国立科博専報，(48), pp. 139–148, 2012 年 3 月 28 日
Mem. Natl. Mus. Nat. Sci., Tokyo, (48), pp. 139–148, March 28, 2012

Distribution and Phylogeny of *Spicaticribra kingstonii*—*rudis* Species Complex

Akihiro Tuji[1,*], Pongpan Leelahakriengkrai[2,] and Yuwadee Peerapornpisal[2]**

[1] Department of Botany, National Museum of Nature and Science, 4–1–1 Amakubo, Tsukuba, Ibaraki 305–0005, Japan
[2] Department of Biology, Chiang Mai University, Thailand, Chiang Mai 50200, Thailand
* E-mail: tuji@kahaku.go.jp

Abstract. *Spicaticribra kingstonii* was found in Ryumon reservoir (Kumamoto prefecture), Fukuji reservoir and Haneji reservoir (Okinawa prefecture) during the phytoplankton survey of 107 Japanese artificial reservoirs at 2009 and 2010. A similar species was found in Mae Jork Lung reservoir, Chiang Mai area, Thailand. It can be identified as *Thalassiosira rudis* by the pattern of central areolae and the position of rimoportulae. A new combination, *Spicaticribra rudis* comb. nov. was proposed for *T. rudis*. A strain of *S. kingstonii* was isolated from Haneji reservoir, and performed molecular analysis for SSU-ITS-LSU region and constructed phylogenetic tree. The phylogenetic tree shows that the root of *S. kingstonii* exists in the *C. meneghiniana* species complex, but the branch length is extremely long and the morphological similarity was so small. *S. kingstonii* is distributed in tropical to subtropical region, and its growth rate should be faster than those of species from cold regions. This could explain the extremely long branch length of *S. kingstonii.*

Key words: phylogeny, *Cyclotella meneghiniana, Spicaticribra kingstonii, Spicaticribra rudis,* Thailand, *Thalassiosira rudis*.

Introduction

Spicaticribra kingstonii Johansen et al. was described from Fontana Lake, a reservoir in the Tennessee River drainage, North Carolina, U.S.A. (Johansen *et al.* 2008). Though this species closely resembles the genus *Thalassiosira*, *Spicaticribra* does not have central strutted processes as does *Thalassiosira*. Johansen *et al.* (2008) also discussed the superficial similarities and differences between *T. pseudonana* and *T. guillardii*. Ludwig *et al.* (2008) described a similar new taxon, *Thalassiosira rudis* Tremarin et al., from Brazil, South America. This species is similar to *S. kingstonii* except for its relatively regular central areolae and the position of the rimportulae. Ludwig *et al*. (2008) discussed the relationship of this species with the *Thalassiosira taxa T. gracilis*, *T. visurgis* and *T. rudolfii*. However, the relationship between these taxa and other genera has not been clarified.

Tanaka and Nagumo (2009) and Tanaka (2010) reported *S. kingstonii* from Lake Ikeda, Kagoshima Prefecture, and Lake Fukuji, Okinawa prefecture in southern Japan. Since the distribution of this species is limited to three separated areas, we could not discuss the distribution of this species.

Materials and Methods

1. Japanese artificial reservoirs

Reservoir water samples from 107 Japanese artificial reservoirs, shown in Figure 1, were collected in August 2009. Additionally, samples from 50 selected sites within the 107 total sites were also collected from March to April 2010. One to two liters of reservoir water was

** Present address: Biology section, Department of Science, Faculty of Science and Technology, Chiang Mai Rajabhat University, Thailand.

