	Stechlin, Scharmützel
Aphanizomenon issatschenkoi	6	Langer
Anabaena crassa	1	Langer
Anabaena flos-aquae	2	Langer, Scharmützel
Anabaena lemmermanii	3	Nehmitz
Anabaena planktonica	22	Langer, Scharmützel, Stechlin
Anabaenopsis elenkinii	2	Langer
Anabaena bergii	3	Melang
Anabaena sp.	1	Stechlin

nizomenon ovalisporum (Forti) (37), and *A. flos-aquae* (33). CYN results in liver, kidney, intestinal, and lung damage (13) and inhibits protein synthesis (40).

Overall knowledge of the cyanobacterial sources of PSP toxins, ATX, and CYN is scarce. To identify the producers of such toxins, we isolated and investigated 92 *Aphanizomenon*, *Anabaena*, and *Anabaenopsis* strains from five northeast German water bodies dominated by cyanobacteria of the order *Nostocales*. All strains were morphologically and phylogenetically classified and screened for the presence of toxin-encoding genes and for the ability to produce cyanobacterial toxins using a polyphasic approach including enzyme-linked immunosorbent assay (ELISA) and liquid chromatography with tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Isolation of strains and morphological characterization. Ninety-two *Aphanizomenon*, *Anabaena*, and *Anabaenopsis* strains were isolated from five lakes in northeast Germany (Table 1). The isolated filaments were washed 5 times and placed in microtiter plates containing 300 μl Z8 medium (20). After successful growth, they were placed in 50-ml flasks containing 20 ml Z8 medium. All strains investigated in this study were maintained at 22°C and a photon flux density of 80 μmol of photons $m^{-2}s^{-1}$. Strains were classified based on morphological traits according to Komárek (17), Komárková-Legnerová and Eloranta (19), and Komárek and Komárkova (18). Morphological studies were conducted using an Olympus BX51 light microscope and color view imaging system (Olympus, Germany).

Genomic DNA extraction/PCR amplification and sequencing. Fresh culture material of all cyanobacterial strains was frozen and thawed three times and then boiled for 5 min and subsequently centrifuged for 5 min (13,000 rpm or ~16,000 × *g*). The supernatants were discarded. Each pellet was resuspended in 100 μl distilled water and vortexed for 1 min. Genomic DNA was extracted using the MoleStrips DNA blood kit and the DNA-Cyano protocol on GeneMole (Mole Genetics, Lysaker, Norway) according to the manufacturer's instructions.

All PCRs were performed on a Peltier thermal cycler PTC 200 (MJ Research, Inc., San Francisco, CA) using the *Taq* PCR core kit (Qiagen GmbH, Hilden, Germany). The reaction mixture contained 0.1 μl *Taq* DNA polymerase (5 U/μl), 0.5 μl deoxynucleoside triphosphate mix (10 mM), 2 μl Qiagen PCR buffer, 1 μl each forward and reverse primer (10 μM), and 1 μl genomic DNA, yielding a total volume of 20 μl. The primers PCβf and PCαr were used to amplify the intergenic spacer and flanking regions of the *cpcB* and *cpcA* genes of the phycocyanin operon (PC-IGS) (27). PCR was also used to check whether the isolated strains were potential producers of either CYN or PSP toxins. The peptide synthetase (PS) gene of the cylindrospermopsin gene cluster was detected according to the PRC method of Schembri et al. (36) using M13 and M14 as primers. To simplify and accelerate detection of the saxitoxin gene cluster, we designed two primers, sxtaf (GCGTACATCCAAGCTGGACTCG) and sxtar (GTAGTCCAGCTAAGGCACTTGC), which amplify a part of the *sxtA* gene of the saxitoxin gene cluster. The *sxtA* gene encodes a polyketide synthase (PKS)-like structure (16).

The cycling protocol for the PC-IGS fragment was one cycle of 5 min at 94°C and then 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C with a final elongation step of 72°C for 10 min. The protocol for *sxtA* gene fragments was one cycle of 4 min at 94°C and then 30 cycles of 10 s at 94°C, 20 s at 55°C, and 1 min at 72°C. PCR products were visualized by 1.5% agarose gel electrophoresis with ethidium bromide staining and UV illumination.

