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Phylogenetic Study by the Morphological and Molecular Analyses of Japanese Planktonic *Anabaena* Species

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Abstract Molecular analyses of nineteen species of planktonic *Anabaena* strains collected from eutrophic lakes and ponds in Japan were conducted and the phylogenetic relationships compared. Using cultured strains with clearly identified morphological characters, there was no discrepancy between the identification results from the morphological study and the cluster classification results from the DNA analyses. The cultured strains were divided into four clusters based on the information from the 16S rDNA and *rbcLX* (*rbcL* and *rbcX*) analyses. The forms and dimensions of vegetative cells and akinetes as well as the relative location of akinetes to heterocytes are important for distinguishing planktonic *Anabaena* species. We also propose a new combination, *Sphaerospermum oumianum* (M. Watanabe) Tuji et Niiyama comb. nov. for *Anabaena oumiana*.

Key words: *Anabaena*, *Anabaena oumiana*, culture, Japan, morphology, planktonic, *rbcL*, *rbcX*, rDNA, *Sphaerospermum oumianum* (M. Watanabe) Tuji et Niiyama comb. nov.

Introduction

To examine morphological variations among Japanese *Anabaena* strains, we collected many plankton samples from different localities throughout Japan, obtaining more than a hundred strains. Nine species with coiled trichomes have already been classified from the results of microscopic observation (Watanabe *et al*., 2004). In this study, molecular analyses of planktonic *Anabaena* strains with either coiled or straight trichomes were conducted and the phylogenetic relationships among them compared.

As Komárek points out (Komárek, 2006), it is not common for the names of cultured strains to be corrected or updated based on subsequent studies, or misidentifications of cultured strains to be corrected in collections list and databases. Phylogenetic trees derived from molecular analysis of misidentified cultured strains thus may not reflect new results from morphological studies. To prevent such a discrepancy from occurring, in the present study we used our own strains, which had clearly defined origins and clearly identified ecological and morphological characters.

Materials and Methods

Cultured strains

The forty-eight strains used in this study are shown in Table 1. Most of the strains were collected from eutrophic lakes and ponds in Japan, as shown in Fig. 1, during 1990 to 1991 and 2001 to 2002. They were isolated and maintained in the stock room of the Department of Botany, National Museum of Nature and Science as TAC (Tsukuba Algal Collection). These strains are now deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES), Japan, excluding TAC111. Isolation was done by the pipette washing method under a binocular. Throughout this study, 10 ml of NaCB medium (Ichimura and Watanabe, 1977) contained in a screw cap test tube (18

Table 1. Species names, culture identifications (ID), localities, DDBJ accession numbers, and original publications for morphology of strains used in this study