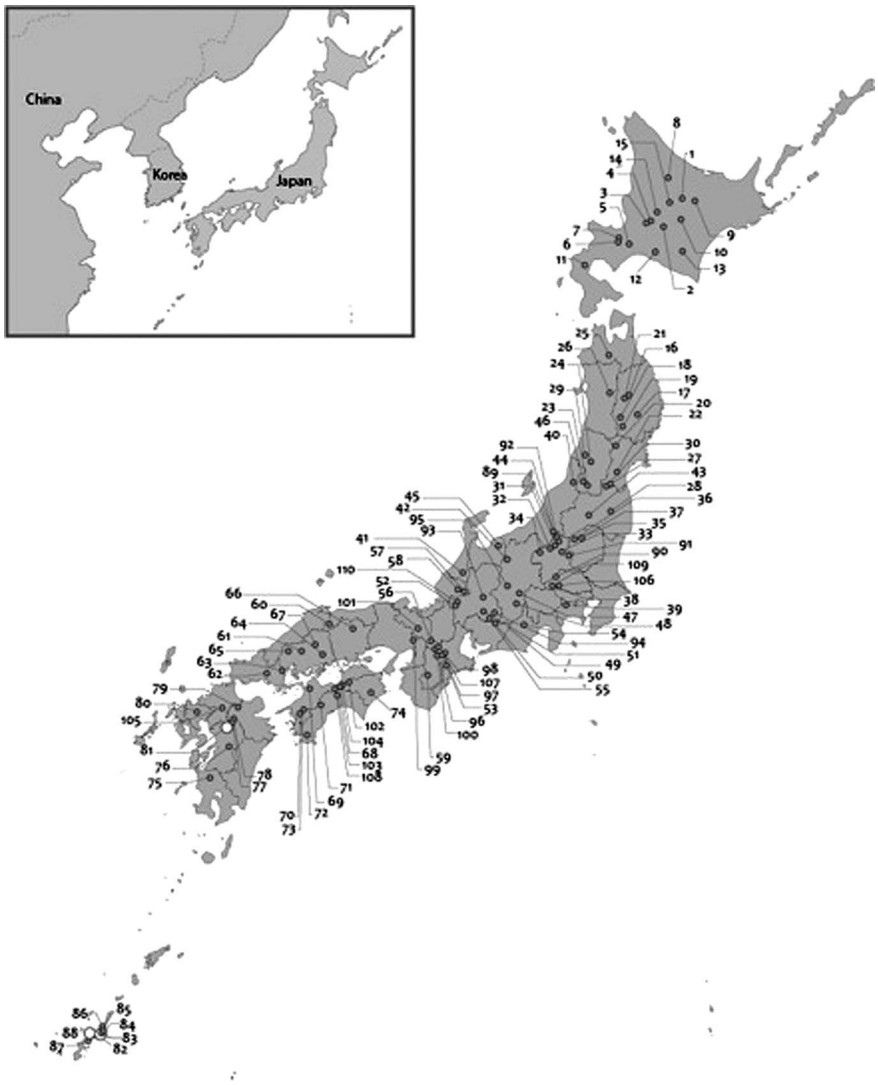


Fig. 1. Site map of Japanese reservoirs examined in this study. 1: Taisetsu, 2: Kanayama, 3: Katsurazawa, 4: Ashibetsu, 5: Izarigawa, 6: Hoheikyo, 7: Jozankei, 8: Iwaonai, 9: Kanoko, 10: Tokachi, 11: Pirika, 12: Nibutani, 13: Satsunaigawa, 14: Takisato, 15: Chubetsu, 16: Shijushida, 17: Tase, 18: Yuda, 19: Ishibuchi, 20: Naruko, 21: Gosho, 22: Kamahusa, 23: Shirakawa, 24: Sagae, 25: Aseishigawa, 26: Tamagawa, 27: Shichikashuku, 28: Miharu, 29: Gassan, 30: Surikamigawa, 31: Fujiwara, 32: Aimata, 33: Sonohara, 34: Shinaki, 35: Ikari, 36: Kawamata, 37: Kawaji, 38: Futase, 39: Miyagase, 40: Oishi, 41: Tedorigawa, 42: Omachi, 43: Okawa, 44: Sagurigawa, 45: Unazuki, 46: Yokokawa, 47: Miwa, 48: Koshibu, 49: Shintoyone, 50: Yahagi, 51: Maruyama, 52: Yokoyama, 53: Hachisu, 54: Nagashima, 55: Origawa, 56: Amagase, 57: Kuzuryu, 58: Managawa, 59: Sarutani, 60: Sugesawa, 61: Haji, 62: Shimajigawa, 63: Yasaka, 64: Hattabara, 65: Nukui, 66: Tomata, 67: Haizuka, 68: Yanase, 69: Ishitegawa, 70: Nomura, 71: Odo, 72: Nakasujigawa, 73: Kanogawa, 74: Nagayasuguchi, 75: Tsuruda, 76: Midorikawa, 77: Shimoke, 78: Matsubara, 79: Yabakei, 80: Kyuragi, 81: Ryumon, 82: Fukuji, 83: Arakawa, 84: Aha, 85: Fungawa, 86: Benoki, 87: Kanna, 88: Haneji, 89: Yagisawa, 90: Shimokubo, 91: Kusaki, 92: Naramata, 93: Iwaya, 94: Agigawa, 95: Misogawa, 96: Muro, 97: Shorenji, 98: Takayama, 99: Hitokura, 100: Nunome, 101: Hiyoshi, 102: Ikeda, 103: Sameura, 104: Shingu, 105: Terauchi, 106: Urayama, 107: Hinachi, 108: Tomisato, 109: Takizawa, 110: Tokuyama.

filtered using PTFE membrane filters having 1.0 μm openings (JAWP04700, Millipore) and was dried with an incubator (ITD-20E, ALP) at 60°C.