Amplified PC-IGS and *sxtA* products were purified through Qiaquick PCR purification columns (Qiagen, Hilden, Germany), and the DNA was eluted in elution buffer according to the manufacturer's protocol. Sequencing of the purified PC-IGS and *sxtA* products was performed using the same primers as for PCR. For each PCR product, both strands were sequenced on an ABI 3100 Avant genetic analyzer using the BigDye terminator V.3.1 cycle sequencing kit (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) according to the manufacturer's instructions.

Phylogenetic analysis. Sequences of the PC-IGS locus in all *Anabaena*, *Aphanizomenon*, and *Anabaenopsis* strains were analyzed using Seqassem version 04/2008 (14). The Align (version 03/2007) MS Windows-based manual sequence alignment editor (14) was used to obtain DNA sequence alignments, which were then corrected manually. Segments with highly variable and ambiguous regions and gaps making proper alignment impossible were excluded from the analyses. A PC-IGS set containing 544 positions was used. *Nostocaceae Cyanobiont* (AY181211) was employed as the outgroup in the PC-IGS tree. Sixteen additional *Aphanizomenon* and *Anabaena* sequences derived from GenBank were included in the PC-IGS analyses. A set containing 555 positions was used for *sxtA* analysis.

Phylogenetic trees for PC-IGS and *sxtA* were constructed using the maximum likelihood (ML) algorithm in PAUP* v.10b (39). In the ML analyses, evolutionary substitution models were evaluated using the AIC criterion in Modeltest v.3.06 (32). GTR+I+G was found to be the best-fitting evolutionary model for the PC-IGS gene. The K81uf evolutionary model was used for *sxtA*. Due to limited computer capacity, ML analyses of all trees were performed with 100 bootstrap replicates using PAUP* v.10b (39).

Cyanotoxin analysis. (i) ELISA. All 92 *Aphanizomenon*, *Anabaena*, and *Anabaenopsis* strains were tested for PSP toxins and CYN by using the Abraxis saxitoxin ELISA and Abraxis cylindrospermopsin ELISA kits (Abraxis LLC, Warminister, PA) following the manufacturer's instructions. The test is an indirect competitive ELISA designed to detect saxitoxin or cylindrospermopsin based on specific antibody recognition. Before analysis, 5 ml of culture material from each cyanobacterial strain was frozen and thawed three times to extract the toxins. The ELISA results do not distinguish between dissolved and cell-bound toxins. The color reaction of the ELISA test was evaluated at 450 nm using a Biotek Synergi 2 microtiter plate reader (Biotek, Bad Friedrichshall, Germany).

(ii) LC-MS/MS. Between 10 and 20 ml of culture material of each strain was filtered through 0.45-μm-pore RC 55 membrane filters (Whatman, Dassel, Germany). Filters were extracted twice with 1.5 ml of acetonitrile-water-formic acid (75:14.9:0.1) at room temperature. Each extraction step included 10 min ultrasonication followed by shaking for 1 h and centrifugation. The supernatants were combined and dried by vacuum centrifugation.

LC-MS/MS sample analyses were carried out on an Agilent 1100 series high-pressure liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany) coupled to a API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA) equipped with a turbo ion spray interface. The extracts were separated using a 5-μm, 2- by 250-mm TSKgel Amide-80 column (Tosohaas, PA) at 30°C. The mobile phase consisted of water (A) and acetonitrile-water (95:5) (B), both containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5); the flow rate was 0.2 ml min^{-1}. The following gradient program was used for analysis of multiple toxins (cylindrospermopsin, anatoxin-a, and paralytic shellfish poisons): 75% B for 5 min, 75% to 65% B over 1 min, hold for 13 min, 65 to 45% B over 4 min, and hold for 10 min. (5). The injection volume was 10 μl.

The mass spectrometer was operated in the selected reaction monitoring mode for detection and quantification of the following toxins as described by Dell'Aversano et al. (5): cylindrospermopsin (CYN); anatoxin-a (ATX); saxitoxin (STX); neosaxitoxin (NEO); decarbamoylsaxitoxin (dcSTX); decarbamoylneosaxitoxin (dcNEO); gonyautoxins 1 (GTX1), 2, 3, 4, and 5; decarbamoylgonyautoxin 3 (dcGTX-3); and *N*-sulfogonyautoxins 1 (C1) and 2. Standard curves were established for all the toxins. CYN and PSP toxin standards obtained from the National Research Council (Halifax, Canada) and anatoxin-a standards from Tocris (United Kingdom) were analyzed in line with the unknowns (one calibration curve after 10 unknowns).