Species	Culture ID TAC ID	NIES ID	Localities	DDBJ accession ID *rbcL*	DDBJ accession ID 16S	Original publication for morphology
Anabaena kisseleviana	TAC34	NIES807	Lake Kasumigaura, Ibaraki, Japan	AB551484	AB551437	
Anabaena flos-aquae	TAC99	NIES1668	Lake Suwa-ko, Nagano, Japan	AB551485	AB551438	
Anabaena crassa	TAC111	-	Shigure Dam, Tokyo, Japan	AB551486	AB551439	
Anabaena mucosa	TAC425	NIES1677	Lake Toro-ko, Hokkaido, Japan	AB551487	AB551440	Niiyama, 1996; Watanabe *et al*., 2004
Anabaena smithii	TAC431	NIES820	Hirosakijo-hori, Aomori, Japan	AB551488	AB551441	Niiyama, 1996
Anabaena crassa	TAC436	NIES1652	Lake Akan-ko, Hokkaido, Japan	AB551489	AB551442	Niiyama, 1996
Anabaena lemmermannii	TAC437	NIES808	Lake Akan-ko, Hokkaido, Japan	AB551490	AB551443	Niiyama, 1996
Anabaena lemmermannii	TAC438	NIES1673	Lake Akan-ko, Hokkaido, Japan	AB551491	AB551444	Niiyama, 1996
Anabaena affinis	TAC439	NIES1639	Lake Shirarutoro-ko, Hokkaido, Japan	AB551492	AB551445	Niiyama, 1996
Anabaena crassa	TAC443	NIES1653	Lake Kasumigaura, Ibaraki, Japan	AB551493	AB551446	Niiyama, 1996 (as *Anabaena spiroides*)
Anabaena flos-aquae	TAC446	NIES1672	Lake Kasumigaura, Ibaraki, Japan	AB551494	AB551447	
Anabaena heterospora	TAC447	NIES1697	a pond in Watarase flood-control zone, Tochigi, Japan	AB551495	AB558957	Watanabe, 2006
Anabaena ucrainica	TAC449	NIES826	Lake Sagami-ko, Kanagawa, Japan	AB551496	AB551448	Watanabe *et al*., 2004
Anabaena smithii	TAC450	NIES822	Lake Akan-ko, Hokkaido, Japan	AB551497	AB551449	Niiyama, 1996
Anabaena elliptica	TAC453	NIES1667	Lake Shirakaba-ko, Nagano, Japan	AB551498	AB551450	Watanabe, 2007
Anabaena affinis	TAC454	NIES1642	Lake Tsukui-ko, Kanagawa, Japan	AB551499	AB551451	
Anabaena ucrainica	TAC455	NIES1696	Lake Tsukui-ko, Kanagawa, Japan		AB551452	Watanabe *et al*., 2004
Anabaena aphanizomenoides	TAC456	NIES1643	a pond, Ibaraki, Japan	AB551500	AB551453	
Anabaena oumiana	TAC464	NIES1678	Lake Inba-numa, Chiba, Japan	AB551501	AB551454	Watanabe *et al*., 2004
Anabaena planctonica	TAC472	NIES1683	Nigo-ike pond, Hyogo, Japan	AB551502	AB551455	
Anabaena crassa	TAC474	NIES1657	Nigo-ike pond, Hyogo, Japan	AB551503	AB551456	
Anabaena flos-aquae	TAC475	NIES1674	Nigo-ike pond, Hyogo, Japan	AB551504	AB551457	
Anabaena reniformis	TAC478	NIES1690	Shin-ike pond, Hyogo, Japan		AB551458	Watanabe *et al*., 2004
Anabaena reniformis	TAC481	NIES1693	Tatsugaya-ike pond, Hyogo, Japan	AB551505	AB551459	Watanabe *et al*., 2004
Anabaena reniformis	TAC484	NIES1694	Lake Tega-numa, Chiba, Japan	AB551506	AB551460	Watanabe *et al*., 2004
Anabaena crassa	TAC485	NIES1658	Lake Biwa-ko, Shiga, Japan		AB551461	
Anabaena crassa	TAC494	NIES1665	a pond, Niigata, Japan		AB551462	
Anabaena circinalis	TAC496	NIES1646	Lake Shiroyama-ko, Kanagawa, Japan	AB551507	AB551463	
Anabaena circinalis	TAC500	NIES1651	Lake Shiroyama-ko, Kanagawa, Japan		AB551464	
Anabaena circinalis	TAC503	NIES1878	Lake Shiroyama-ko, Kanagawa, Japan	AB551509	AB551465	
Anabaena akankoensis	TAC505	NIES1875	Lake Akan-ko, Hokkaido, Japan		AB551466	Watanabe, 2003; Watanabe *et al*., 2004
Anabaena circinalis	TAC507	NIES1880	Lake Inba-numa, Chiba, Japan	AB551510	AB551467	
Anabaena oumiana	TAC509	NIES1904	Funada-ike pond, Chiba, Japan		AB551468	Watanabe *et al*., 2004
Anabaena crassa	TAC514	NIES1894	Lake Inba-numa, Chiba, Japan		AB551469	
Anabaena crassa	TAC515	NIES1895	a pond, Osaka, Japan	AB551511	AB551470	
Anabaena crassa	TAC520	NIES1884	Lake Suwa-ko, Nagano, Japan	AB551512	AB551471	Watanabe *et al*., 2004

Table 1. (Continued)

