Journal of Marine Science and Technology

Volume 22 | Issue 1 Article 11

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Acknowledgements

We thank Dr. Sheng-Ping L. Hwang for providing technical suggestions and constructive discussions. This study was supported by grants (NSC 102-2611-M-019-006, NSC 102-2611- M-019-021-MY2, and NSC 102-2313-B-019-018-MY2) from the National Science Council (R.O.C.). Additional support was also received from the Center of Excellence for the Oceans, National Taiwan Ocean University. L.-K. Kang was supported by an NSC postdoctoral fellowship (NSC101-2811- M-019-005).

Journal of Marine Science and Technology, Vol. 22, No. 1, pp. 89-96 (*2014*)
DOI: 10.6119/JMST-013-0412-1

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Key words: ammonium transporter, nitrogen, phytoplankton, East China Sea.

ABSTRACT

The sequence database of ammonium transporter (*Amt1*) genes in eukaryotic phytoplankton was expanded by obtaining new sequences from cultured strains and natural populations collected in the East China Sea (ECS). From unialgal cultures, 6 new *Amt1* sequences belonging to the Bacillariophyceae, Dinophyceae, and Prasinophyceae were obtained. A phylogenetic analysis revealed that AMT1s of higher plants were most closely related to those in green algae. In addition, haptophyte and diatom AMT1s formed distinct monophyletic clades. Diatom AMT1s were further divided into 3 orthologous subclasses, and active gene duplications were observed in subclass III. As for mixed-species sequencing using ECS samples, 78 sequences homologous to *Amt1* were retrieved, and diatom AMT1s were the dominant group. Our results indicated that eukaryotic phytoplankton *Amt1* genes can be cloned at both the DNA and the RNA levels in marine environments, and this information on *Amt1* diversity will be useful for quantifying *Amt1* expression in the ocean.

I. INTRODUCTION

Nitrogen is one of the limiting resources for marine phytoplankton, particularly in the open ocean [9]. In marine environments, nitrogenous nutrients are present in several forms (e.g., nitrate, ammonium, and urea), and their concentrations vary with space and time. Since marine phytoplankton are a diverse group of organisms with different nitrogen requirements, species compositions and their nitrogen environments mutually affect each other in forming the observed environmental heterogeneity and species succession [5, 7, 22]. Knowledge of how different species vary in nitrogen utilization strategies is thus necessary to evaluate the nitrogen stress of phytoplankton from the level of individual cells to the entire community.

To evaluate nitrogen deficiencies in eukaryotic phytoplankton, several genes of nitrogen-utilization pathways were proposed as potential markers. These genes include nitrate transporter (*Nrt2*) [14, 16, 17, 26], nitrate reductase (*Nr*) [1, 2], ammonium transporter (*Amt1*) [13, 16], and glutamine synthetase (*glnII*) [28]. Among them, *Amt1* encodes an ammonium transporter (AMT1) protein that facilitates the movement of ammonium ions across cell membranes. Ammonium transporter genes are widely found in eukaryotic phytoplankton including diatoms [2, 13], green algae [11, 27], haptophytes [16, 31], a pelagophyte [4], and a prasinophyte [23]. A sequence analysis indicated that these algal sequences are closely related to the high-affinity AMT1 family in higher plants (for a review see [24]). In a diatom (*Cylindrotheca fuciformis*) and haptophyte (*Isochrysis galbana*), the highest *Amt1* mRNA levels were detected in nitrogen-starved cells, followed by nitrate-grown, and then ammonium-grown cells [13, 16]. These data demonstrate that the AMT1 system is highly regulated at the transcript level, and its expression is potentially a useful marker of the utilization of nitrogenous nutrients.

In unicellular algae, the genome of a single species often contains several forms of *Amt1* genes, and these forms may have different functions or efficiencies in ammonium uptake [2, 10, 32]. For example, *Chlamydomonas reinhardtii* contains 8 forms in its *Amt1* gene family, and expressions of these genes are all responsive to different nitrogen conditions [11]. Among them, *Amt1.1* and *Amt1.2* are strongly induced by a nitrogen deficiency, whereas *Amt1.4* is expressed in the presence of organic nitrogen sources, such as arginine [20].