Nucleotide sequence accession numbers. The sequence data were submitted to the EMBL Nucleotide Sequence Database under the accession numbers listed in Table 2 (see also Table S1 in the supplemental material).

TABLE 2. *Aphanizomenon*, *Anabaena* and *Anabaenopsis* strains from different freshwater lakes evaluated in this study by ELISA, LC-MS/MS, and genetic properties

Species and strain	Lake	Detection of[a]:					Accession no.	
		CYN		PSP toxins		ATX (LC-MS/MS)	*sxtA*	PC-IGS
		ELISA	LC-MS/MS	ELISA	LC-MS/MS			
Aphanizomenon								
A. gracile								
AB200816	Scharmützel	−	−	+	+	−	FN552384	FN552296
AB2008/18	Scharmützel	−	−	+	+	−	FN552385	FN552297
AB2008/19	Scharmützel	−	−	+	+	−	FN552386	FN552298
AB2008/21	Scharmützel	−	−	+	+	−	FN552387	FN552299
AB2008/23	Scharmützel	−	−	+	+	−	FN552388	FN552300
AB2008/29	Scharmützel	−	−	+	+	−	FN552389	FN552304
AB2008/31	Scharmützel	−	−	+	+	−	FN552390	FN552305
AB2008/47	Melang	−	−	+	+	−	FN552391	FN552312
AB2008/48	Melang	−	−	+	+	−	FN552392	FN552313
AB2008/49	Melang	−	−	+	+	−	FN552393	FN552314
AB2008/50	Melang	−	−	+	+	−	FN552394	FN552315
AB2008/51	Melang	−	−	+	+	−	FN552395	FN552316
AB2008/59	Melang	−	−	+	+	−	FN552396	FN552318
AB2008/65	Melang	−	−	+	+	−	FN552397	FN552320
A. flos-aquae								
AB2008/63	Stechlin	−	−	−	−	−	FN552398	FN552366
ST122	Stechlin	−	−	−	−	−	FN552399	FN552361
ST128	Stechlin	−	−	−	−	−	FN552400	FN552362
ST130	Stechlin	−	−	−	−	−	FN552401	FN552363
A. issatschenkoi								
AB2008/08	Langer	−	−	−	−	−	FN552405	FN552374
AB2008/09	Langer	−	−	−	−	−	FN552406	FN552375
AB2008/11	Langer	−	−	−	−	−	FN552407	FN552376
Anabaena planktonica								
ST16	Stechlin	−	−	−	−	−	FN552402	FN552326
ST182	Stechlin	−	−	−	−	−	FN552403	FN552328
ST195	Stechlin	−	−	−	−	−	FN552404	FN552331
Anabaenopsis elenkinii								
AB2008/61	Langer	−	−	−	−	−	FN552408	FN552382

[a] +, detected; −, not detected.

RESULTS

Identification of toxin producers. As determined by ELISA and LC-MS/MS, 14 *A. gracile* strains from Lakes Melang and Scharmützel were able to produce PSP toxins (Table 2). The other 78 *Anabaena*, *Aphanizomenon*, and *Anabaenopsis* strains tested negative for PSP toxins by ELISA and LC-MS/MS (Table 2; see Table S1 in the supplemental material). LC-MS/MS revealed that each of the 14 *A. gracile* strains detected in Lakes Scharmützel and Melang produced four PSP toxin variants—gonyautoxin 5 (GTX5), saxitoxin (STX), decarbamoyl-saxitoxin (dcSTX), and neosaxitoxin (NEO)—but at different ratios (Fig. 1). Of the 7 strains from Lake Scharmützel, GTX5 was the most prevalent PSP toxin variant (50.8 to 82.3%), while NEO was the most prevalent PSP toxin variant (45.6 to 70.5%) of the 7 strains from Lake Melang (Fig. 1). The GTX5 fraction was only 11.4 to 29.8% in Lake Melang strains, and the NEO fraction ranged from 2.3 to 10.3% in Lake Scharmützel strains. STX and dcSTX were found in all 14 strains, but at lower ratios of 7.4 to 31.9% and 0.7 to 14.3%, respectively (Fig.