Species	Culture ID TAC ID	NIES ID	Localities	DDBJ accession ID *rbcL*	DDBJ accession ID 16S	Original publication for morphology
Anabaena crassa	TAC525	NIES1887	Lake Suwa-ko, Nagano, Japan	AB551513	AB551472	
Anabaena crassa	TAC529	NIES1891	Lake Biwa-ko, Shiga, Japan		AB551473	Watanabe *et al.*, 2004
Anabaena pseudocompacta	TAC538	NIES1939	Lake Hachiro-gata, Akita, Japan		AB551474	
Anabaena reniformis	TAC543	NIES1942	a pond, Osaka, Japan	AB551514	AB551475	Watanabe *et al.*, 2004
Anabaena spiroides	TAC551	NIES1950	Mishima-ike pond, Shiga, Japan		AB551476	Watanabe *et al.*, 2004
Anabaena circinalis	TAC553	NIES1909	Lake Hachiro-gata, Akita, Japan		AB551477	Watanabe *et al.*, 2004
Anabaena minispora	TAC554	NIES1922	Lake Mikata-ko, Fukui, Japan		AB551478	
Anabaena planctonica	TAC561	NIES1934	Lake Shinotsu-ko, Hokkaido, Japan	AB551515	AB551479	
Anabaena oumiana	TAC568	NIES1931	a pond, Tottori, Japan	AB551516	AB551480	
Anabaena mendotae	TAC583	NIES1920	Lake Kusyu-ko, Hokkaido, Japan	AB551517	AB551481	
Anabaena mendotae	TAC584	NIES1921	Lake Kusyu-ko, Hokkaido, Japan	AB551518	AB551482	
Anabaena circinalis	TAC585	NIES1929	Lake Kusyu-ko, Hokkaido, Japan	AB551519	AB551483	

Fig. 1. Localities of strains cultured and used in this study.

mm×150 mm) was used. The cultures were illuminated by cool-white fluorescent lamps, with a photon flux density of *ca*. 20 μmol/m^2/sec, a photoperiod of 8 hours light and 16 hours dark, and a temperature of 18°C or 20°C.

Morphological observations such as the forms and dimensions of vegetative cells, heterocytes, akinetes and trichomes as well as the relative location of akinetes to heterocytes were conducted under the microscope using 100 times objective oil immersion lenses. The morphological observations were performed for the cultured strains listed on Table 1, excluding TAC99 and TAC111.

DNA extraction and amplification

DNA was extracted using GenomicPrep (Amersham Biosciences, NJ) from cultured strains. The PCR of 16S rDNA was performed with prokaryote universal primers: forward primer 8f (Lane *et al*., 1985) and primer 1480 for heterocytous cyanobacteria (Gugger *et al*., 2002) using a thermal cycler (iCycler) with Ex Taq

DNA polymerase (Takara, Tokyo, Japan). The PCR of *rbcLX* (*rbcL* and *rbcX*) was performed with the CW and CX primer for *Anabaena* species (Rudi *et al*., 1998). PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH) following the instruction manual. The cycle sequencing samples were purified by ethanol precipitation. Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The obtained sequences were assembled using ChromasPro (Technelysium Pty Ltd., Tewantin).

Phylogenetic reconstruction

The 16S rDNA and *rbcLX* sequences were aligned manually with the alignment editor "BIOEDIT" (Tom Hall, CA) on a Windows XP operating system. Phylogenetic inferences were made using two software packages: PAUP 4.0* (phylogenetic analyses using parsimony) (Swofford, 2002) and MrBayes (V. 3.0b4) (Huelsenbeck and Ronquist, 2001). The data sets were subjected to the model test program (V. 3.06) to ascertain the appropriate model of evolution for the data sets. The Bayesian analysis of the 16S DNA data sets was conducted on GTR+invariable+gamma (GTR+I+G) as the best-fit model. The Bayesian analysis of the *rbcLX* data sets was also conducted on GTR+invariable+gamma (GTR+I+G) as the best-fit model. We ran the Bayesian search using these models during 1,000,000 generations, and saved every 1000th tree.

Fig. 2. Molecular phylogeny of *Anabaena* taxa using 16S rDNA. The scale bar indicates 0.1 nucleotide substitutions per site. Numbers above the branches indicate Bayesian posterior probabilities. Bold lines mean >.95 Bayesian posterior probabilities.

Results

We succeeded in sequencing 48 strains for 16S rDNA (Fig. 2) and 35 strains for *rbcLX* (Fig. 3). The succession rate of *rbcLX* was relatively lower than that of 16S rDNA. A long insertion was found in seven strains between the *rbcX* and *rbcS* region. The region of this insertion was the same place reported by Rudi *et al.* (1998). This insertion was omitted from the data set for Bayesian analysis.

The forms and dimensions of the vegetative cells, heterocytes, akinetes and trichomes were measured in each strain, i.e., the width and length of vegetative cells (Figs. 4, 5), width, length and length/width ratios of akinetes (Figs. 6–8), diameter of the trichome coils (Fig. 9) and number of cells between the heterocytes and akinetes (Fig. 10). The order of the strain numbers in Figs. 4–10 corresponds to those of Fig. 2.