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Table 1. Locations of sampling stations and dominant phytoplankton genera observed.

Date	Cruise	St.	Location		Dominant genera (> 20 μm)
			Longitude (E)	Latitude (N)	
Apr. 7~9 2006	ORII 1343	14	123°09'	25°05'	Not determined
Aug. 24~26 2006	ORII 1372	1	122°13'	25°25'	*Guinardia* (55%), *Chaetoceros* (9%), *Helicotheca* (9%)
		4	120°05'	26°10'	*Guinardia* (26%), *Rhizosolenia* (21%), *Skeletonema* (16%)
		8	123°03'	27°11'	*Skeletonema* (28%), *Asterionellopsis* (25%), *Pseudonitzschia* (21%)

In the diatom, *Cylindrotheca fusiformis*, both *Amt1.1* and *Amt1.2* are highly upregulated under N starvation. However, *Amt1.1* is considered more efficient than *Amt1.2* based on higher overall transcript levels [2, 13]. Correctly identifying the function for each *Amt1* form will help clarify strategies used by phytoplankton to survive nutrient stress.

In this study, we expended the *Amt1* database of eukaryotic phytoplankton by obtaining sequences from unialgal cultures belonging to the Bacillariophyceae, Dinophyceae, and Prasinophyceae. The observed grouping patterns of various forms complemented existing concepts of *Amt1* evolution. We also obtained *Amt1* sequences from natural samples collected in the East China Sea (ECS). Information on *Amt1* diversity in natural phytoplankton will be useful in designing polymerase chain reaction (PCR) primers to detect *Amt1* expression in marine environments [18].

II. MATERIALS AND METHODS

1. Phytoplankton Strains and Maintenance

Unialgal cultures of the diatom, *Chaeotoceros affinis* CCMP160, and the dinoflagellate, *Heterocapsa triquetra* CCMP 448, were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). Unialgal cultures of the diatom, *Skeletonema costatum* Strain Kao, and the prasinophyte, *Tetraselmis chui* clone TA, were provided by Dr. H.-M. Su of Tungkang Marine Laboratory, Pingtung, Taiwan. The diatom culture of *Thalassiosira weissflogii* was provided by Dr. E. Cosper [8]. All these cultures were grown at 20°C in f/2-enriched seawater medium [12] under a light: dark 12: 12-h photoperiod with a light intensity of 145 μE m^{-2} s^{-1}. To clone *Amt1* genes, nitrogen-starved cells were obtained by harvesting stationary-phase cells grown in a low-nitrate medium containing 88 μM nitrate.

2. Cruises and Sampling

Two research cruises were conducted in the ECS in 2006 onboard the *R/V Ocean Researcher II*. The first cruise visited a single station (St. 14) on April 7~9, and the second cruise visited 3 stations (Sts. 1, 4, and 8) on August 24~26 (Table 1). Phytoplankton samples were collected with a 20-μm-mesh plankton net with a mouth diameter of 0.5 m. At each station, an oblique tow was performed from 5 m deep to the sea surface in a duration of 10 min at a ship speed of 1 knot. After a tow, samples were removed from the receiving bottle (cod end), and filtered through a 200-μm mesh. Subsequently, plankton in the filtrate were collected with a 20-μm-mesh screen. Part of the sample was preserved with acidic Lugol's solution [25] and stored at 4°C for microscopic examination. The rest of the sample was transferred to cryotubes (Nalgene Nunc, Rochester, NY, USA), and stored in liquid nitrogen for RNA and DNA isolation.

3. Total RNA and Genomic DNA Isolation

Samples frozen in liquid nitrogen were thawed by adding RLT buffer (Qiagen, Valencia, CA, USA) with 1% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and then homogenized by supersonic disruption (Sonicator Ultrasonic Processor XL, Heat System Ultrasonics, Framingdale, NY, USA). Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The RNase-free DNaseI set (Qiagen) was applied for on-column digestion of residual DNA during RNA purification. On the other hand, some other frozen samples were used to extract genomic DNA with the phenol-chloroform method and the addition of cetyltrimethylammnoium bromide [6]. Concentrations of RNA and DNA were determined with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) at wavelengths of 260 and 280 nm.