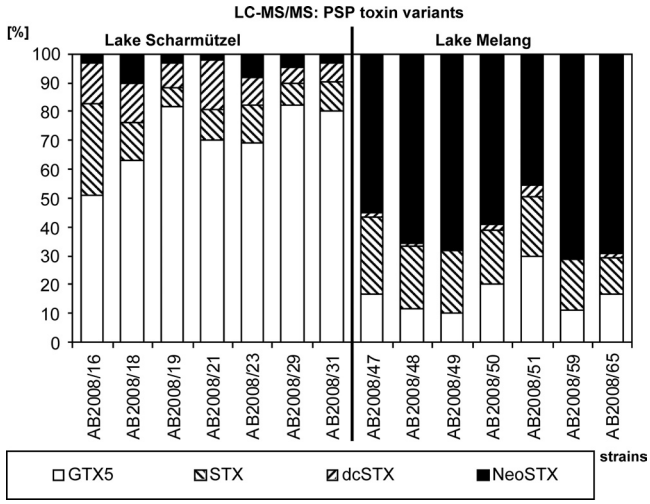

FIG. 1. Ratio (%) of PSP toxin variants in 14 *A. gracile* strains from Lakes Scharmützel and Melang, as determined with LC-MS/MS.

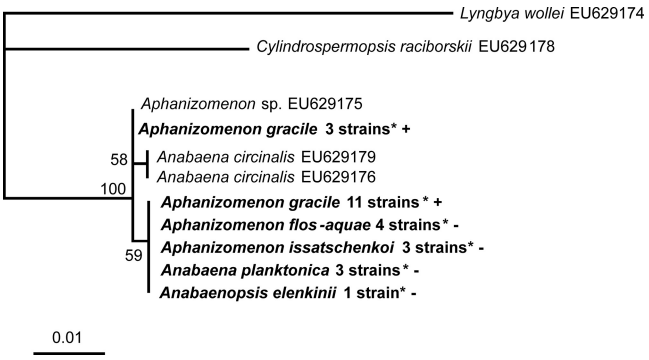

FIG. 2. Maximum likelihood tree based on partial *sxtA* sequences of 30 cyanobacterial strains. Strains from this study are marked in bold. Bootstrap values above 50 are included. The bar indicates 1% sequence divergence. +, PSP toxin producer; −, PSP toxin not detected; *, accession number listed in Table 2.

1). According to ELISA and LC-MS/MS, all 92 *Anabaena* and *Aphanizomenon* strains tested negative for CYN and ATX.

Amplification of *sxtA* and the PS gene. All 92 *Aphanizomenon*, *Anabaena*, and *Anabaenopsis* strains were investigated for the presence of the saxitoxin gene cluster. Amplification of the *sxtA* gene was observed in all 14 PSP toxin-producing *A. gracile* strains and in certain non-PSP toxin-producing strains of *A. flos-aquae* ($n = 4$), *A. planktonica* ($n = 3$), *A. issatschenkoi* ($n = 3$), and *A. elenkinii* ($n = 1$) from Lakes Melang and Scharmützel. All *sxtA* sequences were aligned with sequences taken from GenBank (NCBI), and a phylogenetic tree was constructed to demonstrate the similarity of our sequences to *sxtA* sequences from known PSP toxin-producing strains (Fig. 2). No amplification of the *sxtA* gene was detected in the other 67 *Aphanizomenon*, *Anabaena*, and *Anabaenopsis* strains. None of the 92 strains exhibited amplification of the PS gene of the CYN gene cluster.

Morphological and phylogenetic characterization. Based on their morphological features, the isolated strains were identified as *A. gracile*, *A. flos-aquae*, *A. issatschenkoi*, *A. bergii*, *Anabaena crassa* (Lemm.) Kom.-Legn. et Cronb., *Anabaena flos-aquae* Breb. ex Born. et Flah., *Anabaena lemmermanii* Richt., *Anabaena planktonica* Brunnthaler, and *Anabaenopsis elenkinii* Miller (Fig. 3).