2. Thailand artificial reservoirs

One liter of water sample from each of 4 sites in the Chiang Mai area reservoirs shown in Table 1 was collected and fixed with weak formalin (about a 0.5% final concentration of formaldehyde). Each settled for one day, and concentrated samples were kept with formalin (about a 2% final concentration of formaldehyde). Sediment samples fixed with formalin were also used. Fixed samples were cleaned using concentrated nitric acid. Cleaned materials were filtered and dried up using the same method described above.

3. Scanning electron microscopy (SEM) observation

Filters were cut into small pieces (about 5-mm square) and attached to the SEM stub using carbon adhesive tape. These stubs were sputtered with platinum, and examined using a SEM (JSM-6390 with LaB_6 gun, JEOL).

4. Isolation and culture

Water samples were collected from the Haneji artificial reservoir on 8th July 2011. The electric conductivity at the time of collection was 179μS/cm, the pH was 8.3, and the water temperature was 28.7°C. Each 0.1-ml sample was added to 1 ml of d-medium as described in Tuji (2000) in three multi-well plates having 48 wells. The cultured strain was illuminated by hand-made lights using red LEDs with a photon flux density of ca. 50 μmol/m^2/sec, a photoperiod of L/D＝24/0 hours, and a temperature of ca. 28°C. After two weeks preculture, *Spicaticribra kingstonii* was isolated by the pipette-washing method and cultured using d medium in one multi-well plate having 12 wells with the same light conditions and several

Table 1. List of Thailand reservoirs in Chiang Mai area.

Specimen No. TNS-AL-	Sampling date	Site	Area
57402	2010/3/5	MaeKuang Dam	Chiang Mai, Thailand
57408	2010/3/5	Huay Tung Tao reservoir	Chiang Mai, Thailand
57409	2010/3/5	Mae Jork Lung reservoir	Chiang Mai, Thailand
57411	2010/3/5	Mae Jork Lung reservoir	Chiang Mai, Thailand
57414	2010/3/5	Pond under Mae Jork Lung reservoir	Chiang Mai, Thailand
57415	2010/3/5	Aung Krew reservoir pond	Chiang Mai, Thailand