4. Reverse Transcription, PCR Amplification, and DNA Sequencing

Total RNA was reverse-transcribed into complementary (c)DNA using random hexamers (Promega, Madison, WI, USA) and ImpromII reverse transcriptase (Promega) at 25°C for 10 min and 55°C for 1 h. Next, single-stranded cDNA was used in the PCR to amplify target gene fragments on a Biometra thermocycler (Gotingen, Germany). To clone the *Amt1* genes, 2 sets of degenerate primers for amplifying *Amt1* genes were designed from the conserved regions of homologous amino acid sequences in algae and higher plants. The first set included the forward primer, AmtF1 (TSTTYCYIATGCAMK IIGGITT YGCIATG), and the reverse primer, AmtR3 (CIGG RTTR AAICCRWACCAICCIAWCC) [16], and the second set included AmtF2 (CARCYIGGITTYGCIATGCTIKRIGC IGG) and AmtR5 (CCRIWKAYRAHBGCIGCCCAIGG), where I is inosine, R = A or G, Y = C or T, M = A or C, K = G or T, W = A or T, and S = C or G. Nested PCRs were per-

formed with AmtF1 and AmtR5 primers in the first round and AmtF2 and AmtR3 primers in the second round. In addition, 0.5 μg of total genomic DNA extracted from individual stations was also used as the template for the PCR. PCR products were then purified from a low-melting-point agarose gel and cloned into pGEM-T vectors (Promega, Madison, WI, USA). DNA sequencing of the cloned fragments was performed using an ABI Prism 377A DNA sequencer with the PRISM Ready Reaction BigDye Termination Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). ECS sequences were labeled using the following format: SxxyAMTzz, where xx is the station name (xx = 01, 04, 08, or 14), y is cDNA (R) or genomic DNA (G) used as the template, and zz is the clone number.

5. Phylogenetic Tree Construction

DNA sequences of *Amt1* genes were translated to deduced amino acid sequences using DNAstar software (Lasergene, Madison, WI, USA). The amino acid sequences were aligned using ClustalW [29], and highly fragmented regions were manually edited with the Bioedit Sequence Alignment Editor vers. 5.0.9 (Department of Microbiology, North Carolina State University) [19]. The resultant alignment files were used to construct phylogenetic trees using the PHYLIP software (the *PHYLogency Inference Package*, available at http://evolution.genetics.washington.edu/phylip.html). Pairwise distances were calculated using the Kimura formula within the PROTDIST program, and Neighbor-joining trees were generated with the NEIGHBOR program. Bootstrap values were obtained with 1000 bootstrap replicates to estimate the degree of confidence. Sequences obtained in this study were deposited in GenBank under the accession numbers JN807467~JN807550.

6. Phytoplankton Identification and Enumeration

DNA Lugol's-preserved samples were placed on Sedgewick-Rafter counting slides (Hausser Scientific, Horsham, PA, USA), and examined with a Nikon Optiphot-2 microscope (Tokyo, Japan) at a magnification of 100x. All cells on the counting slide were examined and enumerated. If there were fewer than 200 cells on a counting slide, additional counting slides were prepared from the same sample, and were similarly examined to obtain statistically meaningful results. Phytoplankton cells were identified to the generic level based on Tomas [30].

III. RESULTS

1. *Amt1* Sequences from Cultivated Organisms

The degenerate primers for the *Amt1* generated 6 gene fragments from cDNAs of *Tetraselmis chui*, *Heterocapsa triquetra*, *Chaetoceros affinis*, *Skeletonema costatum*, and *Thalassiosira weissflogii* with lengths ranging 618~645 bp. After removing the primer sequences, these gene fragments contained an open reading frame of 187~198 amino acids, and the NCBI Blast server (blastp) suggested that they all belonged to the AMT1

Table 2. GenBank accession numbers and genome coordinates of ammonium transporter genes included in the phylogenetic tree in this study.