Phylogenetic relationships of the investigated strains are presented in the maximum likelihood tree of the PC-IGS region (Fig. 4). Four main clusters were found. Cluster I contained *A. bergii* and *Anabaenopsis elenkinii* strains, and cluster II contained *A. issatschenkoi* strains. *Anabaena* spp. were grouped together in cluster III. Cluster IV contained sequences of *Anabaena* spp. and *Aphanizomenon* spp. (Fig. 4). Subcluster IVa included *Anabaena* spp. and *A. flos-aquae* strains, and subcluster IVb contained *A. gracile* and *A. flos-aquae* strains but no *Anabaena* sequences. The 34 *A. gracile* strains investigated were distributed in subclusters 1 and 2 of subcluster IVb. Subcluster IVb1 comprised seven *A. gracile* strains from Lakes Melang and Scharmützel, including one PSP toxin-producing strain from Lake Scharmützel. Sequences of *A. flos-aquae* and *Aphanizomenon* obtained from GenBank were also located here. Subcluster IVb2 comprised one *Aphanizomenon* sp. sequence obtained from GenBank and 27 *A. gracile* strains from Lakes Melang, Langer, and Scharmützel, including 13 PSP toxin-producing strains from Lakes Melang and Scharmützel.

DISCUSSION

This study demonstrated that 14 *A. gracile* strains from two northeast German lakes (Melang and Scharmützel) are PSP toxin producers. This is the first evidence implicating *A. gracile* as a PSP toxin producer in German water bodies. The only previously known PSP toxin-producing *A. gracile* strain was isolated from Lake Crato in Portugal (31). PSP toxin production has also been detected in several *A. flos-aquae* strains (25, 30). However, after morphological and genetic reevaluation, two PSP toxin-producing *A. flos-aquae* strains (NH5 and LMECYA 31) were reclassified as *Aphanizomenon* sp. and *A. issatschenkoi*, respectively (21, 22, 25). PSP toxin-producing *A. flos-aquae* strains have also been isolated from a Chinese lake (Dianchi) and a Portuguese reservoir (Crestuma-Lever) (9, 23). In both cases, classification as *A. flos-aquae* is not unequivocal because of the lack of morphological descriptions. The 18 *A. flos-aquae* strains investigated in our study were unable to produce PSP toxins.

LC-MS/MS confirmed the presence of the same four PSP toxin variants (STX, NEO, GTX5, and dcSTX) in all 14 PSP toxin-producing *A. gracile* strains. However, the strains from Lake Melang mainly produced NEO, one of the most toxic PSP toxin variants (15, 38). Mouse bioassays have shown that this toxin is 14 times more toxic than GTX5, the variant mainly produced in the strains from Lake Scharmützel (15, 38).

The same four PSP toxin variants were found by Dias et al. (6) in *A. issatschenkoi* strain LMECYA 31. At 22°C, which corresponded to our culture conditions, Dias et al. (6) observed predominant production of GTX5 in this strain. The Portuguese *A. gracile* strain LMECYA 40 only produced two PSP toxin variants, STX and NEO, and *A. flos-aquae* strains isolated from Chinese Lake Dianchi produced three PSP toxin variants, STX, NEO, and GTX5 (23, 31). Australian *Anabaena circinalis* and American *Lyngbya wollei* strains exhibited a higher number (up to 9) of PSP toxin variants (29, 41).

Amplification of parts of the *sxtA* gene in our 14 PSP toxin-producing *Aphanizomenon gracile* strains confirmed the presence of a saxitoxin gene cluster in these strains. Interestingly, we also found the *sxtA* gene in 11 non-PSP toxin-producing *A. issatschenkoi*, *A. flos-aquae*, *A. elenkinii*, and *A. planktonica* strains. According to Li et al. (22) and Liu et al. (23), strains of *A. issatschenkoi* and *A. flos-aquae* have been confirmed as PSP toxin producers.

