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PHYLOGEOGRAPHY OF THE FRESHWATER RAPHIDOPHYTE *GONYOSTOMUM SEMEN* CONFIRMS A RECENT EXPANSION IN NORTHERN EUROPE BY A SINGLE HAPLOTYPE[1]

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Gonyostmum semen **is a freshwater raphidophyte that has increased in occurrence and abundance in several countries in northern Europe since the 1980s. More recently, the species has expanded rapidly also in north-eastern Europe, and it is frequently referred to as invasive. To better understand the species history, we have explored the phylogeography of *G. semen* using strains from northern Europe, United States, and Japan. Three regions of the ribosomal RNA gene (small subunit [SSU], internal transcribed spacer [ITS] and large subunit [LSU]) and one mitochondrial DNA marker (cox1) were analyzed. The SSU and partial LSU sequences were identical in all strains, confirming that they belong to the same species. The ITS region differentiated the American from the other strains, but showed high intra-strain variability. In contrast, the mitochondrial marker cox1 showed distinct differences between the European, American, and Japanese strains. Interestingly, only one cox1 haplotype was detected in European strains. The overall low diversity and weak geographic structure within northern European strains supported the hypothesis of a recent invasion of new lakes by *G. semen*. Our data also show that the invasive northern European lineage is genetically distinct from the lineages from the other continents. Finally, we concluded that the mitochondrial cox1 was the most useful marker in determining large-scale biogeographic patterns in this species.**

Key index words: **cox1; *Gonyostomum semen*; ITS; LSU; phylogeography; raphidophyte SSU**

Abbreviations**: cox1, mitochondrial gene of the cytochrome c oxidase subunit 1; ITS, internal transcribed spacer; LSU, large subunit of the ribosomal DNA region; SSU, small subunit of the ribosomal DNA region**

Raphidophytes are planktonic autotrophic protists, which form blooms in both marine and limnic environments. Many raphidophyte species are considered harmful, causing for instance mass mortality in fish due to toxin production (Edvardsen and Imai 2006). *Gonyostomum semen* (Ehrenberg) Diesing is the most common raphidophyte species in freshwater environments, and is considered to have a cosmopolitan distribution (Bourrelly 1985) based on microscopic identification. Nevertheless, this species is usually rare in phytoplankton communities worldwide. Interest in *G. semen* has risen in the scientific community recently, mainly because this species has increased in abundance and occurrence in northern Europe since the 1980s and more recently in Central Europe (Cronberg et al. 1988, Lepistö et al. 1994, Poniewozik et al. 2011, Rengefors et al. 2012, Lebret et al. 2013, Karosienė et al. 2014, Hagman et al. 2015). In 2002, *G. semen* was observed in as much as 27% of all monitored Swedish lakes (Rengefors et al. 2012). In addition, this raphidophyte is a nuisance microalga, and forms dense blooms during extended periods of time (Lebret et al. 2012b). Although *G. semen* is not known to be toxic, it expels slimy threads from trichocysts, inducing skin irritation in bathers and thereby affecting recreational activities (Sörensen 1954, Cronberg et al. 1988).

Although Cronberg et al. (1988) first suggested that *G. semen* may have increased since the late 1950s, the expansion of *G. semen* was first described in a Finnish study during a period of 10 years (1978–1989; Lepistö et al. 1994). Lepistö et al. (1994) found that the number of studied lakes with *G. semen* increased from 11 to 42 of 110 sampled, and that it was found in a wider range of lake types than

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observed in the past. Previously, reports of *G. semen* were restricted to humic lakes and ponds (reviewed in Cronberg et al. 1988 and Willén 2003). However, dense populations have more recently been observed in nonhumic oligotrophic lakes (Laugaste 1992), eutrophic ponds (Pithart et al. 1997) and large deep reservoirs (Lecohu et al. 1989, Negro et al. 2000).

Several factors have been suggested to favor the development of *G. semen* in northern European lakes. Cronberg et al. (1988) suggested that mass blooms were connected to low pH. However, recent studies showed that the species is also able to form blooms in more alkaline lakes (Rengefors et al. 2012), and that *G. semen* strains can grow in a wide range of pH (Sassenhagen et al. 2015). By analyzing data from the Swedish national lake inventory program (Rengefors et al. 2012, Trigal et al. 2013) it was found that high water color, high dissolved organic carbon concentration, and low pH were strong predictors for *G. semen* occurrence. Moreover, when performing a trend analysis, Rengefors et al. (2012) found that temperature was the most important variable driving biomass temporal variations, and suggested this as a driver of *G. semen* expansion.

Thus, the increase in occurrence and abundance of *G. semen* is likely due to a changing environment and to more favorable conditions with an interaction of all these factors. Due to its increase in occurrence and expansion of its habitat range, *G. semen* is now viewed to be invasive in Norway, Sweden, and Finland (Lebret et al. 2012a, 2013, Rengefors et al. 2012, Hagman et al. 2015). According to Valery et al. (2008), an invasive species is a species that has gained competitive advantage after the disappearance of a natural obstacle, which can either result in dispersal to a new environment or that the species has increased its distribution range by adapting to new conditions, allowing it to spread and form dominant populations.

To date, little is known concerning the evolutionary and phylogeographic history of the species. Lebret et al. (2013) showed that the Fenno-Scandinavian populations belong to a single metapopulation, suggesting that it has likely expanded recently. However, it is unknown how this population is related to other lineages of *G. semen* and when the expansion occurred. Phylogenetic analyses using molecular tools could provide new insights into the species' history and expansion. In macroorganisms, phylogeographic studies have enhanced the understanding of the evolutionary history, expansion, or invasion patterns of species, as well as gene flow between regions (Sakai et al. 2001). For free-living microbes, phylogeographic studies are becoming more common but are still available for only a handful of species with a focus on toxin producers. For instance, Lilly et al. (2005) used a phylogeographic approach coupled to morphological comparison to determine the source of the newly introduced population of *Alexandrium minutum* (Dinophyceae) to Australia.

Despite the increasing number of studies concerning *G. semen*, almost no genetic information is currently available. Only two sequences of the small subunit (SSU) of the ribosomal DNA (rDNA) were available on GenBank at the start of this study (accession numbers: DQ408616 and AB512123). The aim of this study was thus to investigate the phylogenetic relationships among *G. semen* strains. We analyzed strains from lakes in northern Europe, Japan, and the United States. Four DNA markers; the mitochondrial gene cytochrome c oxidase subunit 1 (cox1), the entire small subunit rDNA (SSU), the internal transcribed spacer (ITS) including ITS1, 5.8S and the ITS2, and the partial large subunit of the rDNA (LSU) were sequenced. We aimed to compare the variability in the four markers and their potential to identify phylogeographical patterns in *G. semen*. In accordance with the hypothesis that *G. semen* has expanded in the last four to five decades in Norway, Sweden, and Finland, we expected little difference among sequences of those strains, and larger difference between the northern European (Norway, Sweden, and Finland) and the American and Japanese strains.

METHODS

Strains and cultivation of G. semen. In total, 62 strains were sequenced, including 60 strains of *G. semen* (30 from Sweden, 10 from Norway, eight from Finland, 10 from United States and two from Japan), one of *Gonyostomum latum* (Iwanoff) (Japan) and one unknown raphidophyte strain from the United States, which was a priori identified as *G. semen* (Table 1). The *G. semen* strains from Finland, Norway, and Sweden ($n = 48$), were isolated from 15 locations (14 lakes including lake Helgasjön sampled at two distinct stations; Fig. 1), with one to four strains per location (Table 1). The isolations and cultivation of the strains were performed according to Lebret et al. (2013). The Norwegian strain NIVA-7/05 isolated from Vansjø was obtained from the Norwegian Institute for Water Research (NIVA) culture collection. The Japanese strains (*G. semen* and *G. latum*) were obtained as extracted DNA from the National Institute for Environmental Studies (NIES) culture collection in Japan. The American strains were obtained from the Center of Marine Science in Wilmington, North Carolina (United States). All strains were grown in modified Wright's cryptophyte medium (Guillard and Lorenzen 1972) with an addition of selenium to a final concentration of 1.2 $\mu g \cdot L^{-1}$. The cultures were harvested by centrifugation at concentrations of ~2,000 cells $\cdot$ mL^{-1} according to Lebret et al. (2012b) and the pellets were stored at −80°C until DNA extraction.