The cultured strains can be divided into four clusters, **A**, **B**, **C** and **D**, using the information from the 16S rDNA and *rbcLX* analyses (Figs. 2, 3). In addition, cluster **A** can be divided into the subclusters **A′** and **A″** based on 16S rDNA analysis. Subcluster **A′** contains *Anabaena circinalis* and *A. planctonica*, and subcluster **A″** contains *A. crassa*, *A. ucrainica*, *A. elliptica*, *A. smithii*, *A. mucosa* and *A. minispora*. However, the division

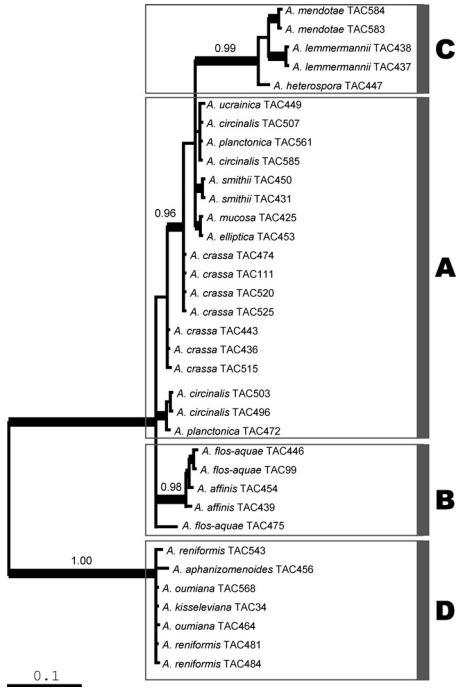


Fig. 3. Molecular phylogeny of *Anabaena* taxa using *rbcL* and *rbcX* DNA. The scale bar indicates 0.1 nucleotide substitutions per site. Numbers above the branches indicate Bayesian posterior probabilities. Bold lines mean >.95 Bayesian posterior probabilities.

between **A′** and **A″** is not supported by *rbcLX* analysis (Fig. 3). Cluster **B** contains *A. affinis*, *A. flos-aquae*, *A. pseudocompacta* and *A. spiroides*. Cluster **C** contains *A. lemmermannii*, *A. mendotae* and *A. heterospora*. Although *A. akankoensis* is also included in Cluster **C**, this species is somewhat different from the other members of Cluster **C**. Vegetative cells of *A. akankoensis* overlap with the other members of Cluster **C** in dimension, but differ from them in shape. In addition, the akinetes of *A. akankoensis* are sometimes somewhat curved and develop at both sides or scarcely one side of the heterocytes and at one or two cells distant from the heterocytes. When this occurs, the *A. akankoensis* is considered to be in cluster **C′**. Cluster **D** contains *A. oumiana*, *A. kisseleviana*, *A. reniformis* and *A. aphanizomenoides*.

The width of the vegetative cells is 7.0–16.0 μm in Cluster **A′**, 5.0–13.0 μm in Cluster **A″**, 3.5–8.0 μm in Cluster **B**, 3.2–7.0 μm in Cluster **C**, 6.3–8.8 μm in Cluster **C′** and 3.0–8.9 μm in Cluster **D** (Fig. 4). Cluster **A** tends to have the largest value and Cluster **D** the smallest one. Clusters **B** and **C** are between Cluster **A** and Cluster **D**.

The length of the vegetative cells is 3.8–14.5 μm in Cluster **A′** and 2.5–21.0 μm in Cluster **A″**; however, it was 2.5–12.0 μm in Cluster **A″** excluding TAC453, 3.5–8.0 μm in Cluster **B**, 2.0–8.2 μm in Cluster **C** (5.0–11.5 μm in Cluster **C′**) and 2.5–10.0 μm in Cluster **D** (Fig. 5). There was little difference in the lengths of the vegetative cells between each cluster.