The *sxtA* gene encodes a polyketide synthase (PKS)-like structure (16). PKS is involved in the synthesis of secondary metabolites like microcystin, cylindrospermopsin, and saxitoxin (16, 28, 36). Moustafa et al. (26) demonstrated that assembly of the saxitoxin gene cluster in the cyanobacterium *A. circinalis* involved multiple horizontal gene transfers from different bacterial and cyanobacterial sources. The same authors hypothesize that various former PSP toxin-producing *A. circinalis* strains most likely lost the ability to produce PSP toxins over time, leading to a coexistence of PSP toxin-producing and non-PSP toxin-producing strains (26). This mechanism implies

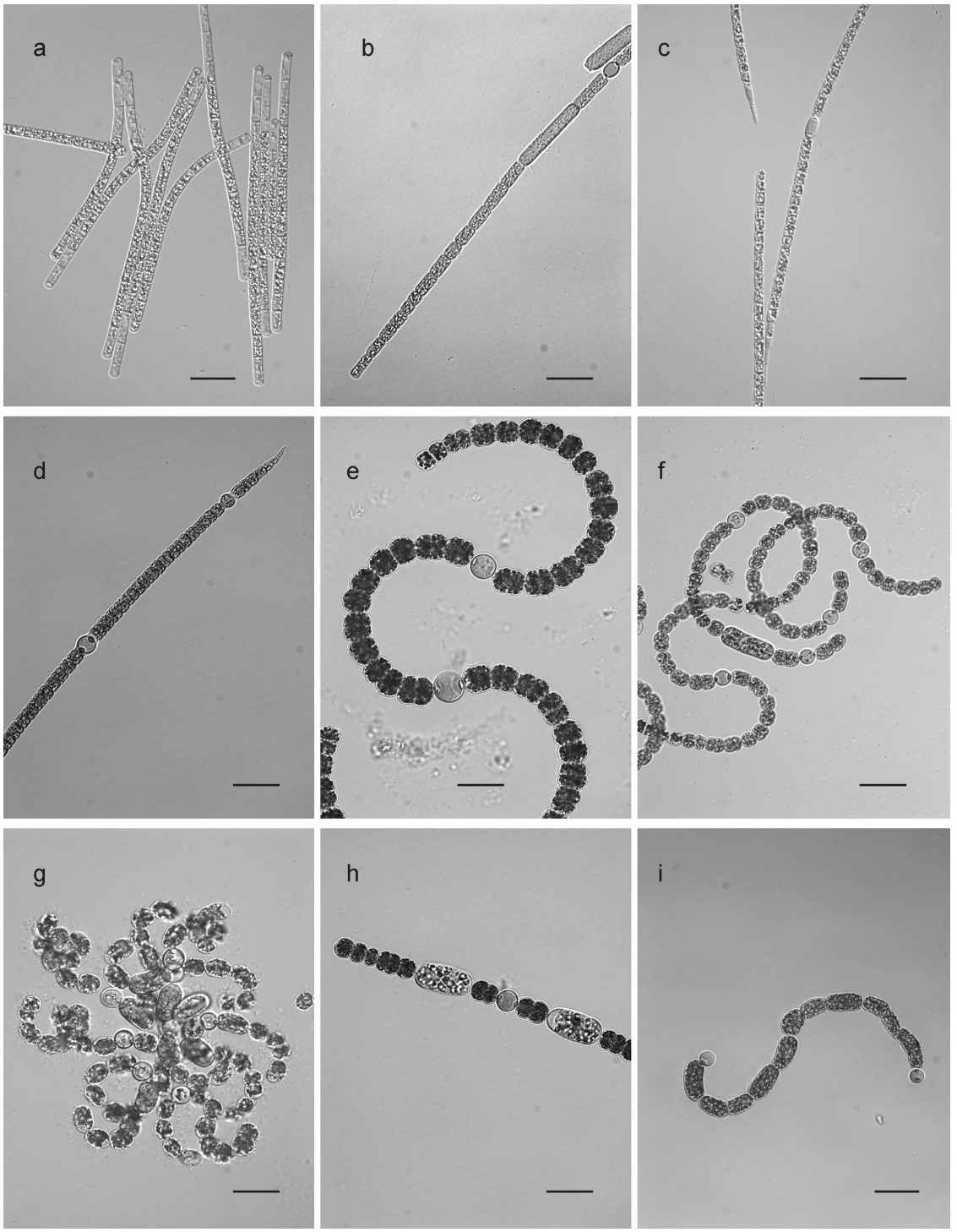

FIG. 3. Micrographs of *Nostocales* strains investigated in this study. (a) *Aphanizomenon flos-aquae*; (b) *Aphanizomenon gracile*; (c) *Aphanizomenon issatschenkoi*; (d) *Anabaena bergii*; (e) *Anabaena crassa*; (f) *Anabaena flos-aquae*; (g) *Anabaena lemmermanii*; (h) *Anabaena planktonica*; and (i) *Anabaenopsis elenkinii.* Scale bars indicate 25 μm.

that the non-PSP toxin-producing *A. gracile* strains in our study have lost all or part of the saxitoxin gene cluster.

Previous 16S rRNA gene, internal transcribed spacer 1 (ITS1), and *rbcLX* sequence studies by Gugger et al. (10), Lyra et al. (24), and Rajaniemi et al. (34) have demonstrated that planktonic *Aphanizomenon* spp. and *Anabaena* spp. are genetically heterogeneous and form intermixed clusters in phylogenetic trees. Our phylogenetic study using PC-IGS sequences supports these findings in the case of *Anabaena* spp. and *A. flos-aquae*. Clusters III and IVa of our phylogenetic tree comprise