Organism	GenBank[a]	JGI protein ID	Protein name
Plants			
Arabidopsis thaliana	NP_193087		AMT1.1
	NP_176658		AMT1.2
	NP_189073		AMT1.3
	NP_194599		AMT1.4
	NP_189072		AMT1.5
Chlorophytes			
Chlamydomonas reinhardtii	AAL85345		AMT1.1
	AAM94623		AMT1.2
	EDO97333		AMT1.3
	EDP08718		AMT1.4
	EDP01915		AMT1.5
	EDO97275		AMT1.6
	EDP07394		AMT1.7
	EDP03492		AMT1.8
Volvox carteri f. *nagriensis*		69712	AMT1.2
		84428	AMT1.3
		65690	AMT1.4
		61118	AMT1.5
		41671	AMT1.6
		77636	AMT1.7
		67734	AMT1.8
		75428	AMT1.10
Tetraselmis chui	**JN807467**		AMT1
Dinoflagellates			
Heterocapsa triquetra	**JN807468**		AMT1
Haptophytes			
Emiliania hyxleyi		74037	AMT1.1
		467882	AMT1.2
		62984	AMT1.3
		433750	AMT1.4
Isochrysis galbana	ABD94149		AMT1
Diatoms			
Chaetoceros affinis	**JN807469**		AMT1.1
	JN807470		AMT1.3
Cylindrotheca fuciformis	Q94KI7		AMT1.1
	Q5EXJ7		AMT1.2
Fragilariopsis cylindrus		275907	AMT1.1
		212054	AMT1.2
		225282	AMT1.3
		209552	AMT1.4
		257791	AMT1.5
Phaeodactylum tricornutum	XP_002180943	27877	AMT1.1
	XP_002183725	54981	AMT1.2
		1862	AMT1.3
		10881	AMT1.4
		13418	AMT1.5
		1813	AMT1.6
		11128	AMT1.7
Skeletonema costatum	**JN807471**		AMT1
Thalassiosira pseudonana		108565	AMT1.1
		147331	AMT1.2
		126112	AMT1.3
		137441	AMT1.4
		101250	AMT1.5
		104856	AMT1.6
Thalassiosira weissflogii	**JN807472**		AMT1

[a] Accession numbers in boldface type are new sequences obtained in this study.