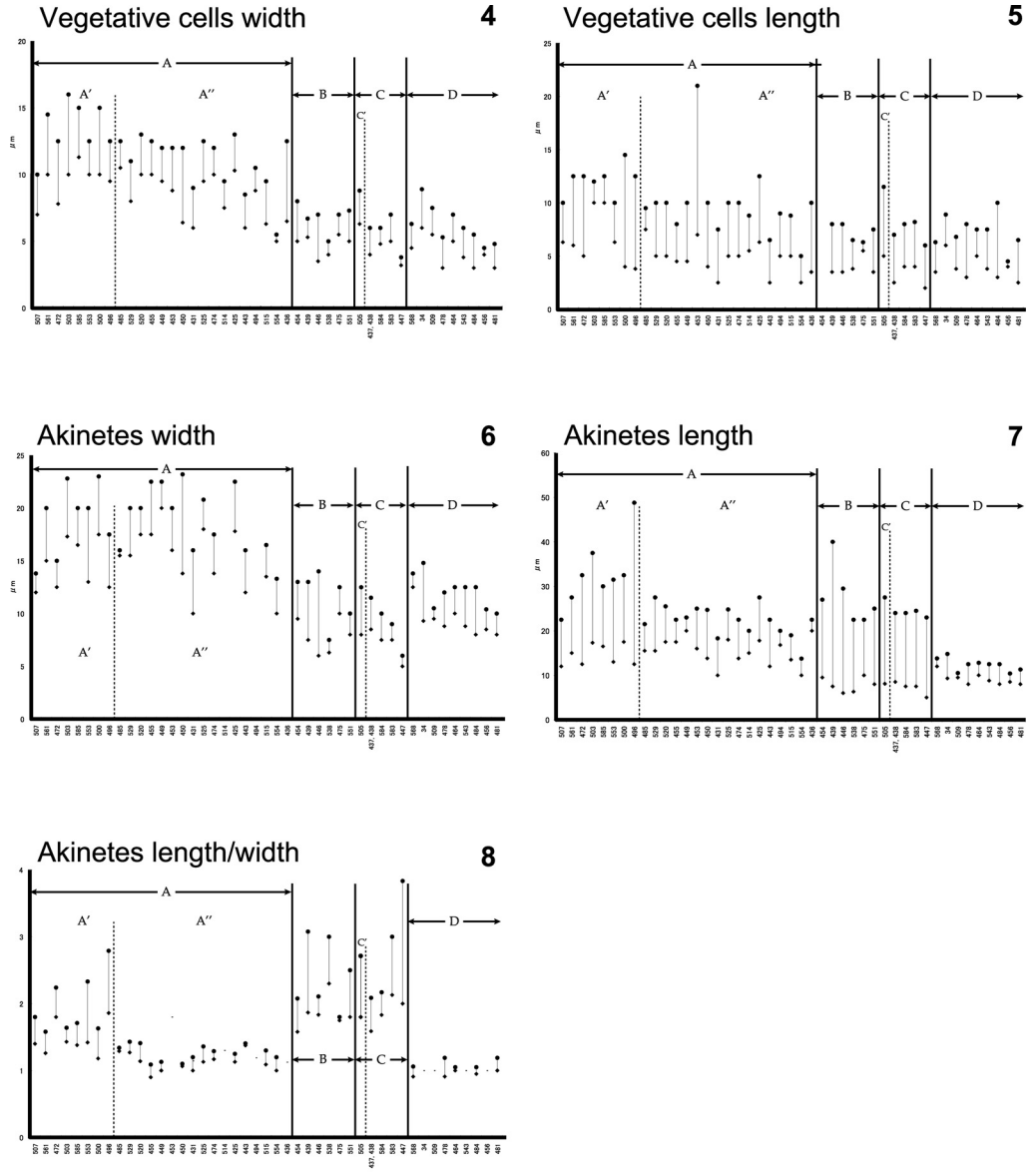
The width of the akinetes was 12.0–23.0 μm in Cluster **A′**, 10.0–23.2 μm in Cluster **A″**, 6.0–14.0 μm in Cluster **B**, 5.0–11.5 μm in Cluster **C** (8.0–12.5 μm in Cluster **C′**) and 8.0–14.8 μm in Cluster **D** (Fig. 6). Cluster **A** tended to have the largest value. There was little difference between the other four clusters.

The length of the akinetes was 17.5–48.8 μm in Cluster **A′** (−37.0 μm excluding TAC496), 10.0–27.5 μm in Cluster **A″**, 11.0–40.0 μm in Cluster **B** (−30 μm excluding TAC439), 10.0–24.5 μm in Cluster **C**, 18.0–27.5 μm in Cluster **C′** and 8.0–14.8 μm in Cluster **D** (Fig. 7). Cluster **A** has the largest value and Cluster **D** the smallest one. There was little difference between Clusters **B** and **C**.