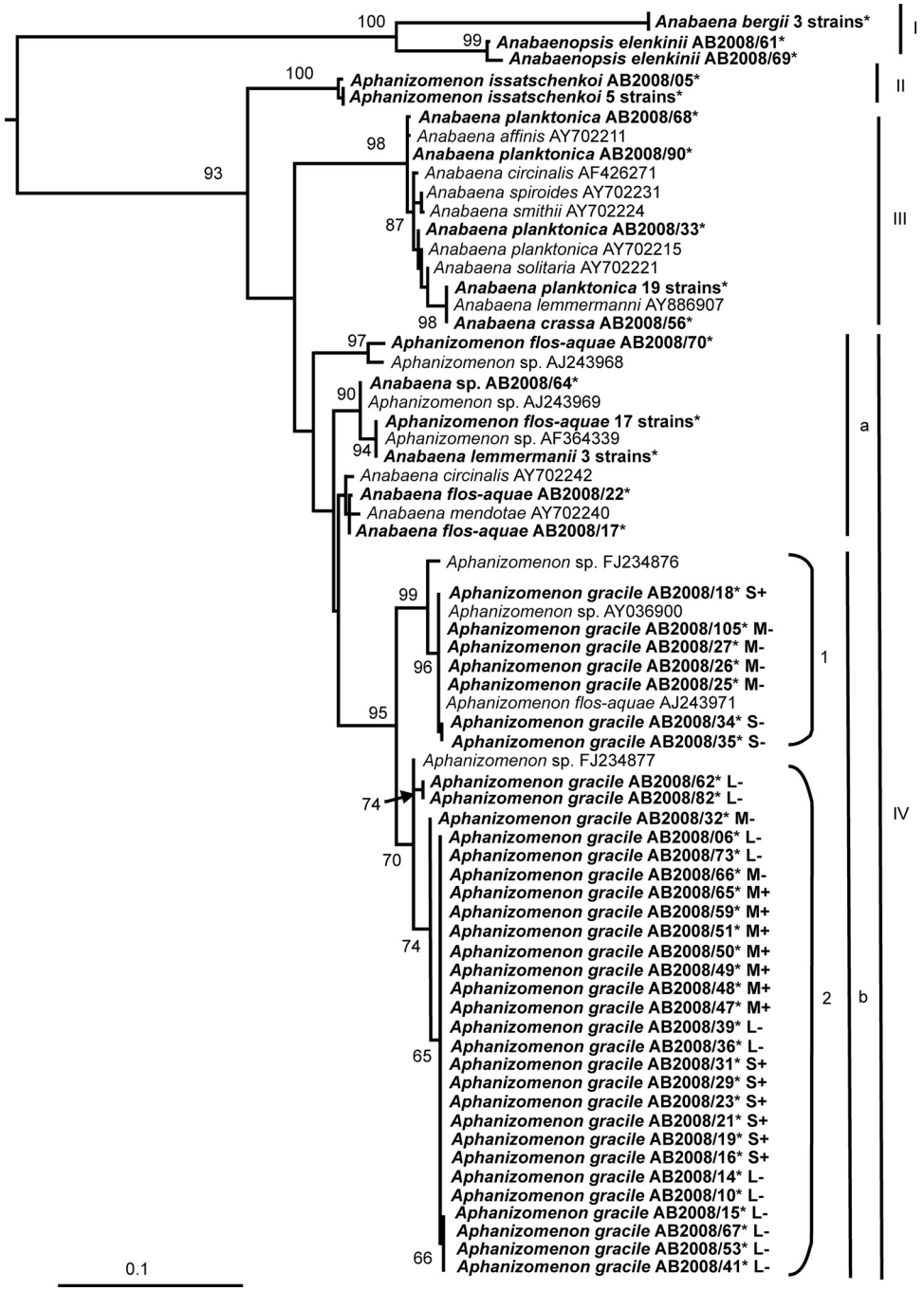

FIG. 4. Maximum likelihood tree based on partial PC-IGS sequences of 107 cyanobacterial strains. Strains from this study are marked in bold. Bootstrap values above 50 are included. The scale bar indicates 10% sequence divergence. L, Lake Langer; M, Lake Melang; S, Lake Scharmützel. +, PSP toxin producer; −, PSP toxin not detected; ⁎, accession number listed in Table 2 (and see Table S1 in the supplemental material).

Anabaena and *A. flos-aquae* sequences. In cluster IVb, our *A. gracile* strains are clearly separated from the *Anabaena* spp. and *Aphanizomenon flos-aquae* strains. Considering that *A. flos aquae* CCAP 1401 (accession no. AJ243971), which is included in cluster IVb, was renamed *A. gracile* in the Czech Culture Collection of Autotrophic Organisms (CCALA), morphological classification of our strains as *A. gracile* seems well supported.

Our PSP toxin-producing and non-PSP toxin-producing *A. gracile* strains could not be distinguished using the phylogenetic tree of PC-IGS. In cluster IVb, toxic strains are grouped together with nontoxic strains. Although toxic strains from Lakes Melang and Scharmützel were characterized by different ratios of GTX5 and NEO, no phylogenetically similar pattern was found in the PC-IGS tree.

Identification of the PSP toxin-producing *A. gracile* strains isolated from Lake Melang explains the presence of paralytic