DNA extraction. DNA was extracted using a CTAB-based protocol as described by Lebret et al. (2012b). The DNA concentration of the samples was estimated by measuring the absorbance of a subsample diluted ten times at 260 nm using a spectrophotometer (Ultraspec 3000; Pharmacia Biotech, Cambridge, England). For each sample, the quality of the DNA was determined using the 260/280 nm absorbance ratio. Only samples of high DNA quality (i.e., with a 260/280 ratio above 1.8) were used for downstream analyses. The DNA samples were stored at −80°C until DNA sequencing preparation.

DNA sequencing. Four DNA regions were sequenced, the cytochrome-c oxydase subunit 1 (*cox1*, mitochondrial gene), and the small subunit (entire SSU), the internal transcribed spacer (ITS1-5.8-ITS2), and the large subunit (LSU, partial sequence) of the rRNA gene. For the LSU and ITS regions,

TABLE 1. Summary of data for the algal strains used in the phylogenetic analysis, and successfully sequenced DNA markers for each strain.

Species	Country	Lake/ location	Coordinates	Isolation date	Strains	DNA markers			
						SSU	LSU	ITS	*cox1*
Gonyostomum semen	Sweden	Bergträsket	65°54′N; 23°02′E	July 2010	BE06	X	X		
		Mjöträsket	65°55′N; 23°05′E	July 2010	MJ01	X	X	X	X
					MJ07	X	X	X	X
					MJ39	X	X		
		Sidensjön	63°53′N; 19°48′E	July 2010	SI03			X	X
					SI22	X	X		X
					SI27	X	X	X	X
		Ekholmssjön	59°52′N; 17°03′E	August 2010	EK04	X	X		
					EK11	X	X	X	X
					EK15				X
		Lillsjön	58°48′N; 17°26′E	August 2010	LL05			X	
					LL06	X	X	X	X
					LL11	X	X	X	X
					LL16	X	X		
		Stråken	57°07′N; 14°34′E	August 2010	ST10			X	
					ST16			X	
					ST17	X	X		
					ST18	X	X		
		Helgasjön Stat. 2	56°58′N; 14°42′E	August 2010	HEII23	X	X	X	X
					HEII25	X	X	X	
					HEII27	X	X		X
		Helgasjön Stat. 1	56°55′N; 14°49′E	August 2010	HEI02	X	X		X
					HEI08				X
					HEI12	X	X	X	X
		Liasjön	56°26′N; 13°59′E	July 2010	LI01	X	X		
					LI06	X	X	X	X
					LI10			X	X
		Bökesjön	55°34′N; 13°26′E	October 2008	BO32	X	X	X	
				July 2009	BO178	X	X	X	
				October 2009	BO302	X	X	X	X
	Norway	Lundebyvatn	59°32′N; 11°28′E	July 2010	LU01	X	X		X
					LU02				X
					LU07	X	X	X	X
		Gjølsjøen	59°26′N; 11°41′E	July 2010	GJ03	X	X		X
					GJ09	X	X		X
					GJ17	X	X	X	X
		Isesjøen	59°17′N; 11°14′E	July 2010	IS09	X	X		X
					IS12	X	X	X	X
					IS19	X	X		
		Vansjø	59°43′N; 10°67′E	2005	NIVA-7/05	X	X		X
	Finland	Pikku-Torava	60°25′N; 23°47′E	July 2010	PI10			X	
					PI12	X	X		X
					PI13				X
					PI14				X
		Kylänalanen	60°24′N; 23°45′E	July 2010	KY03	X	X		X
					KY05	X	X		X
					KY17	X	X	X	X
					KY20			X	
	Japan	Fukushima	–	October 2002	NIES-1009	X	X	X	X
		Kitaibaraki Ibaraki	–	July 2004	NIES-1380	X	X	X	X
	United States	Minnesota	46°55′N; 88°29′W	–	GS1103-4	X	X	X	X
					GS1103-5	X	X	X	X
					GS1103-6	X	X	X	X
					GS1103-9	X	X	X	X
					GS1103-12	X	X	X	X
					GS1103-13	X	X	X	X
					GS1103-14				X
					GS1103-15				X
					GS1103-16	X	X		X
		North Carolina	–	–	GS0405-B3	X	X		
Gonyostomum latum	Japan	Tsukuba Ibaraki	–	August 2005	NIES-1808			X	
Raphidophyte	United States	North Carolina			GS0906-1	X		X	

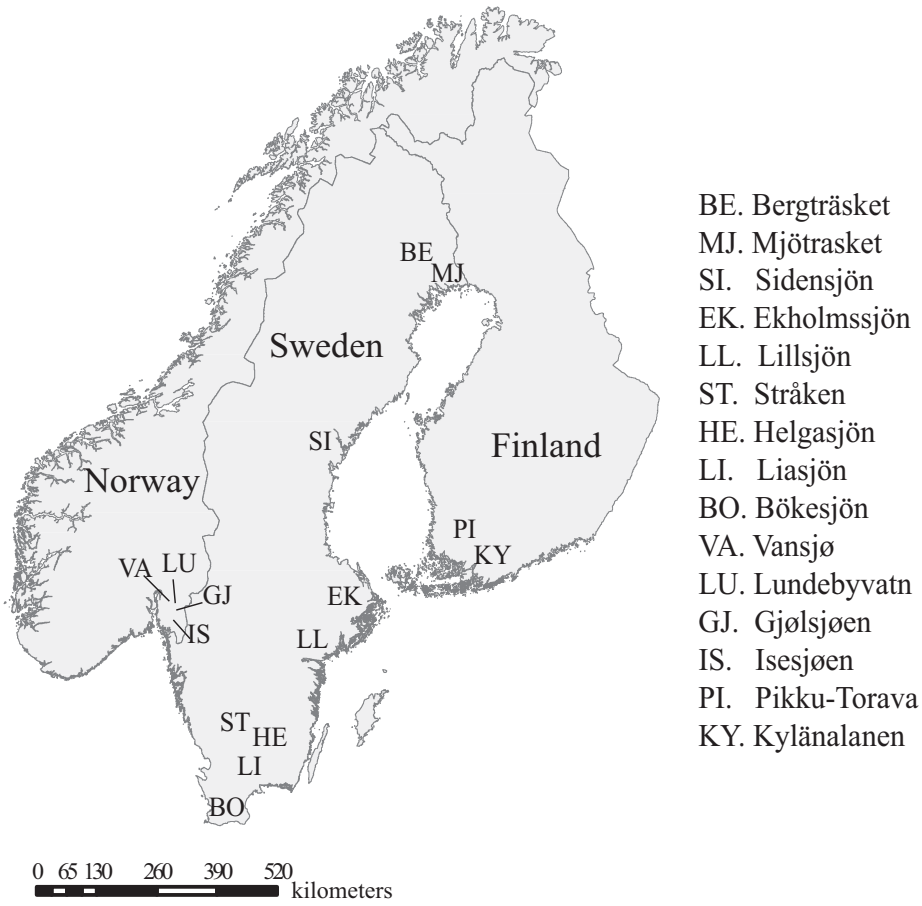

FIG. 1. Sampling locations in northern Europe where the *Gonyostomum semen* strains were isolated.