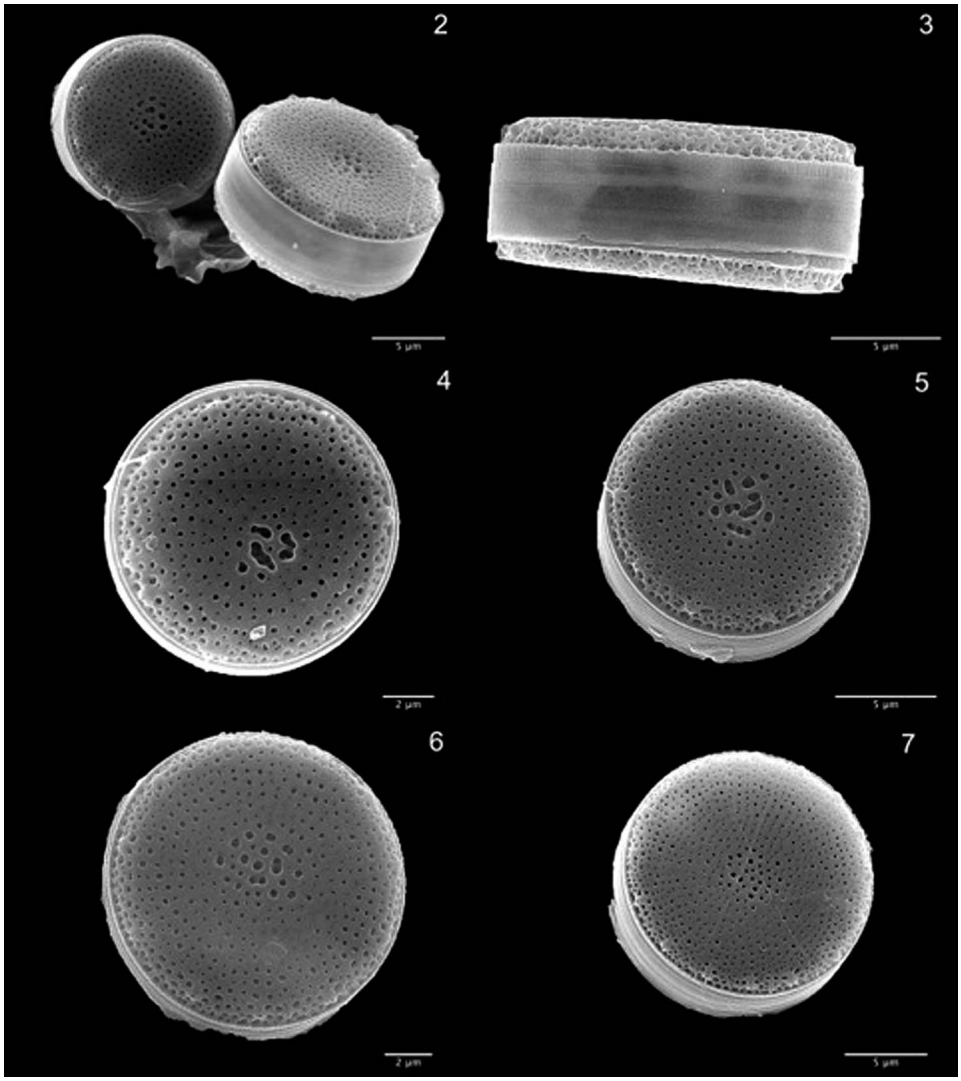
Table 2. List of primers used for PCR and sequences in this study.

Gene	Primer	Sequence 5′-3′	Direction	Reference
SSU region	SR1	TACCTGGTTGATCCTGCCAG	Forward	Nakayama, T. *et al.* (1996)
	443r	RCGSRCCTGCTGCTGCCTTCCTTG	Reverse	Beszteri *et al.* (2001)
	550f	TAGGTCTGGCAATTGGAATGAG	Forward	
	SR5	ACTACGAGCTTTTTAACTGC	Reverse	Nakayama,T. *et al.* (1996)
	997r	AAAACATCCTTGGWARATGCT	Reverse	Beszteri *et al.* (2001)
	SR9	AACTAAGAACGGCATGCAC	Reverse	Nakayama,T. *et al.* (1996)
	SR10	AGGTCTGTGATGCCCTTAGA	Forward	Nakayama,T. *et al.* (1996)
	SR12	CCTTCCGCAGGTTCACCTAC	Reverse	Nakayama,T. *et al.* (1996)
ITS region	ITS5	GGAAGTAAAAGTCGTAACAAGG	Forward	White *et al.* (1990)
LSU region	D1R	ACCCGCTGAATTTAAGCATA	Forward	Scholin *et al.* (1994)
	D2C	CCTTGGTCCGTGTTTCAAGA	Reverse	Scholin *et al.* (1994)
	Euk34r	GCATCGCCAGTTCTGCTTACC	Reverse	Liu *et al.* (2009)
	LSU-R2	ATTCGGCAGGTGAGT	Reverse	Takano et Horiguchi (2006)

different temperature conditions (10–30°C).