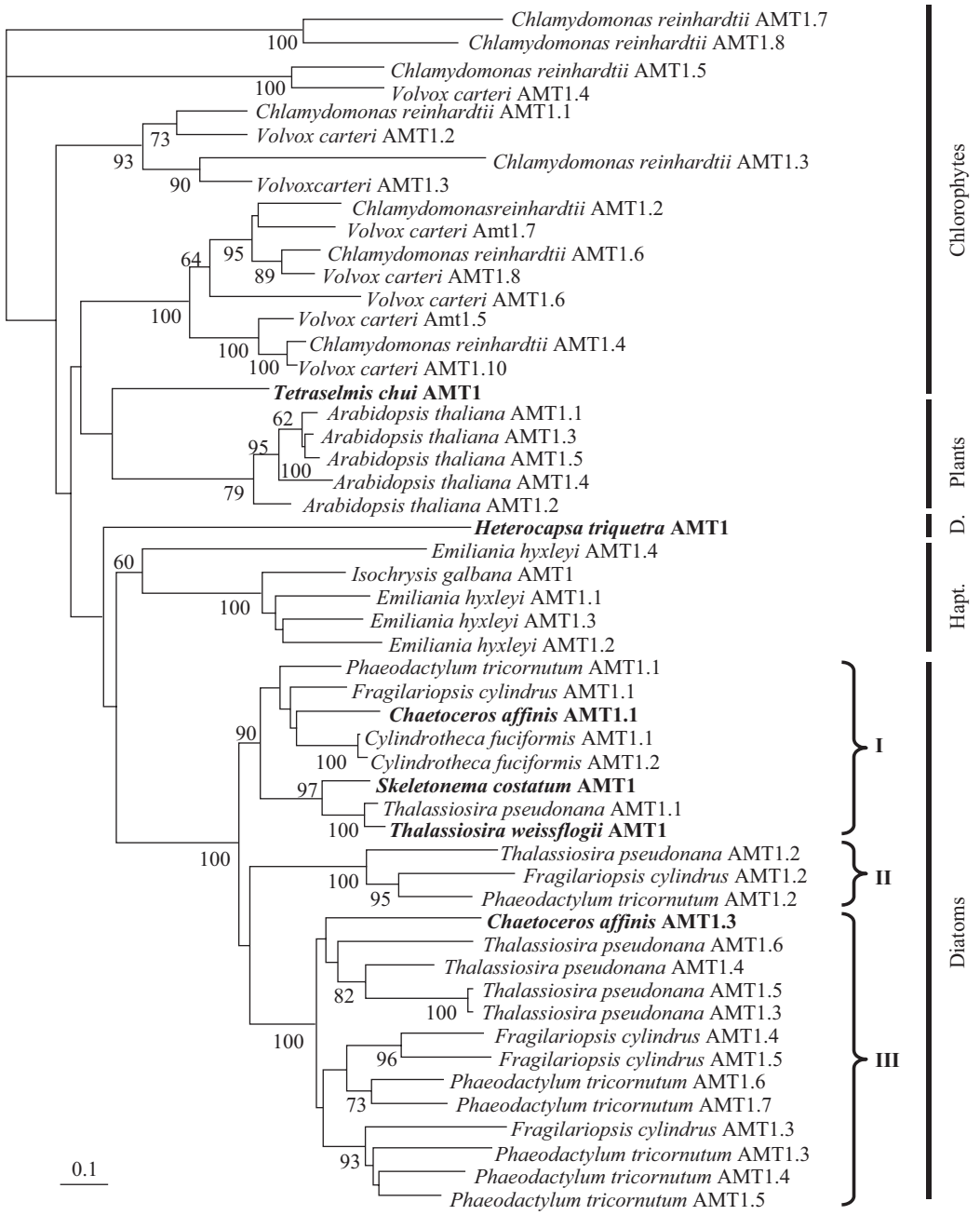

Fig. 1. Phylogenetic tree of ammonium transporter (*AMT1*) amino acid sequences in eukaryotic phytoplankton and higher plants. *AMT1* sequences obtained in this study are shown in boldface type. Numbers at the nodes are bootstrap values based on 1000 resamplings, and only values of > 60% are shown. The scale bar represents an estimated number of amino acid substitutions per position. Accession numbers are provided in Table 2. D, dinoflagellates; Hapt, haptophytes.

family (pfam00909). These deduced amino acid sequences were aligned with known AMT1 sequences, including multiple forms from individual genome projects of algae and plants, to determine their taxonomic associations (Fig. 1). The phylogenetic analysis revealed that *T. chui* and *H. triquetra* AMT1 sequences were not grouped with any known algal clades. In contrast, the other 4 diatom AMT1 sequences clustered together with known diatom AMT1 sequences and formed a single bootstrap-supported clade. Within the diatom branch, the amino acid sequences of AMT1 shared a mean identity of 0.62 with one another, but the mean identity between diatoms and other algae decreased to 0.41. In addition, diatom AMT1s formed 3 phylogenetically distinct subclasses (Fig. 1). Subclass I included 8 AMT1 sequences which shared an average of 0.75 identity with the first discovered AMT1.1 sequence in *C. fuciformis*. Subclass II included 3 AMT1.2s which shared an average identity of 0.72 with one another, and subclass III included the other 13 diatom AMT1s.