shellfish poisoning toxins there (3). However, PSP toxin-producing *A. gracile* strains were also detected in Lake Scharmützel, although no PSP toxins were detected there in a survey conducted in 1995 to 1997 (3). According to Rücker et al. (35), *A. gracile* biovolumes in Lakes Melang and Langer are much higher than those in Lake Scharmützel. When the proportion of PSP toxin-producing *A. gracile* in the phytoplankton community is as low as that in Lake Scharmützel, the PSP toxin concentration in water samples is probably below the detection limit. Although paralytic shellfish poisoning toxins were detected in Lake Langer in 2007 (J. Fastner, unpublished data), we did not find any PSP toxin-producing cyanobacterial strains there. Therefore, an extensive survey would be needed in order to detect PSP toxin-producing cyanobacteria in Lake Langer.

The same applies to ATX and CYN. In our study, PCR, ELISA, and LC-MS/MS did not reveal the presence of ATX or CYN producers among the investigated strains, although ATX and CYN had been detected in Lakes Langer and Melang in the 1990s (3, 7). ATX producers have yet to be detected in German water bodies. The only known CYN producer in northeast German water bodies is *A. flos-aquae* (33). Because *A. gracile* exhibited the highest correlation coefficients between biovolume and CYN concentration in a broad range of German water bodies, it is viewed as a prime candidate (35, 43).

In conclusion, this is the first study identifying *A. gracile* as a PSP toxin producer in German water bodies. As non-PSP toxin-producing *A. issatschenkoi*, *A. flos-aquae*, *A. planktonica*, and *Anabaenopsis* sp. strains also possessed the *sxtA* gene of the saxitoxin gene cluster, it is very likely that other nostocalean species in German water bodies can also produce paralytic shellfish poisoning toxins.

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