PCR were performed in a final volume of 25 μL, with 1.5 μL of $MgCl_2$ (25 mM), 2.5 μL of PCR 10× buffer, 2.5 μL of dNTP's (1.25 mM), 1 μL of each primer (forward and reverse; 10 μM), 2.5 units of AmpliTaq® DNA-polymerase (Life Technologies, Stockholm, Sweden) and 25 ng of genomic DNA. The ITS region was amplified with the primer pair, ITS1 (forward: 5′-TCCGTAGGTGAACCTGCGG-3′; White et al. 1990) and ITS4b (reverse: 5′-TCCTCCGCTTAATTATATGC-3′; Tesson et al. 2013). The PCR was performed according to Tesson et al. (2013) using a Verity thermocycler (Applied Biosystems, Foster City, California, USA). For the ITS marker, the PCR products were separated on a 1% agarose gel, and the band containing the expected-size fragment (~800 bp) was cut from the gel and purified using a EZNA gel extraction kit (Omega Bio-Tek, Stockholm, Sweden) according to the manufacturer's instructions.

The LSU region was amplified with the forward primer 1274 (5′-GACCCGTCTTGAAACACGGA-3′) previously used by Wylezich et al. (2010) for Raphidophyceae. The reverse primer LSUGSR1 (5′-AGCAACCGAGATTCAACGCCG-3′) was designed for this study to obtain good quality sequences of *G. semen*. The PCR temperature profile was: 1 min at 94°C; followed by 30 cycle of 30 s at 94°C, 30 s at 54°C, and 2 min at 72°C; and finished with 5 min at 72°C. PCR products were precipitated with 11 μL of NH_4Ac (8 M) and 37.5 μL of 95% ethanol. After 15 min of incubation at room temperature, the samples were centrifuged for 30 min at 2,000*g*. The liquid phase was then removed and the pellet was washed with 50 μL of 70% ice-cold ethanol, after which the ethanol was removed. The pellet was resuspended in 20 μL deionized MilliQ water.

For the LSU and ITS regions, the sequencing was performed using a BigDyeTM terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer instructions for an ABI3100 sequencer (Applied Biosystems). The sequencing was performed with the same primers used in the PCR and in duplicate for each strain.

To assess the intragenetic diversity of the ITS region, the ITS PCR products of four strains (BO32, KY03, LU07, and MJ07) were cloned and sequenced by Macrogen (the Netherlands, Amsterdam). For each strain, 12 colonies were picked randomly and sequenced.

PCR amplification and sequencing of the SSU and cox1 regions were performed at the Genomic Core Facility of the Sahlgrenska Academy, Gothenburg University (Gothenburg, Sweden). The PCR reactions were carried out in a final volume of 5 μL containing 2.5 μL of AmpliTaq Gold® 360 Master Mix, Life Technologies, Stockholm, Sweden 0.4 μL of each primer (forward and reverse, 10 μM), and 20 ng of genomic DNA, using a GeneAmp® PCR system 9700 (Applied Biosystems). The SSU region was amplified and sequenced using four primers to cover the full region of ~1,700 bases (two external primers and two primers covering the central part of the SSU). First, the SSU was amplified and sequenced using the following external primers 4,616 (forward: 5′-AACCTGGTTGATCCTGCCAG-3′) and 4,618 (reverse: 5′-TGATCCTTCTGCAGGTTCACCTAC-3′). The PCR was performed using the following temperature profile: 5 min at 94°C; and 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; terminated by 7 min at 72°C. Then, primers were designed to cover the central part of the SSU, using the sequences obtained from the first SSU sequencing. These internal primers were SSUmGSF1 (forward: 5′-AAGGAGTGTGTACCTGCATC-3′) and SSUmGSR1 (reverse: 5′-AAGGTCAGTACTCGTTGCATGCATC-3′). The PCRs were

performed using the temperature protocol: 1 min at 94°C; followed by 35 cycles with 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C; finished by 10 min at 72°C.

The mitochondrial gene cox1 was amplified and sequenced using the primer pair LCO1490 (forward: 5′-GGT CAACAAATCATAAAGATATTGG-3′) and HCO2198 (reverse: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Riisberg et al. 2009). The PCRs were performed using the following temperature protocol 3 min at 94°C; followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 65°C; finalized by 10 min at 65°C.

For the SSU and cox1 regions, the PCR products were purified using magnetic beads (Ampure, Agencourt, Brea, California, USA), and eluted in 15 μL of distilled water. The sequencing preparation was carried out using BigDye® Terminator v 3.1 Cycle Sequence Kit (Life Technologies, Applied Biosystems) according to the manufacturer's instructions in a GeneAmp® PCR system 9700 (Applied Biosystems). The sequence-PCR products were purified using magnetic beads (Ampure, Agencourt), and eluted in High Dye Formamide. The sequence reaction products were analyzed with a 3730 DNA Analyzer (Applied Biosystems). The sequencing was performed with the same primers used in the PCR and in duplicate for each strain.

Phylogenetic analyses. The sequences were visualized using BioEdit v.7.1.3.0 (Hall 1999), edited manually and aligned using the ClustalW model in MEGA v.5.2.2 (Tamura et al. 2011). Sequences of other raphidophyte species, the chlorophyte *Dunaliella* sp. and the haptophyte *Emiliana huxleyi* (used as an outgroup) were downloaded from GenBank and were added to the alignments. The three ITS regions (ITS1, 5.8S, and ITS2) were delimited by comparison with the available sequence of *Vacuolaria virescens* (Raphidophyceae; AF409125) from GenBank. The phylogenies of SSU, LSU, cox1, and ITS (cloned sequences) were estimated with a Bayesian method using MrBayes (Ronquist et al. 2012), with the general time reversible evolutionary model with gamma-distributed rate variation across sites. All the Bayesian analyses were performed with two Markov chains for 2,000,000 generations, the chain was sampled every 500 generations. The first 25% of the samples were discarded as a burn-in phase. The trees generated by MrBayes were visualized in Treeview 1.6.6 (Page 1996). The phylogenies were also estimated with Maximum Likelihood analyses using the Kimura 2-parameter model with gamma-distributed rates among sites using MEGA v.5.2.2 (Tamura et al. 2011). For the *cox1* region, network analyses were performed to visualize the differentiation among *G. semen* sequences. These analyses were done using a median joining method in Network 4.6 (Bandelt et al. 1999) and a statistical parsimony method in TCS 1.21 (Clement et al. 2000).

RESULTS

A total of 43 strains were successfully sequenced for the cox1, and 47 for the SSU, 34 for the ITS, and 46 for the LSU regions (Table 1). All poor quality sequences (i.e., with unclear peaks) were removed from the analyses.

The SSU rRNA gene was fully sequenced with 1,680 base pairs (bp), and the alignment and phylogenetic analyses were based on a sequence frame of 1,461 bp to obtain sequences of the same size as the sequences from other raphidophyte species (obtained from GenBank) and to allow proper phylogenetic comparison. All *G. semen* sequences of the SSU were identical (GenBank accession number: KP200894). The sequences of *G. semen* diverged from other raphidophyte sequences by 2% for the freshwater species *Vacuolaria virescens* and *Merotrichia bacillata*, and 4% and 5% for *Chattonella marina* (marine) and *Heterosigma akashiwo* (marine), respectively. This result was confirmed with the phylogenetic analysis using Bayesian analysis (Fig. 2).

For the LSU of the rRNA gene, only partial sequences were obtained with a length of 406 bp and all the *G. semen* sequences, including the ones from Japan and United States, were identical (GenBank accession number: KP230756). The sequences of *G. semen* diverged from other raphidophytes ranging from 4% for *V. virescens* to 8% for *H. akashiwo*. These results are supported by the phylogenetic analyses (Fig. S1 in the Supporting Information).