5. Molecular analysis

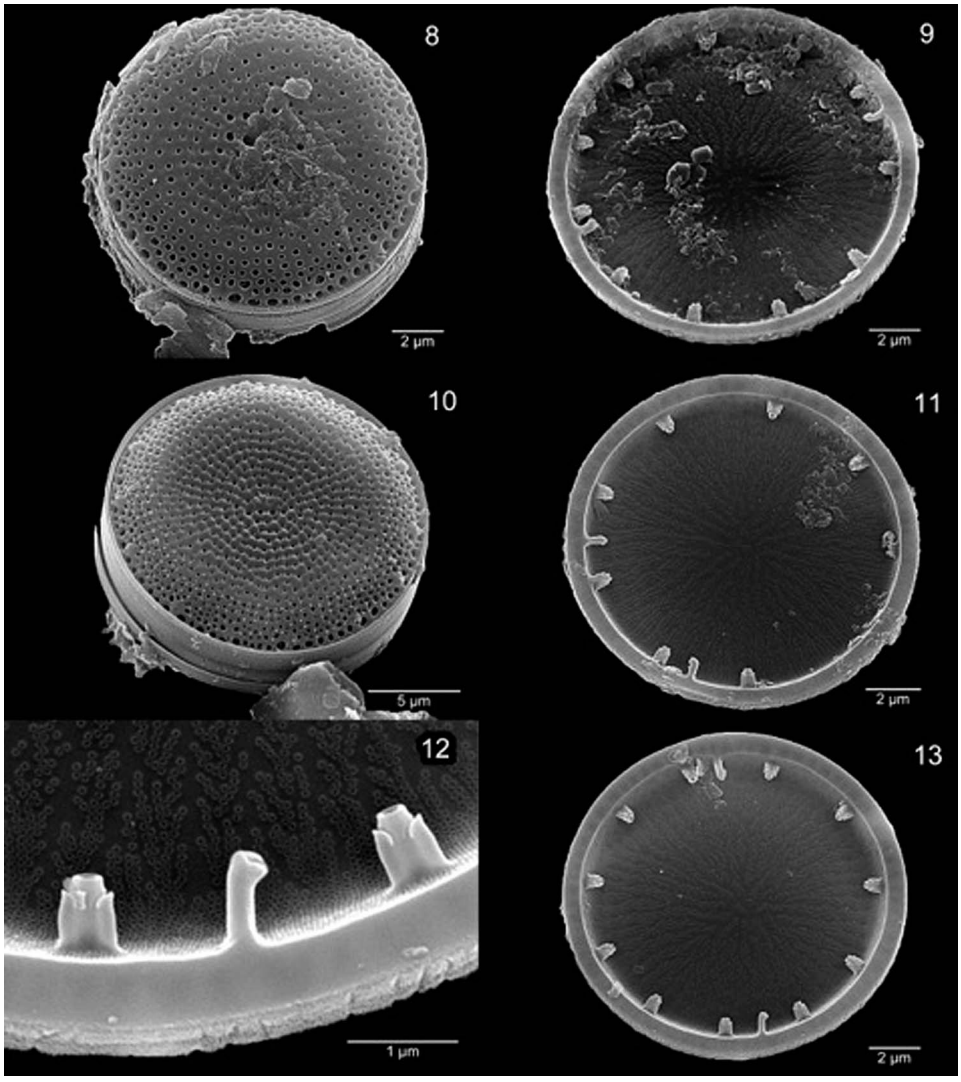
The cells were concentrated using a centrifuge, and DNA was extracted using the Chelex method (Walsh *et al.* 1991). PCR of the SSU-ITS-LSU regions was performed using a thermal cycler (iCycler, BioRad) with Ex Taq polymerase (Takara) using a primer set of SR-1 forward and LSU-R2 reverse (Table 2). The PCR product was purified with ExoSAP-IT (USB Corporation, Cleveland) in accordance with the manufacturer's instructions. The cycle sequencing samples were purified by ethanol precipitation. Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) using the primers in Table 2. The obtained sequences were assembled using Chromas PRO software (Technelysium Pty Ltd).



Figs. 2–7. *Spicaticribra kingstonii* from Japanese reservoirs. Figs. 2, 3, 5, 7: Fukuji R.; Fig. 4: Ryumon R.; Fig. 6: Haneji R.

6. Phylogenetic reconstruction

The Basic Local Alignment Search Tool (BLAST) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to find sequences similar to the sequence of *S. kingstonii*. Phylogenetic and molecular evolutionary analyses of the obtained sequences were conducted using the MEGA 5 computer program (Tamura *et al*. 2011). Alignments were checked manually. A Maximum Likelihood (ML) tree was constructed using software with the best fits model (Tamura three-parameter using a discrete Gamma distribution (+G) with five rate categories and by assuming that a certain fraction of sites is evolutionarily invariable (+I)) based on the lowest BIC score (Bayesian Information Criterion) and the substitution nucleotide matrix parameters calculated by the software. One thousand bootstraps were generated.