The length/width ratio was 1.2–2.8 in Cluster **A′**, 0.9–1.8 in Cluster **A″**, 1.6–3.1 in Cluster **B**, 1.6–3.8 in Cluster **C**, 1.8–2.7 in Cluster **C′**, and about 1.0 in Cluster **D** (Fig. 8).

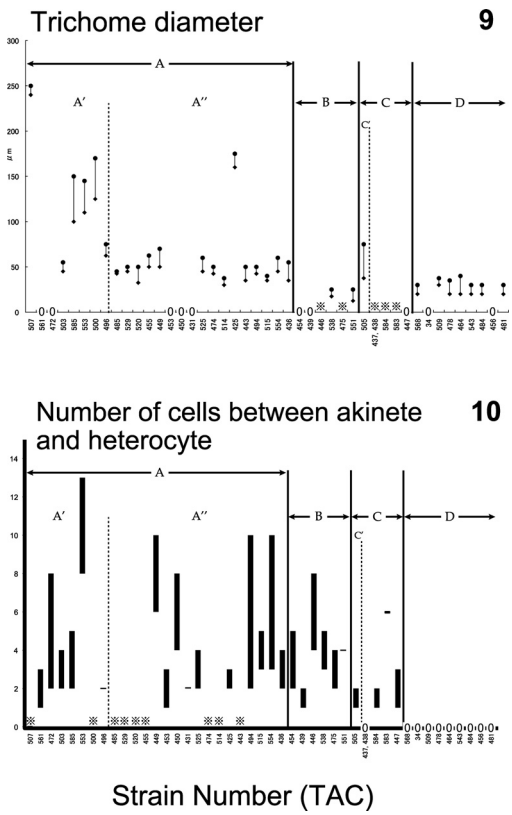
Both coiled (regularly or irregularly) and straight trichomes were observed in every cluster (Fig. 9). The diameter of the coils was 30–60 μm in many strains in Cluster **A**, but reached to 100–175 μm and to 250 μm in Cluster **A′**, about 20–25 μm in Cluster **B**, 37–75 μm in Cluster **C′**, and *ca*. 20–30 μm in Cluster **D**. Cluster **C** contained straight and irregularly coiled trichomes, and no regularly coiled ones.

There were 1–13 cells between the akinete and heterocyte in Cluster **A**, 1–8 in Cluster **B**, 1–6 in



Figs. 4–10. Relationships between morphological characters and clusters found in 16S rDNA.

Fig. 4. Vegetative cells width of cultured strains. Fig. 5. Vegetative cells length of cultured strains. Fig. 6. Akinetes width of cultured strains. Fig. 7. Akinetes length of cultured strains. Fig. 8. Akinetes length/width ratios of cultured strains. Fig. 9. Trichomes diameter of cultured strains. If the trichome is straight or irregularly coiled, then the coil diameter is shown as zero or an asterisk is indicated, respectively. Fig. 10. Relative location of the akinetes to the heterocytes. Asterisks mean that no heterocyte was observed in the cultured strains, and zero means that the akinete is adjacent to the heterocyte.



Cluster **C**, excluding TAC437 whose value is zero, and 1–2 in Cluster **C′**. However in Cluster **D**, the value was zero, i.e., the akinete was always adjacent to the heterocyte.

The characteristics of the four clusters gained in this study are as follows according to the results from the morphological observations reported above, and in consideration of the previous studies on the Japanese planktonic *Anabaena* species (Watanabe, 1992, 1996a, 1996b, 1998, 2003, 2006 and 2007; Niiyama, 1996; Watanabe *et al*., 2004),

Cluster **A**: Vegetative cells spherical, hemispherical, barrel-shaped or somewhat elliptical, 5.0–16.0 μm wide, 2.5–14.5(−21.0) μm long (Figs. 4, 5); akinetes 10.0–23.0 μm wide, 18.0–37.5(−48.0) μm long, and oval to cylindrical with length/width ratios 1.2–2.8 in Cluster **A′**, and spherical to wide oval with length/width ratios 0.9–1.8 in Cluster **A″** (Figs. 6–8); trichomes regularly coiled or straight (Fig. 9); akinetes distant from heterocytes, the number of cells between akinetes and heterocytes 1–13. Cluster **A** can be divided into two subclusters **A′** and **A″** (Fig. 2). Subcluster **A′** contains *Anabaena circinalis* and *A. planctonica*, and subcluster **A″** contains *A. crassa*, *A. ucrainica*, *A. elliptica*, *A. smithii*, *A. mucosa* and *A. minispora*. Although *A. ucrainica* is treated as a synonym of *A. mucosa* by Komárek and Zapomelova (2007), we regard them as distinct from each other (Watanabe, 1996a, 2007; Watanabe *et al*., 2004).