The direct sequencing of the ITS region resulted in sequences of 729 bp. Among the European strains, some sequence variations were detected at the main polymorphic sites determined by the cloning of the ITS region (Tables 2 and 3; see next paragraph). However, no distinct biogeographical pattern could be detected among the European strains. The Japanese and American strains appeared overall to have less intra-strain variability than the European strains. Among the Japanese strains, variability among ITS copies was detected for 10 sites (double peaks or deletion/insertion), and 15 sites for the American strains (Tables 2 and 3). Only small differences were observed when comparing Japanese and European strains, mainly due to lower intra-strain variability in the Japanese *G. semen*. Similarly, the American *G. semen* had little intra-strain polymorphism. However, American strains differed more from the European and Japanese strains than these from each other, due to the presence of five additional polymorphic sites (Tables 2 and 3).

A total of 47 sequences of the ITS were successfully obtained from the cloning of four strains to determine the intra-strain polymorphism among gene copies. The sequencing yielded 815 bp sequences (GenBank accession numbers: KP230757 to KP230803) covering the ITS1 (282 bp), the conserved 5.8S (158 bp), and ITS2 (373 bp) rDNA regions. Within the 47 cloned sequences from *G. semen*, high variability was detected among the copies with a total of 99 polymorphic sites (Fig. 3A). The variation between the clone sequences ranged between 0% and 4%. Among the 47 colonies selected for sequencing, 45 unique sequences were identified; the sequences were identical in the clones 2 (LU7-2) and 3 (LU7-3 and in the clones 7 (LU7-7) and 12 (LU7-12) of the strain LU7 (Fig. 4). According to the phylogenetic analyses, all the clones belonged to the same clade and were closely related (Fig. 4). In addition, no clear geographic patterns were detected among the ITS clones of the four strains originating from distinct European lakes

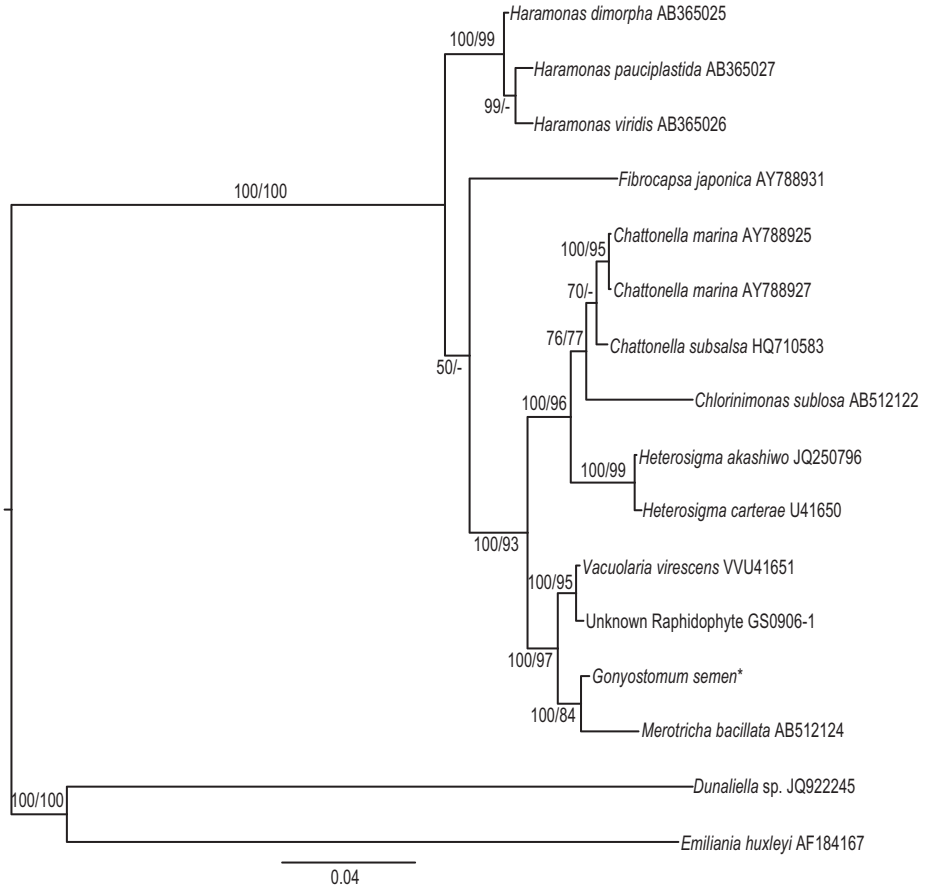

FIG. 2. Bayesian phylogenetic tree of the small subunit of the ribosomal DNA (SSU). The Bayesian analysis was performed using the general time reversible (GTR) evolutionary model with gamma-distributed rate variation across sites, and two Markov chains with 2,000,000 generations. The numbers at the nodes indicate the clade posterior probabilities (percentages) from Bayesian inference (BI) and from maximum likelihood (ML) analyses (BI/ML) (*includes all isolates [$n = 46$], as the sequences were identical, GenBank accession number: KP200894).

(Fig. 4). The ITS sequences obtained from cloning diverged on average by 46% from *G. latum* (GenBank accession number: KP230804), 47% from *V. virescens*, and 50% from *H. akashiwo*.

Sequences with up to 690 bp were obtained for the *cox1* region (accession numbers: KP230751 to KP230755). The alignment and phylogenetic analyses including other raphidophytes were performed using 558 bp. The *cox1* region of *G. semen* differed by ~20% from the other raphidophyte species. The *cox1* region was variable among *G. semen* isolates with 53 polymorphic sites (Fig. 3B). Among the northern European strains, only one haplotype was detected; however, 2.5% and 7.6% divergence was detected with the American and Japanese strains, respectively. In addition, the cox1 sequences of the American and Japanese strains diverged by 2%. The Bayesian phylogenetic analyses showed that *G. semen* strains belong to one clade (Fig. 5). The American and Japanese strains formed one cluster isolated from the European strains. The sequence of the strain GS1103-15 had 2 bp differences with the other American strains from the same location. The haplotype network of the *cox1* region for *G. semen* confirmed these results, showing clear differentiation among geographic locations with no shared haplotypes among locations (Fig. 6). The European strains all belonged to one single haplotype highly differentiated from the strains isolated in Japan and United States according to the median joining and statistical parsimony methods (results from the parsimony analysis not shown). In addition, the American and Japanese haplotypes were clearly genetically separated for the cox1 DNA region by nine mutations.

DISCUSSION

The freshwater raphidophyte *G. semen* has increased in abundance and occurrence in northern Europe during the last decades, becoming a key phytoplankton species in many lakes. Evidence from population genetic analyses and monitoring data (Rengefors et al. 2012, Lebret et al. 2013, Hagman et al. 2015) suggests that *G. semen* has invaded new lakes rather than only increased in number, and that this invasion is recent (past four or five decades). Our phylogenetic analyses showed that strains from the northern European lakes belong to a single lineage that differs from Japanese and American *G. semen* strains. Moreover, low genetic variation in the *cox1* region among the northern European strains provides further support of a recent expansion from a common source lineage most likely dis-

TABLE 2. Summary of the main polymorphic sites observed in the ITS1 region for each strain of *Gonyostomum semen* detected from the sequences obtained from direct sequencing; two sites are not included as the polymorphism was observed in only one strain for each site.