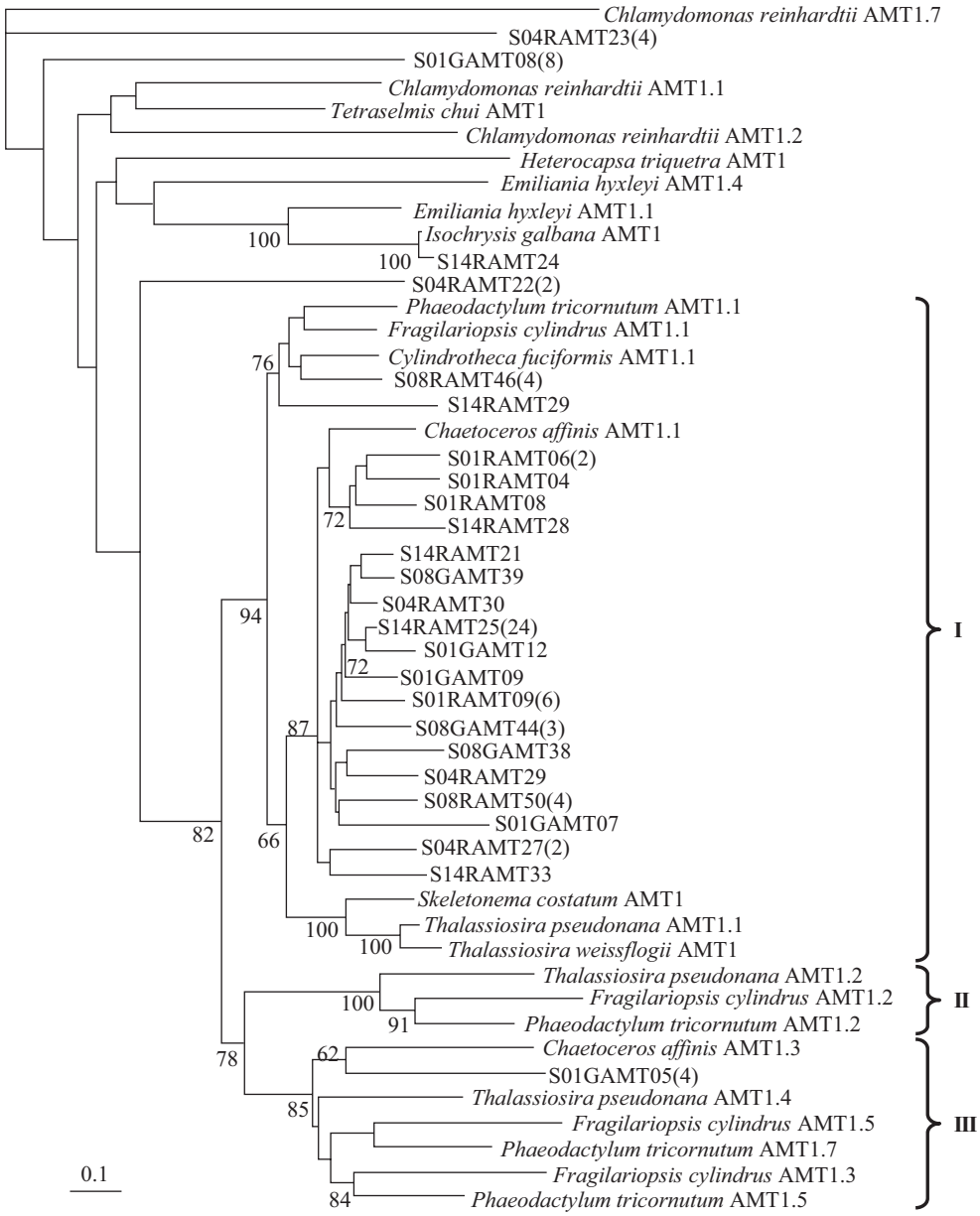

Fig. 2. Phylogenetic analysis of ammonium transporter (AMT1) amino acid sequences obtained by mixed-species sequencing in the East China Sea. Selected diatom AMT1 sequences with known taxonomic associations were used as a basis for comparison. This phylogenetic tree is a condensed version of a more-detailed analysis, and individual operational taxonomic units (OTUs) in the present alignment are represented by the most-conserved AMT1 sequence in that clade (Table 3). The scale bar represents the estimated number of amino acid substitutions per position. Numbers in parentheses are sequence numbers belonging to the respective OTUs. Numbers at the nodes are bootstrap values based on 1000 resamplings, and only values of > 60% are shown.