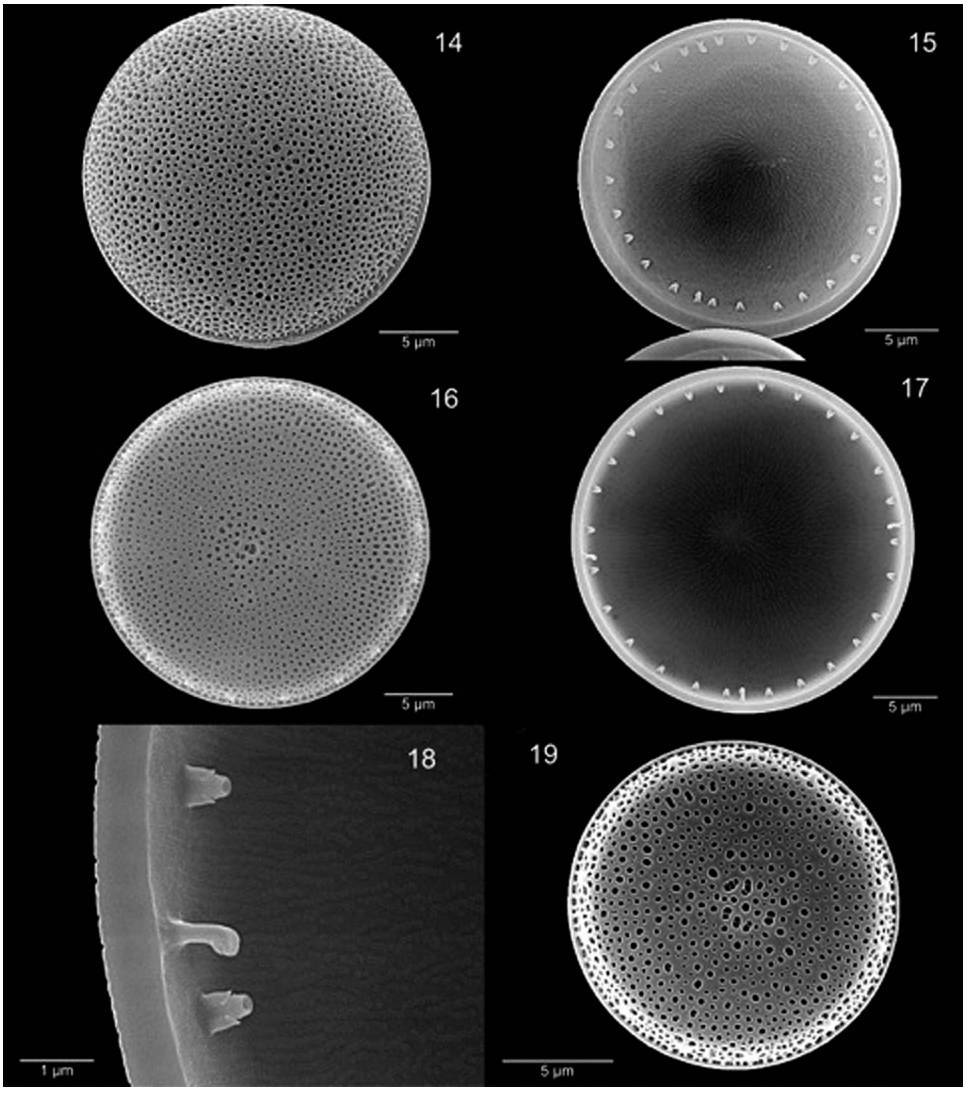


Figs. 8–13. *Spicaticribra rudis* from Mae Jork Lung reservoir, Chiang Mai area, Thailand. Figs. 8, 9, 11–13: TNS-AL-57409; Fig. 10: TNS-AL-57411.

Results

1. SEM observation

Spicaticribra kingstonii is found in three Japanese reservoirs. It was found in Ryumon reservoir in Kumamoto prefecture and Fukuji reservoir in Okinawa prefecture in August 2009 and in Haneji reservoir in Okinawa prefecture in both August 2009 and March 2010. These three reservoirs are located in southern Japan. This species has not been observed in the northern or middle areas of Japan. SEM photographs from Japanese samples are shown in Figs. 2–7. *S. kingstonii* found in this study agree with the descriptions in Tanaka & Nagumo (2009) and Tanaka (2010). SEM photographs from the isolated strain of *S. kingstonii* are shown in Figs. 14–19. The sexual reproduction and initial valves are also observed (Figs. 14–15). Japanese individuals always have irregular central areolae, especially large individuals, though the North American individuals presented in Johansen *et al*. (2008) sometimes had regular central areolae



Figs. 14–19. *Spicaticribra kingstonii*. Strain examined for molecular analysis. FIgs 14, 15: Initial valve.