		ITS1 positions																				
Country	Strains	67	76	78	81	90	97	117	130	135	148	193	214	219	225	237	241	250	256	270	271	272	277
Sweden	MJ01	C/T	A/-	G	G/C	A	T	T/g	C/T	G/C	T/G	A/g	T	C	A	-/t	T/a	G/a	G/C	C	C	T	T
	MJ07	C/t	A/-	G	G	A	T/c	T	C/T	G/C	T/G	A/g	T	C	A	-/t	T/a	G/A	G/c	C/A	C/g	T/c	T/-
	SI03	C/t	A/-	G/c	G/C	A	T/c	T/g	C/T	G/C	T/G	A/g	T	C/a	A	-/t	T/a	G/A	G/c	C/a	C/g	T/c	T/-
	SI27	C/t	A/-	G/c	G/C	A	T/c	T/g	C/T	G/C	T/G	A/g	T	C/a	A	-/t	T/a	G/a	G/c	C/a	C/g	T/c	T/-
	EK11	C/t	A/-	G	G/C	A	T	T/g	C/T	G/C	T/G	A/G	T	C	A	-/t	T	G/A	G/c	C/a	C/g	T/c	T/-
	LL05	C/T	A/-	G	G/C	A	T/c	T	C/T	G/C	T/G	A/G	T	C	A	-/t	T	G/A	G/c	C/a	C/g	T/c	T/-
	LL06	C/T	A/-	G	G/C	A	T/c	T/g	C/T	G/C	T/G	A/g	T	C	A	-/t	T	G/a	G/c	C/A	C/G	T/c	T/-
	LL11	C	A/-	G	G/C	A	T/C	T	C/T	G	T/G	A/g	T	C	A	-/t	T	G/A	G/c	C/a	C	T/c	T/-
	ST10	C/t	A/-	G/C	G/C	A	T/c	T/G	C/T	G/C	T/G	A/g	T	C/a	A	-/t	T	G/a	G/c	C/a	C/g	T/c	T/-
	ST16	C/T	A/-	G/C	G/C	A	T/c	T/G	C/T	G	T/G	A/G	T/g	C/a	A	-/t	T/a	G/A	G/c	C/a	C/g	T/c	T/-
	HEII23	C/T	A/-	G/C	G/C	A	T/c	T/g	C/T	G/C	T/G	A/g	T	C/a	A	-/t	T	G/A	G/c	C/a	C/g	T/c	T/-
	HEII25	C/T	A/-	G	G/C	A	T/c	T/G	C/T	G/C	T/G	A/g	T	C/a	A	-/t	T	G/A	G/c	C/A	C/G	T/c	T/-
	HEI12	C/T	A/-	G/c	G/C	A	T/c	T/g	C/T	G/C	T/G	A/g	T/g	C/a	A	-/t	T/a	G/a	G/c	C/a	C/g	T/c	T/-
	LI06	C/T	A/-	G/C	C/G	A	T/c	T/g	C	G/C	T	A/g	T/g	C/A	A	-	T/a	G/a	G	C/a	C/g	T	T/-
	LI10	C/T	A/-	G/C	G/C	A	T/c	T/G	C/T	G/C	T/G	A/g	T/g	C/a	A	-/t	T/a	G/a	G/c	C/a	C/g	T/c	T/-
	BO32	C/t	A	G/c	G/C	A	T/c	T	C/t	G/C	T/g	A/g	T/g	C	A	-/t	T/a	G/a	G/c	C/a	C/g	T/c	T/-
	BO178	C/T	A	G/C	G/C	A	T	T/G	C/T	G/C	T/g	A/g	T/g	C/a	A	-/t	T/a	G/a	G	C/a	C/G	T/c	T/-
	BO302	C/T	A	G/C	G/C	A	T	T/G	C/t	G/C	T/g	A/g	T/g	C/a	A	-/t	T/a	G/a	G	C/a	C/g	T/c	T/-
Norway	LU07	C/t	A/-	G/c	G/C	A	T/c	T/g	C/T	G	T/G	A/G	T	C/a	A	-/t	T	G/a	G/c	C	C	T	T
	GJ17	C/T	A/-	G/c	G/C	A	T/c	T/g	C/T	G/C	T/G	G/A	T	C/A	A	-	T/A	G/A	G	C	C/G	T	T
	IS12	C/t	A/-	G	G/C	A	T/c	T	C/T	G/C	T/G	A/g	T	C	A	-/t	T	G/a	G/c	C/a	C/g	T/c	T/-
Finland	PI10	C/T	A/-	G	G/C	A	T/c	T/g	C/T	G/C	T/G	A/g	T	C/a	A	-/t	T/a	G/A	G/c	C/a	C/g	T/c	T/-
	KY17	C/T	A	G/C	G/C	A	T/c	T/G	C/T	G/C	T/G	A/g	T/g	C/A	A	-/t	T/a	G/A	G	C/A	C/g	T/c	T/-
	KY20	C/T	A/-	G/C	G/C	A	T/c	T/g	C/T	G/C	T/G	A/g	T/g	C/a	A	-/t	T/a	G/A	G/C	C/a	C/g	T/c	T/-
Japan	NIES-1009	C/T	A	G	C	A	T/C	T/G	C	G	T	A/G	T/g	C	A	-	T	G	G	C	C	T	T/-
	NIES-1380	C	A	G	C	A	T/C	T/G	C	G	T	A/G	T/G	C	A	-	T	G	G	C	C	T	T/-
United States	GS1103-4	C/t	A	G	C	A/g	T/c	T/G	C	G	T	a/G	T/g	C	A/t	-	T	G	G	C	C	T	T/-
	GS1103-5	C/t	A	G	C	A/g	T/c	T/g	C	G	T	a/G	T/g	C	A/t	-	T	G	G	C	C	T	T/-
	GS1103-6	C/t	A	G	C	A/G	T/c	T/g	C	G	T	a/G	T/g	C	A/t	-	T	G	G	C	C	T	T/-
	GS1103-9	C/t	A	G	C	A/G	T/c	T/g	C	G	T	a/G	T	C	A/T	-	T	G	G	C	C	T	T/-
	GS1103-12	C	A	G	C	A/G	T/c	T/g	C	G	T	a/G	T	C	A/T	-	T	G	G	C	C	T	T/-
	GS1103-13	C	A	G	C	A/G	T/c	T/g	C	G	T	a/G	T	C	A/T	-	T	G	G	C	C	T	T/-

The numbers refer to the nucleotide position; the nucleotide in capital shows the dominant one on the sequence; -: deletion.

TABLE 3. Summary of the polymorphic sites observed in the 5.8S and the ITS2 rDNA regions for each strain of *Gonyostomum semen* detected from the sequences obtained from direct sequencing six sites are not included as the polymorphism was observed in only one strain for each site.