2. *Amt1* Sequence Diversity in the ECS

In total, 78 gene fragments homologous to *Amt1* were obtained from ECS phytoplankton samples (Fig. 2). A comparison between DNA and RNA sequences revealed no intron regions. The taxonomic affiliations of ECS AMT1 sequences were determined by comparing them to the AMT1 phylogenetic tree (Fig. 1), and sequences located in the same clade and sharing identities of > 0.95 were collected as an operational taxonomic unit (OTU) (Table 3). Diatom AMT1s were the dominant group among ECS microplankton, comprising 64 of 79 sequences (Fig. 2). In this subclass, 2 OTUs (S08RAMT46 and S14RAMT29) formed a bootstrap-supported clade with AMT1 sequences of *Phaeodactylum*, *Fragilariopsis*, and *Cylindrotheca*. Next, 4 OTUs, namely, S14RAMT28, S01RAMT08, S01RAMT04, and S01RAMT06, shared an average identity of 0.82 with one another, and formed a clade with a bootstrapping value of 72% (Fig. 2). *C. affinis* AMT 1.1 was the sequence most closely related to this clade, but the bootstrapping support was weak (< 60%). The remaining subclass I OTUs formed multiple branches without a clear

Table 3. Distributions of ammonium transporter 1 (AMT1) sequences among various operational taxonomic units (OTUs) at individual stations during East China Sea cruises in 2006.

OTU	April	August					
	St.14	St. 1		St. 4		St. 8	
	RNA[a]	RNA	gDNA[b]	RNA	gDNA	RNA	gDNA
Green algae							
S04RAMT24				40%			
S01GAMT08			33%		15%		9%
Unknown							
S04RAMT22				10%	8%		
Diatom subclass I							
S08RAMT46						50%	
S01RAMT06		13%	7%				
S14RAMT21	21%						
S14RAMT25	50%		7%	10%	62%		64%
S01RAMT09		63%	7%				
S08GAMT44					15%		9%
S08RAMT50						50%	
S04RAMT27				20%			
Other subclass I	21%	24%	20%	20%			18%
Diatom subclass III							
S01GAMT05			27%				
n[c]	14	8	15	10	13	8	11

[a] Clones were obtained from an RT-PCR.
[b] Clones were obtained from a PCR using environmental genomic DNA.
[c] The total number of *Amt1* sequences obtained at each station.

association with known diatom AMT1s. In contrast, no OTU from the ECS was clustered with diatom subclass II AMT1s, and only 1 OTU was categorized in subclass III, which was related to *C. affinis* AMT 1.3 (Fig. 2).

Of the 13 sequences that did not group with the diatom clade, S14RAMT24 was classified as haptophyte AMT1 with bootstrap support. The other 12 sequences formed 2 OTUs: S04RAMT23 and S01GAMT08, and they were weakly related to *C. reinhardtii* AMT1.7 (Fig. 2). The OTU S04RAMT22 occupied a branch in between diatoms and haptophytes, and its taxonomical affiliation requires further clarification.

3. Phytoplankton Enumeration

Phytoplankton at St. 1 in the upwelling region were strongly dominated by *Guinardia*. In contrast, *Asterionellopsis*, *Pseudonitzschia*, and *Skeletonema* were abundant at St. 8 in the mid-shelf region. At St. 4 in the coastal region, dominant phytoplankton included *Guinardia*, *Skeletonema*, and *Rhizosolenia* with approximately equal contributions (Table 1). Dinoflagellates were also observed at these stations, but their relative abundances were consistently < 5% of diatoms.