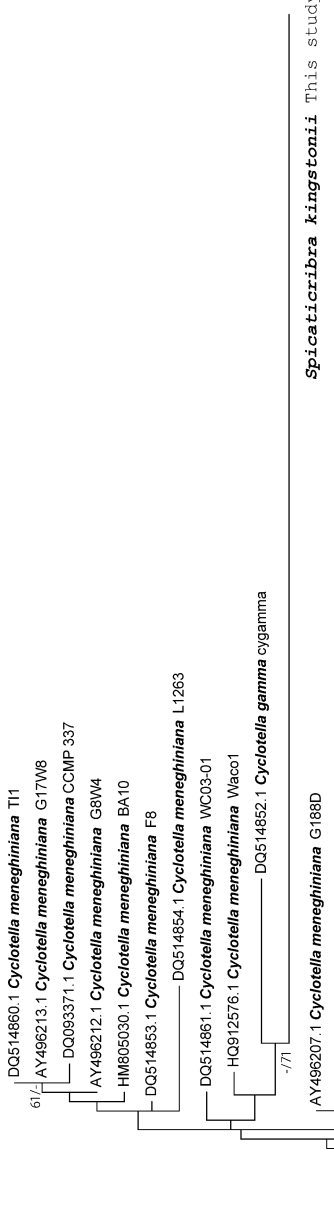


Fig. 20. Phylogenetic position of *Spicaticribra kingstonii* and related taxa determined by Maximum Likelihood (ML) method from 18S rDNA. Accession numbers are followed taxonomic names. Numbers at branches indicate NJ (Neighbor Joining)/ML bootstrap support values (only values higher than 60 are shown).

(Figs. 5, 8, and 10 in Johansen *et al*. 2008).

A similar species was found in Mae Jork Lung reservoir at 700th Anniversary Chiang Mai Sport Complex, Chiang Mai area, Thailand. The electric conductivity at the time of collection in this reservoir was 53 μS/cm, the pH was 8.2, and the water temperature was 28.7°C. Morphological variation of this species is close to North American *S. kingstonii* individuals. Irregular and regular central areolae were also found in this species (Figs. 8–13). The rimoportulae of Thailand individuals are positioned at the frustules' edge and are elongated and strongly curved. This position and this curved character differ from the North American and Japanese individuals and can be used to identify the species as *Thalassiosira rudis* Tremarin *et al.*

2. Molecular analysis and phylogeny of *Spicaticribra kingstonii*

The results of the BLAST search using the 18S rDNA for *S. kingstonii* show high similarity with the *Cyclotella meneghiniana* species complex. Figure 20 shows the ML tree of 18S rDNA for *S. kingstonii* and related taxa, mainly the *C. meneghiniana* species complex. *Thalassiosira pseudonana* is used as an outgroup. This phylogenetic tree shows that the root of *S. kingstonii* exists in the *C. meneghiniana* species complex, but the branch length is extremely long, and 92–104 bp differences exist between *S. kingstonii* and the *C. meneghiniana* species complex. Though we have also constructed phylogenetic trees using several different methods, the results were the same (data not shown). The morphological similarity between *S. kingstonii* and *C. meneghiniana* is very low. *S. kingstonii* does not have chambers, costae, spines or central fultoportula, but *C. meneghiniana* does.

Discussion

Since the differences between *S. kingstonii* and *T. rudis* were only in the pattern of the central areolae and the position of the rimoportulae, *T. rudis* should belong to the genus *Spicaticribra*. The position of rimoportula is well conserved, and so this difference is thought to be a species-level difference.

Spicaticribra rudis (Tremarin, Ludwig, Becker et Torgan) Tuji, Leelahakriengkrai et Peerapornpisal comb. nov.

Basionym: *Thalassiosira rudis* Tremarin, Ludwig, Becker et Torgan in Ludwig in Tremarin, Becker & Torgan, Diat. Res. 23: 391. *f. 1–57*. 2008.

Since the genus *Spicaticribra* is found in southern Japan, Thailand, North America and South America, it seems to be harvested in sub-tropical to tropical regions. The planktonic florae from subtropical and tropical regions are limited. Hence, this genus might be distributed widely.

Because of the wide morphological variation, the *S. kingstonii—rudis* species complex might be a species flock like the *Cyclotella ocellata* species complex (Edlund *et al.* 2003). Because of the isolated freshwater bodies by oceans in subtropical to tropical areas, the endemism in these areas may occur by a different process than in cold regions.