		Positions																							
		5.8S			ITS2																				
Country	Strains	366	402	426	446	456	475	477	510	537	559	585	627	641	645	657	658	659	660	675	716	736	738	740	741
Sweden	MJ01	A	C	T	C/a	C	C	G/a	G	T	T	C	G	A/-	A	a/G	a/G	a/C	/-/C	C	C	T	C/t	C	T
	MJ07	A	C/T	T/c	C	C	C	G	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T/c	C	C	T/C
	SI03	A	C	T	C/a	C	C	G/a	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C/t	C	T/c
	SI27	A	C	T	C/a	C	C	G/a	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C/t	C	T/C
	EK11	A	C	T	C/a	C	C	G	G	T	T/a	C	G	A	A	A/G	A/G	A/C	/-/C	C	C	T	C/t	C	T/c
	LL05	A	C	T	C	C	C	G	G	T	T/a	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T/c	C	C	T/C
	LL06	A	C	T	C/a	C	C	G	G	T	T	C	G	A	A/g	A/g	A/g	A/c	/-/c	C	C	T	C	C	T/C
	LL11	A	C	T	C/a	C	C	G/a	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C	C	T
	ST10	A	C	T	C/a	C	C/g	G/a	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C/t	C	T/C
	ST16	A	C	T	C	C	C	G	G	T	T/a	C	G	A	A	A/G	A/G	A/C	/-/C	C	C	T/c	C/t	C	T
	HEI123	A/t	C/T	T	C/a	C/a	C/g	G/A	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C/t	C	T/C
	HEI125	A	C	T	C/a	C	C	G	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C/t	C	T/C
	HEI12	A	C/t	T/c	C/a	C/a	C/g	G/A	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T/C	C/t	C	T/C
	LI06	A	C/T	T	C	C/a	C/g	A/g	G	T/a	T	C/T	G	A	A/g	A/g	A/c	A/c	/-/c	C	C	T/c	C	C	T/C
	LI10	A/t	C/t	T	C/a	C/a	C/G	G/A	G	T/A	T	C/T	G	A	A/g	A/g	A/g	A/c	/-/c	C	C/g	T/C	C/t	C	T/C
	BO32	A	C	T	C/a	C	C	G/a	G	T	T/a	C/T	G	A	A	A/G	A/G	A/C	/-/C	C	C	T/c	C/t	C	T/C
	BO178	A	C	T	C/a	C	C	G/a	G	T	T/a	C/T	G	A/-	A	a/G	a/G	a/C	/-/C	C	C/g	T/C	C/t	C	T/C
	BO302	A	C	T	C/a	C	C	G/a	G	T	T	C/T	G	A/-	A	A/G	A/G	A/C	/-/C	C	C/g	T/C	C/t	C	T/C
Norway	LU07	A	C	T	C/a	C/a	C	G/a	G	T	T	C/T	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C	C	T/C
	GJ17	A	C	T	C	C	C	G	G	T/a	T/a	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T	C	C	T/C
	IS12	A/t	C/T	T/c	C/a	C	C	G/a	G	T	T	C	G	A	A/g	A/g	A/g	A/c	/-/c	C	C	T	C	C	T/C
Finland	PI10	A	C	T/c	C	C/a	C/g	G	G	T	T/a	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T/c	C/t	C	T/C
	KY17	A	C	T	C/a	C	C	G	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T/c	C/T	C	T/C
	KY20	A	C	T	C	C	C	G	G	T	T	C	G	A	A	A/g	A/g	A/c	/-/c	C	C	T/c	C/T	C	T
Japan	NIES-1009	A	C	T	C	C	C	G	G	T	T	C	/-	A	A	a/G	a/G	a/C	/-/C	C	C	T	C	C	T
	NIES-1380	A	C	T	C	C	C	G	G	T	T	C	/-	A	A	a/G	a/G	a/C	/-/C	C	C	T	C	C	T
Unites States	GS1103-4	A	C	T	C	C	C	G	A	T	T	C	G	/-	A	G	G	C	C	C/T	C	T	C	C/G	T
	GS1103-5	A	C	T	C	C	C	G	A	T	T	C	G	/-	A	a/G	a/G	a/C	/-/C	C/T	C	T	C	C/G	T
	GS1103-6	A	C	T	C	C	C	G	A	T	T	C	G	/-	A	G	G	C	C	C/T	C	T	C	C/G	T
	GS1103-9	A	C	T	C	C	C	G	A	T	T	C	G	/-	A	G	G	C	C	C/T	C	T	C	C/G	T
	GS1103-12	A	C	T	C	C	C	G	A	T	T	C/T	G	/-	A	a/G	a/G	a/C	/-/C	C/T	C	T	C	C/G	T
	GS1103-13	A	C	T	C	C	C	G	A	T	T	C/T	G	/-	A	a/G	a/G	a/C	/-/C	C/T	C	T	C	C/G	T

The numbers refer to the nucleotide position; the nucleotide in capital shows the dominant one on the sequence; -: deletion.

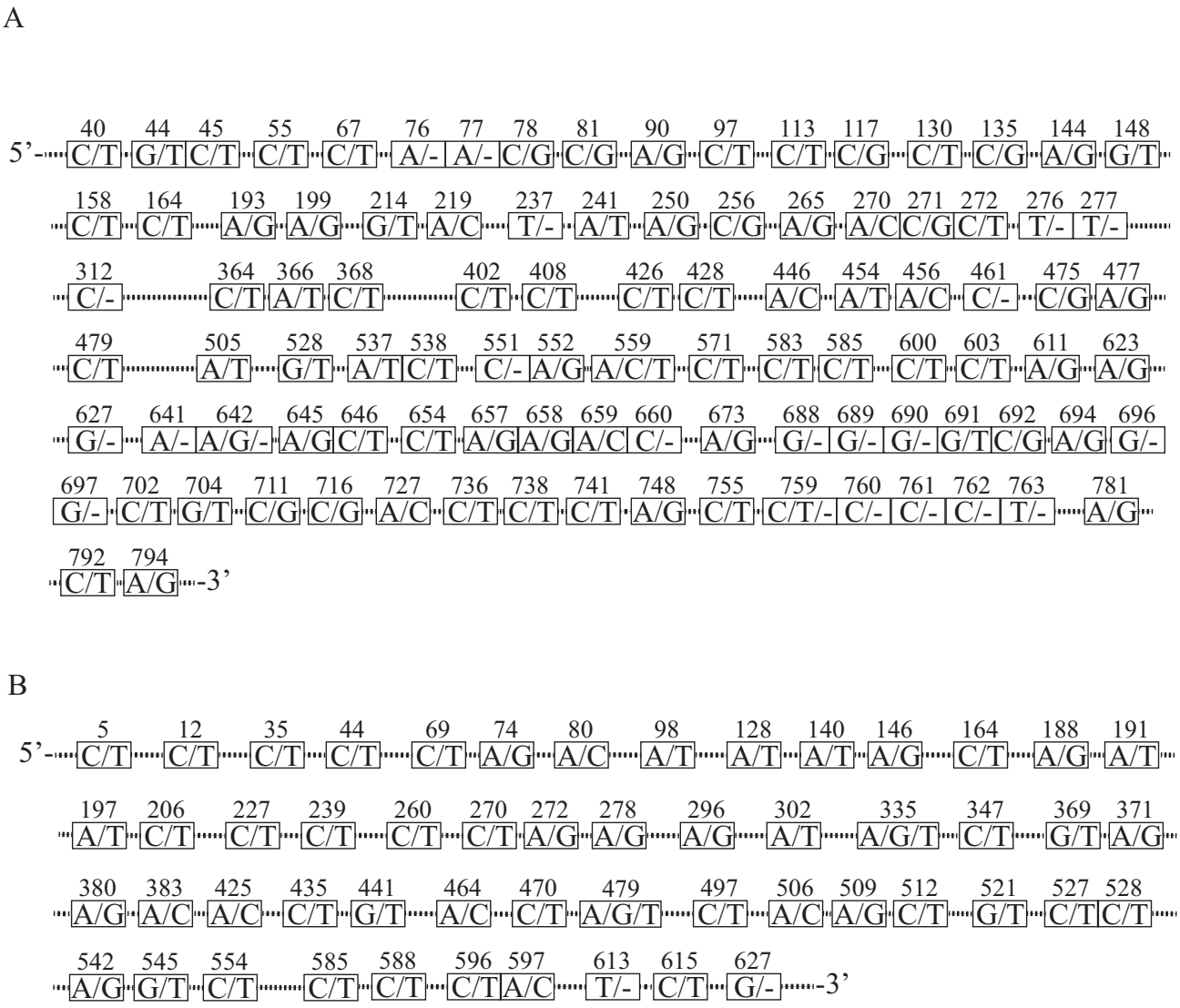

FIG. 3. Scheme of polymorphic sites in the ITS rDNA based on the sequencing of 47 clones (A) and cox1 (B) of *Gonyostomum semen* derived from direct sequencing of 43 strains.

tinct from the Japanese and American lineages identified in this study. Below, we discuss the phylogeographic patterns of this species, particularly in light of its recent expansion.

Recent expansion of G. semen *and phylogeographic patterns.* Analysis of the mitochondrial cox1 marker showed that strains from northern Europe belonged to a single haplotype indicating that the genetic diversity in this region is low, which is consistent with a scenario of a recent colonization. In addition, low diversity and a lack of geographic pattern was found in the ITS marker among the European strains (the intra-strain ITS variability in *G. semen* is discussed below). These results are in concordance with our previous population genetic study (Lebret et al. 2013), showing the presence of a single population in northern Europe. Low genetic diversity has been observed in the invasive range of several other species. For instance, identical ITS sequences were observed in the invasive range of the cyanobacterium *Cylindrospermopsis raciborskii*, while native populations showed higher diversities (Haande et al. 2008). In this study, the sequences of the cox1 region for *G. semen* revealed only a single haplotype in northern Europe ($n = 32$), and two haplotypes in the United States for only nine sequenced isolates from two lakes, suggesting that there may be a higher diversity in the United States. However, the number of strains in the United States or Japan was too low to draw any definitive conclusions.