IV. DISCUSSION

Amt1 sequences obtained in this study (Figs. 1, 2) are clearly members of the AMT1 clade as defined in McDonald *et al*. [23]. Members of AMT1 are found in a wide variety of organisms from eukaryotic algae to plants, and individual species often contains multiple copies of AMT1 with slightly different sequences. In the 3 diatom genomes completely sequenced of *F. cylindrus*, *T. pseudonana*, and *P. tricornutum*, at least 5 AMT1 homologs were discovered in each genome [2]. Similarly, our PCR amplified 2 AMT1 homologs in the diatom, *C. affinis* (Table 2, Fig. 1). With the addition of new sequences generated in this study, the topology of phylogenetic tree indicated that the standard parent-to-offspring inheritance (vertical transfer) is the major driving force of AMT1 evolution in eukaryotic algae (Fig. 1). This conclusion agrees with a broader pattern proposed by McDonald *et al*. [23, 24]. In addition, all diatom AMT1 homologs formed a monophyletic group well-separated from haptophyte AMT1s, indicating that sequence differences followed the divergence of diatoms (orthologs). Within the diatom clade, 3 well-supported subclasses were evident (Fig. 1). Individual subclasses contained a specific form of AMT1 in different species, such as the formation of subclass II by 3 AMT1.2 sequences from *Thalassiosira*, *Fragilariopsis*, and *Phaeodactylum* (Fig. 1). This branching pattern suggests gene family expansion through duplication in an ancestral diatom (paralogs), and members of different subclasses entered various species via vertical transfer.

Gene duplications appeared to be active again in subclass III AMT1s, as a single species often contained numerous closely related sequences (paralogs). This evolutionary pattern observed in diatoms was also found in green algae [11].

In the course of evolution, orthologous genes often retain the same function [21]. In *Chlamydomonas*, *Amt1.1* expression is downregulated by ammonium or nitrate and is very responsive to nitrogen starvation [11]. This expression pattern is similar to that of Arabidopsis *Amt1.1* [11], which encodes the main system responsible for ammonium uptake. In diatom subclass I, *Cylindrotheca Amt1.1* and *Amt1.2* showed transcription patterns similar to those of *Amt1.1* in *Chlamydomonas* and *Arabidopsis* [13]. In addition, *Amt1* fragments of *S. costatum* and *T. weissflogii* were obtained from cultures grown in low-nitrate medium, and they were grouped in subclass I (Fig. 1). According to these similar transcriptional patterns, subclass I AMT1s are very likely the main transporter system responsible for ammonium uptake in diatoms. Subclass I AMT1s thus are potentially suitable nitrogen marker genes for evaluating nitrogen deficiencies in eukaryotic phytoplankton.

Mixed-species sequencing from field samples with degenerate primers usually results in highly divergent sequences. Interestingly, 93% of diatom AMT1s obtained from ECS samples were associated with subclass I (Fig. 2). This result indicates that AMT1 is commonly present and actively expressed in surface waters of the ECS, which suggests that it is an important protein necessary for the survival of diatoms in this habitat. If the amount of PCR products is a reflection of template abundance, groupings of AMT1 sequences from the ECS should have certain relationships with species compositions. For example, microscopic examination indicated that *Chaetoceros* is a dominant group of phytoplankton at St. 1 (Table 1), and 4 related AMT1 sequences were amplified from that station (Fig. 2). For a similar reason, the 24 sequences contained in OTU S14RAMT25 might have come from *Asterionellopsis* or *Pseudonitzschia*, although a reference is lacking on the phylogenetic tree (Table 1, Fig. 2). Apparently, diatom AMT1s with known taxonomic associations are insufficient to identify sequences from natural phytoplankton. Additional cultivated strains need to be sequenced to expand the AMT1 sequence database. Alternatively, single-cell PCRs can be performed for the same purpose by directly selecting visually identifiable species from field samples [18].

Based on data collected in this study, it is clear that *Amt1* genes can be used to clone homologous genes in marine environments at both the DNA and the RNA levels. With careful primer design, a quantitative reverse-transcription-PCR based-platform can be established to quantify expressions of *Amt1* genes in the ocean. One potential problem will be multiple homologs with different functions in a species [2, 10, 32]. A more-detailed study of expression levels and regulatory factors for individual homologs will enhance our ability to interpret data and quantify target messenger RNAs.

ACKNOWLEDGMENTS

We thank Dr. Sheng-Ping L. Hwang for providing technical suggestions and constructive discussions. This study was supported by grants (NSC 102-2611-M-019-006, NSC 102-2611-M-019-021-MY2, and NSC 102-2313-B-019-018-MY2) from the National Science Council (R.O.C.). Additional support was also received from the Center of Excellence for the Oceans, National Taiwan Ocean University. L.-K. Kang was supported by an NSC postdoctoral fellowship (NSC101-2811-M-019-005).

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