Cluster **B**: Vegetative cells spherical to hemispherical, or barrel-shaped, 3.5 μm wide, 3.5–8.0 μm long (Figs. 4, 5); Akinetes elliptical to cylindrical with round ends, sometimes slightly arcuate, 6.0–14.0 μm wide, 11–30(−40) μm long, length/width ratios 1.6–3.1 (Figs. 6–8); trichomes regularly or irregularly coiled, or straight (Fig. 9); akinetes distant from heterocytes, the number of cells between akinetes and heterocytes 1–8 (Fig. 10). Cluster **B** contains *Anabaena affinis*, *A. flos-aquae*, *A. pseudocompacta* and *A. spiroides*. A long insertion (ca. 270 bp) was only found in all seven strains in Cluster **B**.

Cluster **C**: Vegetative cells spherical, barrel-shaped, or elliptical to cylindrical, 3.2–7.0 μm wide, 2.0–8.2 μm long (Figs. 4, 5); akinetes long elliptical to cylindrical with round ends, 5.0–11.5 μm wide, 10.0–24.5 μm long, length/width ratios 1.6–3.8 (Figs. 6–8); trichomes irregularly coiled or straight (Fig. 9); akinetes adjacent to or one to several cells distant from heterocytes (Fig. 10). Cluster **C** contains *Anabaena lemmermannii*, *A. mendotae* and *A. heterospora*.

Cluster **C′**: Vegetative cells lemon-shaped, 6.3–8.8 μm wide, 5.0–11.5 μm long (Figs. 4, 5); akinetes cylindrical with round ends, sometimes somewhat arcuate, 8.0–12.5 μm wide, 18.0–27.5 μm long, length/width ratios 1.8–2.7 (Figs. 6–8); trichomes somewhat regularly with a coiling diameter of 37.0–75.0 μm (Fig. 9); akinetes one to two cells distant from one or both sides of the heterocytes (Fig. 10). Cluster **C′** contains *Anabaena akankoensis*.

Cluster **D**: Vegetative cells spherical, barrel- or kidney-shaped, 3.0–8.9 μm wide, 2.5–10.0 μm

long (Figs. 4, 5); akinetes spherical to oval, 8.0–14.8 μm wide, 8.0–14.8 μm long, length/width ratios almost 1.0 (Figs. 6–8); trichomes regularly coiled with small coiling diameter, 20.0–37.5 μm, or straight (Fig. 9); akinetes always adjacent to one or both sides of the heterocytes (Fig. 10). Cluster **D** contains *Anabaena oumiana*, *A. kisseleviana*, *A. reniformis* and *A. aphanizomenoides*. Komárek and Zapomelova (2007) treat *A. oumiana* as a synonym of *A. torques-reginae,* but in accordance with Watanabe's opinion (Watanabe, 1996a, 2007), the former is treated as a distinct species in this article.

Discussion

Komárek and Anagnostidis (1989) point out that the genus *Anabaena* is heterogeneous from several morphological and ecological viewpoints, and distinguished by the periphytic or planktonic mode of life and particularly by the ability to produce gas vesicles within the vegetative cells. They established two subgenera, the subgenus *Anabaena*, which is periphytic and without gas vesicles in the cells (type species: *Anabaena oscillarioides* Bory ex Born. et Flah. 1886) and the subgenus *Dolichospermum* (type species: *Anabaena flos-aquae* Breb. ex Born. et Flah. 1886), which is planktonic and with gas vesicles. Recently, Wacklin *et al*. (2009) transferred all planktonic *Anabaena* species into the new genus *Dolichospermum*.