Although the evidence from our previous studies supports the hypothesis of a recent expansion of *G. semen* (Rengefors et al. 2012, Lebret et al. 2013), it could not be ruled out that *G. semen* colonized the lakes after the last ice age, and only increased in abundance recently due to more favorable condi-

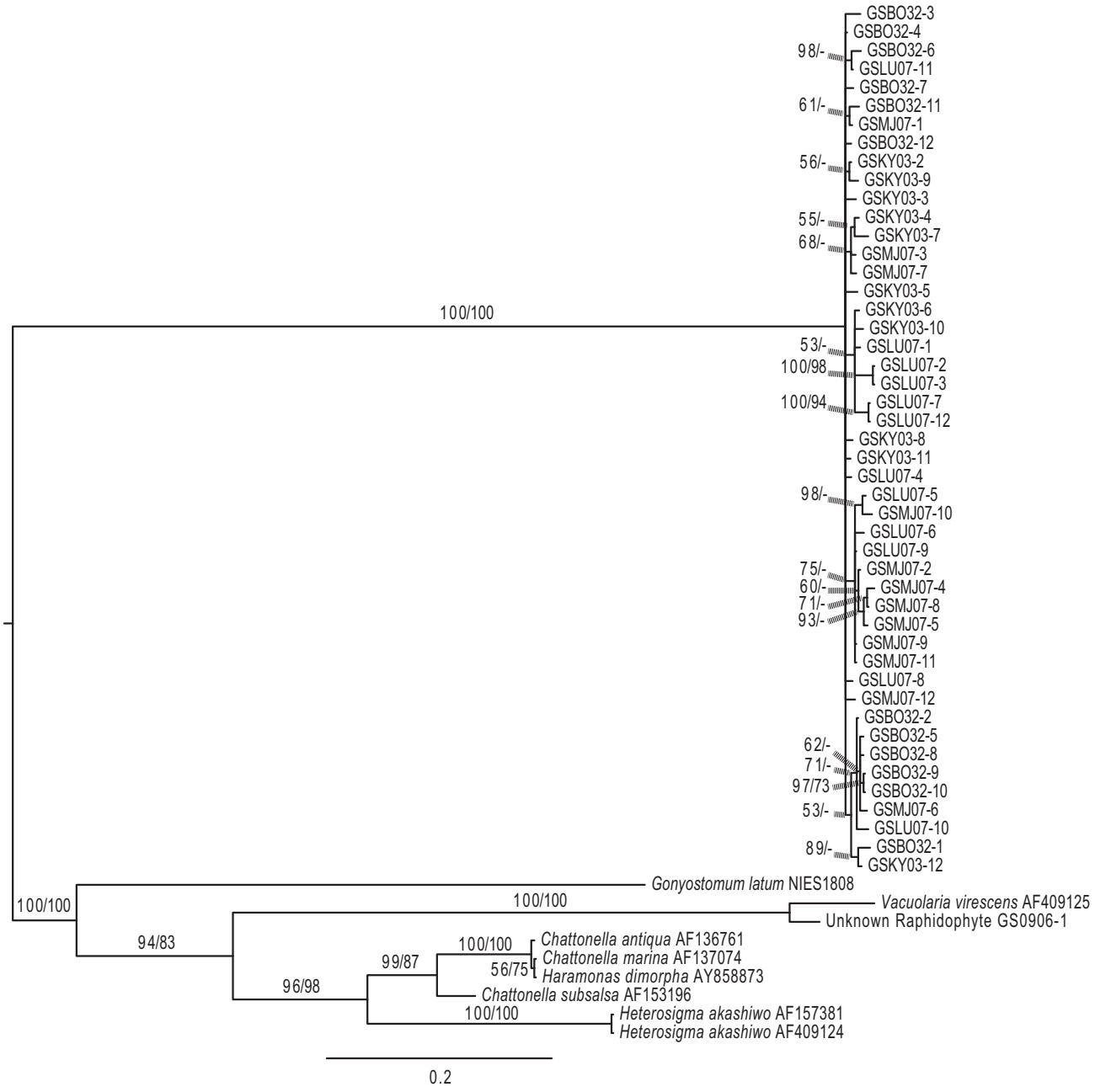

FIG. 4. Bayesian phylogenetic tree of the ITS clones (GenBank accession numbers of *Gonyostomum semen* sequences: KP230757 to KP230803). The Bayesian analysis was performed using the general time reversible (GTR) evolutionary model with gamma-distributed rate variation across sites, and two Markov chains with 2,000,000 generations. The numbers at the nodes indicate the clade posterior probabilities (percentage) from Bayesian inference (BI) and from maximum likelihood (ML) analyses (BI/ML). The posterior probabilities inferior to 50 from the maximum likelihood are shown as "-".

tions. In the latter case, we would have expected several different haplotypes of the *cox1* marker in northern Europe as a result of multiple colonization events or diversification following the regression of the ice cap. For instance, Tahvanainen et al. (2012) identified several different ITS haplotypes for the dinoflagellate *Alexandrium ostenfeldii*, explained by postglaciation colonization of the Baltic Sea and differentiation of populations. Alternatively, *G. semen* might have needed more time to differentiate and exhibit nucleotide polymorphism among the European strains. For example, Siver et al. (2013) showed that differentiation of strains of a *Mallomonas* species occurred millions of years ago based on the phylogeny established from 18S, 28S rDNA and the ribulose-bisphosphate carboxylase (*rbcL*) gene. In addition, the low diversity is unlikely to be the consequence of a life cycle based exclusively on

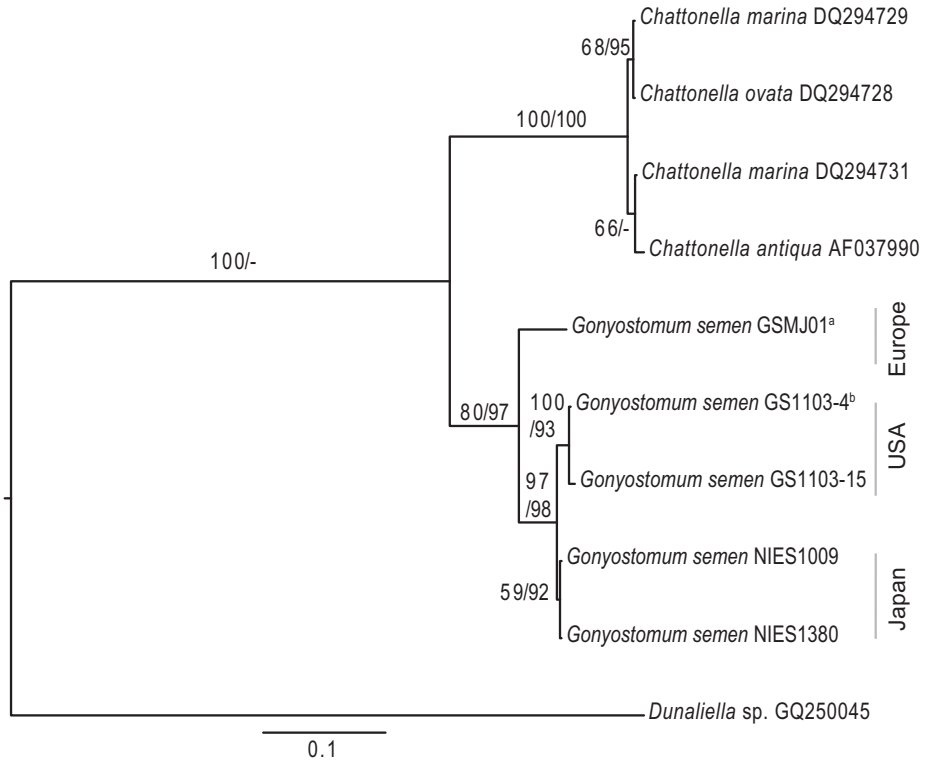

FIG. 5. Bayesian phylogenetic tree of the cox1 (GenBank accession numbers of *Gonyostomum semen* sequences: KP230751 to KP230755). The Bayesian analysis was performed using the general time reversible (GTR) evolutionary model with gamma-distributed rate variation across sites, and two Markov chains with 2,000,000 generations. The numbers at the nodes indicate the clade posterior probabilities (percentage) from Bayesian inference (BI) and from maximum likelihood (ML) analyses (BI/ML). The posterior probabilities inferior to 50 from the maximum likelihood are shown as "-" ([a]includes all European isolates [$n = 32$], as the sequences were identical; [b]includes eight American isolates, as the sequences were identical).