The phylogenetic analysis suggests that the *C. meneghiniana* species complex is an origin of *S. kingstonii*. The branch length of *S. kingstonii* from *C. meneghiniana* is extremely long, which is likely caused by the very fast molecular evolution of *S. kingstonii*. Normally the rates of molecular evolution are the same in similar organisms, and this is the basis of the molecular clock. However, if the growth rate and/or the rate of sexual activity of *S. kingstonii* are very fast, the molecular evolution rate would be fast. *S. kingstonii* is distributed in high temperature water bodies, and its growth rate should be faster than those of species from cold regions. This could explain the extremely long branch length of *S. kingstonii.*

Acknowledgements

The authors would like to thank Dr. Hidetaka Ichiyanagi, Water Resources Environment Technology Center for the sampling at Japanese artificial reservoirs. Part of this work was supported by the research program, " Biodiversity inventory in the Western Pacific region" of the National Museum of Nature and Science, Tokyo, and Watershed Ecology Research Group.

References

Beszteri, B., E. Acs, J. Makk, G. Kovács, K. Márialigeti & K. T. Kiss, 2001. Phylogeny of six naviculoid diatoms based on 18S rDNA sequences. *International Journal of Systematic and Evolutionary Microbiology*, 51: 1581–1586.

Edlund, Mark B., R. M. Willlams & N. Soninkhishig, 2003. The planktonic diatom diversity of ancient Lake Hovsgol, Mongolia. *Phycologia,* 42: 232–260.

Johansen, J. R., J. P. Kociolek & R. L. Lowe, 2008. *Spicaticribra kingstonii*, gen. nov. et sp. nov. (Thalassiosiraceae, Bacillariophyta) from Great Smoky Mountains National Park, USA. *Diatom Research,* 23: 367–375.

Liu, H., I. Proberta, J. Uitzc, H. Claustred, S. Aris-Brosoue, M. Fradab, F. Not & C. Vargasa, 2009. Extreme diversity in noncalcifying haptophytes explains a major pigment paradox in open oceans. *Proceedings of the National Academy of Sciences of the United States of America,* 106: 12803–12808.

Nakayama, T., S. Watanabe, K. Mitsui, H. Uchida & I. Inouye, 1996. The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. *Phycological Research,* 44: 47–55.

Scholin, C. A., M. Herzog, M. Sogin & D. M. Anderson, 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *Journal of Phycology,* 30: 999–1011.

Takano, Y. & T. Horiguchi, 2006. Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell. *Journal of Phycology,* 42: 251–256.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei & S. Kumar, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximuii Johansen, Kociolek et Lowe found from Lake Fukuji, Okinawa Prefeture. *Diatom,* 26: 44–45.

Tanaka, H. & T. Nagumo, 2009. First report of Spicaticribra kingstonii Johansen, Kociolek et Lowe with accompanying centric diatoms found from Lake Ikeda, Japan (Bacillariophyta). *The bulletin of Japanese Society of Phycology,* 57: 86–92.

Tuji, A. 2000. The effect of irradiance on the growth of different forms of freshwater diatoms: Implications for succession of attached diatom communities. *Journal of Phycology,* 36: 659–661.

Walsh, P. S., D. A. Metzger & R. Higuchi, 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques,* 10: 506–513.

White, T. J., T. Bruns, S. Lee & J. W. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315–322 In: *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York.

Spicaticribra kingstonii—*rudis* 種群の分布と系統

辻　彰洋・Pongpan Leelahakriengkrai・Yuwadee Peerapornpisal

日本全国の 107 ダム湖を 2009 年から 2010 年に調査した結果，竜門ダム湖（熊本），福地ダム湖・羽地ダム湖（沖縄）から *Spicaticribra kingstonii* が見いだされた．また，タイ国・チェンマイ地域の Mae Jork Lung ダム湖から極めて似た種が見いだされた．中心部の胞紋および唇状突起の位置からタイ産個体は *Thalassiosira rudis* と同定できた．*T. rudis* について，*Spicaticribra rudis* の新組み合わせを提案した．羽地ダム湖から分離した *S. kingstonii* 株について SSU-ITS-LSU の遺伝子解析と系統樹作成を行ったところ，同種は *Cyclotella meneghiniana* 種群のクレードに入るが，その枝は非常に長かった．また，両者の形態的な類似性は低かった．熱帯から亜熱帯に生息する *S. kingstonii* は，分裂速度や生殖頻度が高いと考えられ，進化速度が速くなり，このような現象が生じたと考えた．