From the molecular analysis, it was demonstrated that *Aphanizomenon* and planktonic *Anabaena* are very close, but the same species of planktonic *Anabaena* are sometimes found in diverse clusters including both *Anabaena* and *Aphanizomenon* (Gugger *et al*., 2002; Rajaniemi *et al.*, 2005; Komárek, 2006). The majority of traditional morphological studies depend on the samples directly collected from the field. On the other hand, many molecular analyses depend on the existing cultured strains. When the names of cultured strains are not corrected or updated based on subsequent studies or misidentifications are not corrected and remain on the collections list, the validity of the species name and the phylogenetic trees derived from molecular analyses will be in question. In this study, we used our own cultured strains with clear origins and clearly identified morphological characters (see Niiyama, 1996; Watanabe *et al*., 2004). There is thus no discrepancy between the identification results from the morphological study and the cluster classification results from the DNA analyses (Figs. 2, 3).

The forms and dimensions of vegetative cells and akinetes as well as the relative location of akinetes to heterocytes are important for distinguishing *Anabaena* species. On the other hand, the form of trichomes such as straight or coiled, and the dimensions of the coils, do not reflect the phylogeny. TAC496, TAC500 and TAC503 can be identified as *Anabaena circinalis* from the forms and dimensions of the vegetative cells and akinetes, but the diameters of the coils do not overlap, and are 62.5–75.0 μm, 125.0–170.0 μm and 45.0–55.0 μm, respectively. The only way *A. circinalis* can be distinguished from *A. planctonica* is that the former has coiled trichomes and the latter has straight ones. It is also difficult to distinguish *A. smithii* from *A. crassa* or *A. ucrainica* by the forms and dimensions of the vegetative cells and akinetes.

Though Cluster **A** has less molecular diversity, it includes many taxa which are common in Japanese lakes and ponds. From consideration of the morphological characters, *Anabaena viguieri* Denis et Fremy and *A. citrispora* M. Watanabe will be in Cluster **A** (Cluster **A′**). Cluster **B** seems to be paraphyletic based on 16S rDNA analysis. However, the result of *rbcLX* analysis of this cluster shows every strain in this cluster includes the same long insertion between *rbcX* and *rbcS* region. This fact suggests the monophyletic group of Cluster **B**. This long insertion is not found in other clusters. In addition, the strains in cluster **D** have a different short insertion (ca. 30 bp) in the same region.

Cluster **C** is a monophyletic cluster based on both 16S rDNA and *rbcLX* analyses. The posterior probabilities (0.98 for 16S rDNA, 0.99 for

rbcLX) are very high for this cluster in both regions. Although Cluster **C′** contains only one species, *Anabaena akankoensis,* Watanabe (2007) considers *A. curva* Hill and *A. solitaria* Klebahn to be in the same morphological group as *A. akankoensis*, and thus both taxa may be members of this cluster.

Cluster **D** remarkably differs from the other three clusters in the morphology. The akinete of *Anabaena* species in Cluster **D** is always adjacent to the heterocyte. In addition, coiled trichomes of the *Anabaena* species in Cluster **D** have a smaller diameter and very compacted coils. During the culture condition, some strains with coiled trichomes of Cluster **D** varied their form from coiled to straight, but not from straight to coiled. Also, the apical ends of the trichomes of several strains of Cluster **D** were narrower at the end than at the beginning of the culture. These characteristics cannot be found in other clusters. Zapomelova *et al*. (2009) proposed the new genus *Sphaerospermum* with the type species *Anabaena reniformis* Lemmerm. They also transferred *A. kisseleviana* Elenkin and *Aphanizomenon aphanizomenoides* (Forti) Horecka et Komárek (=*Anabaena aphanizomenoides* Forti in this study) to this genus. This genus, *Sphaerospermum*, coincides with Cluster **D** in the present study. From the results we obtained both in the morphological and molecular analyses, *A. oumiana* M. Watanabe is also included in *Sphaerospermum*, as Zapomelova *et al*. (2009) suggested.

Here, we propose the new combination below for *Anabaena oumiana.*

Sphaerospermum oumianum (M. Watanabe) Tuji et Niiyama comb. nov.

Basionym: *Anabaena oumiana* M. Watanabe, Bulletin of the National Science Museum, Series B (Botany) **22**: 3, *f. 5–7, 14, 15*. 1996.

Iconotype: figures 5–7 in Watanabe (1996).

Isotype: TNS-AL-53658 in TNS (Department of Botany, National Museum of Nature and Science) collected from Akanoi Bay on August 22, 1994.

Type locality: Lake Biwa, Shiga pref., Japan.

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