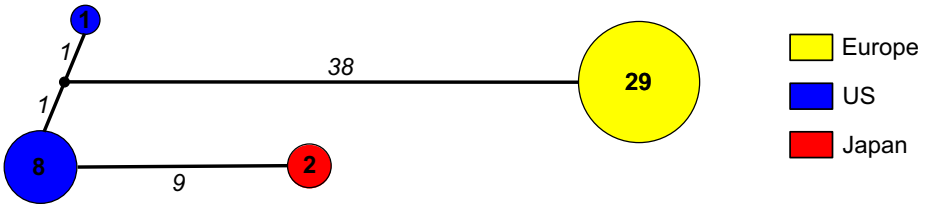

FIG. 6. Haplotype network based on cox1 sequences using median joining in Network. Mutations steps are indicated in italics. Number of analyzed strains is given inside the haplotypes.

asexual reproduction. Previous studies (Cronberg 2005, Figueroa and Rengefors 2006) described the life cycle of *G. semen* and showed that the species undergoes sexual reproduction regularly, allowing genetic recombination and diversification. Nevertheless, on a cautionary note, we cannot exclude that different haplotypes of *G. semen* might be present in the lakes at low abundances which were not detected, or that some haplotypes might have gone extinct, being poorly adapted to the changing environment.

At the transcontinental scale, clear sequence differences were observed among *G. semen* strains. The cox1 markers showed a distinct genetic differentiation between the Japanese, North American, and northern European strains. In addition, clear differences were observed between the ITS sequences from Europe and from United States. These results indicate restricted gene flow at large distances in *G. semen* and that distinct lineages have different geographic distributions, although the low number of strains from the United States and Japan mean that we cannot exclude categorically that the European lineage is not present in these countries. For instance, Boo et al. (2010) found that some haplotypes of *Syruna petersenii* were detected with very low frequency among isolates from different continents. In this study, the fact that the expansion of *G. semen* is restricted to Europe might be partly explained by the presence of distinct lineages. The European lineage could potentially have specific characteristics favoring its expansion in Europe.

Phylogeny of G. semen. The phylogenetic analyses of the LSU and SSU rRNA regions provide strong evidence that *G. semen* is most closely related to other freshwater raphidophyte species (i.e., *M. bacillata* and *V. virescens*) as suggested previously in preliminary analyses by Figueroa and Rengefors (2006). In this study, the strain GS0906-1 was microscopically identified as *G. semen*, while the sequencing of the SSU rRNA gene and the ITS revealed that this strain probably belongs to the *Vacuolaria* genus as it clustered in the phylogenetic analyses with the GenBank sequences of *V. virescens*. This result demonstrates

the importance of molecular tools in species identification and highlights the problem of *G. semen* identification and detection. Consequently, the question of the allegedly global distribution of *G. semen* should be revisited using molecular tools.

Other phylogeographic studies of phytoplankton have shown the presence of cryptic species within identical morphospecies using molecular tools (Montresor et al. 2003). In addition, phylogenies can also reveal that individuals with distinct morphologies have identical DNA sequences, showing the recent divergence of species, or phenotypic plasticity occurring within a species (Logares et al. 2007). Thus, the importance of conserved markers should not be underestimated, as it can reveal important features of the species adaptation and ecology.

Utility of the different markers for the study of G. semen. The SSU and partial LSU rRNA sequences of the different *G. semen* strains were identical, thus no phylogeographical pattern could be observed based on these markers. The SSU rRNA region is a very well-conserved DNA region and thus is commonly used for taxonomy and phylogeny at the species level in phytoplankton (Potter et al. 1997, Edvardsen et al. 2003, Logares et al. 2007). However, the less conservative marker, LSU rRNA gene, can be useful in some species for the detection of geographic patterns. Depending on the species, the LSU region can be well conserved among individuals of the same species, and in other cases show a significant variability (Saravanan and Godhe 2010, Baggesen et al. 2012). For instance, Godhe et al. (2006) successfully used the LSU region to identify geographic patterns over large distances in the marine diatom *Skeletonema marinoi*. The LSU of *G. semen* was not variable, this might be explained by the fact that we only obtained a partial sequence of the LSU, while the complete LSU of *G. semen* might show polymorphism. Thus, sequences of both SSU and partial LSU can be important to confirm morphological identification to species level when the morphological characteristics are ambiguous. For this study, using these two markers we can confirm that all our *G. semen* isolates from northern Europe, United States, and Japan belong to the same species (as a genetic entity).

The ITS region is commonly considered as a good biogeographic marker for phytoplankton studies, as it shows high variability (Coleman 2001, Godhe et al. 2006, Lilly et al. 2007, Rynearson et al. 2009, Tesson et al. 2013). For instance, Godhe et al. (2006) showed that the ITS2 best resolved the biogeographical pattern among northern European strains of the marine diatom *S. marinoi*. In our study, the ITS region showed only small differences among the *G. semen* European strains. However, the direct sequencing of the ITS revealed the presence of multiple copies by the presence of double peaks, which was confirmed by the cloning and sequencing of single colonies for four strains. The ITS region is known to have a high number of copies within a single genome for a wide range of organisms particularly in large phytoplankton species (Zhu et al. 2005), such as *G. semen*. However, the direct sequencing of the ITS region showed that the different strains have the same double peak pattern, suggesting the presence of very similar intra-strain polymorphism. This result also suggests that the distinct copies among strains are most likely very similar.

The presence of several distinct copies of the ITS within a cell complicated the editing of the sequences obtained from direct sequencing, and the interpretation of potential phylogenetic and geographic patterns. Thus, based on the ITS region, it remains challenging to clearly identify genetically different or identical strains of *G. semen* with certainty, and to highlight genetic variations among strains. Nevertheless, distinct sequence differences were detected between the American and the other strains, including the sequences obtained from cloning. Thus, the ITS region can reveal long distance geographic pattern for *G. semen*. At smaller geographic scales, such as within northern Europe, no clear geographic patterns were detected based on the ITS region. This could either be explained by the low diversity following the rapid expansion of the species distribution, or that high intra-strain diversity of the ITS could potentially hide small variability among strains. Thus, the ITS might not be a good marker to identify geographic patterns for raphidophyte species in general.

Mitochondrial DNA, such as the *cox1* gene, is commonly used in phylogeographic studies in plants and animals, but its use has been more limited for free-living microbes. Nevertheless, mitochondrial DNA can potentially be a very informative marker to study biogeographic patterns in a wide range of organisms. For *G. semen*, the sequencing of the cox1 region revealed large differences at large geographic scale. Adding more strains from a larger range of locations could potentially reveal important geographic and dispersal pattern of the species.

Conclusions. The four DNA markers investigated in *G. semen* showed low diversity in Norway, Sweden and Finland, the region in which the species has recently expanded. Also, all strains belonged to a single haplotype different from the lineages on the other continents. Together, this evidence provides strong support of a very recent expansion from a single rather than multiples source of lineages. Finally, we concluded that the mitochondrial *cox1* was the most useful marker in determining large-scale biogeographic patterns in this species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Bayesian phylogenetic tree of the large subunit of the ribosomal DNA (LSU).