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Evolutionary Bioinformatics

Computational Identification of MicroRNAs from the Expressed Sequence Tags of Toxic Dinoflagellate *Alexandrium Tamarense*

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ABSTRACT: Micro ribonucleic acids (miRNAs) represent a class of small noncoding RNAs that play important roles in multiple biological processes by degrading targeted mRNAs or by repressing mRNA translation. In the case of algal lineages, especially dinoflagellates, knowledge regarding the miRNA system is still limited and its regulatory role remains unclear. In the present study, a computational approach was employed to screen miRNAs from the expressed sequence tags (ESTs) of *Alexandrium tamarense*. A total of 18 potential miRNAs were identified according to a range of filtering criteria. In addition, unique evolutionary features, such as miRNA gene duplication and sequence similarity to metazoan miRNAs, implied that the miRNA system in dinoflagellates is complex. Moreover, based on these 18 miRNA sequences, 42 potential target genes showing diverse functions in regulating growth and development were predicted in *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*. Taken together, our data suggest the existence of miRNAs in dinoflagellates and provide clues for further functional studies on these predicted miRNAs.

KEYWORDS: microRNA, computational identification, EST, dinoflagellate

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Introduction

Micro ribonucleic acids (miRNAs) are small (≈19–23 nt) noncoding RNAs that are abundant in most plant and animal species.[1] miRNAs have been shown to post-transcriptionally regulate the genes affecting many different biological processes.[2–5] miRNAs are normally identified through direct sequencing of small RNA libraries with high-throughput sequencing methods. Moreover, many miRNAs from a broad range of species have been predicted by fast-developing computational approaches.[6–8] Because most of the miRNA-predicting algorithms are mainly based on whole genome sequences, the prediction of miRNAs is limited to model species, such as wormwood and some bilaterian animals.[9–11] Expressed sequence tags (ESTs) can be another resource for identifying miRNA precursors, owing to the fact that pre-miRNAs (precursors of miRNAs) always share a similar evolutionary conserved secondary hairpin structure. Several efficient pipelines have been developed to identify miRNAs in numerous species without available genome sequences, including porcine, peach, horsegram, and certain species of insects.[9,12–15] Therefore, the computational approach using EST data has already become a widely-used method for miRNA prediction. Based on the survey of the miRNEST database,[16] 9,980 miRNA candidates were predicted from ESTs of 420 species.[17]

Recently, several studies have focused on miRNAs in protists taxa; their results indicated that miRNA machinery originated from these early-diverging eukaryotes.[18,19] miRNAs have also been identified in some species of protists alga; for

instance, 13 miRNAs have been identified from the diatom *Phaeodactylum tricornutum*,[20,21] 26 miRNAs from the brown alga *Ectocarpus siliculosus*,[22] and 50 miRNAs from the unicellular green alga *Chlamydomonas reinhardtii*.[23] These studies have provided evidence for the existence of miRNAs in algae, and they have also suggested the independent evolution of miRNA systems.[20,24]

Dinoflagellates represent a large and diverse eukaryotic flagellated microalgae group that constitutes important primary producers in marine and freshwater ecosystems.[25] Some dinoflagellate species, such as *Alexandrium* spp., are best known as the source of harmful algal blooms (HABs), or "red tides," with concentrations of up to several million cells per liter of seawater.[26,27] Algal blooms caused by dinoflagellates have a significant impact on the environment through toxin bioaccumulation in the food chain, and they affect coastal ecosystems worldwide.[27,28] Previous studies have shown that dinoflagellates have a nuclear structure that is unique among eukaryotes. Because they have a high content of deoxyribonucleic acid (DNA) (3–250 pg of DNA organized into hundreds of chromosomes), and because they lack histones, it is likely that novel methods of DNA compaction and gene expression control have evolved in these species.[25,27,28] Therefore, gene expression regulators (for example, miRNAs) may exhibit unique features in terms of horizontal gene transfer, gene expression, and gene integration.

Alexandrium tamarense, one of the best-studied dinoflagellates, can form toxic blooms and cause paralytic shellfish poisoning through the production of saxitoxin.[26,29,30] The increasing number of blooms of *Alexandrium tamarense* and other *Alexandrium* species worldwide make it a very important genus for fisheries globally. In the present study, an in silico approach was employed to predict candidate miRNAs from the ESTs of *Alexandrium tamarense*. The aim of this study is to confirm the existence of the miRNA system, determine the features of these potential miRNAs, and further understand their possible functions by predicting the target genes.

Materials and Methods

Dataset of miRNAs and *Alexandrium tamarense* ESTs. All 21,643 previously known mature miRNAs were obtained from the miRNA Registry Database (release 18.0, November 2011).[31,32] These miRNAs were defined as the reference miRNA dataset. A total of 10,885 *Alexandrium tamarense* ESTs were downloaded from the National Center for Biological Information dbEST database in March 2012 (see Supplementary File 3 for the statistical characteristics of these ESTs).

Prediction of putative miRNAs and their precursors. The procedure used to search for putative miRNAs in the present study was modified from previously reported EST-based approaches,[9,15] which is summarized in Figure 1. First, the PatScan software (Argonne National Laboratory, Argonne, IL, USA) was used to identify the matched patterns between all known mature miRNAs and the ESTs of *Alexandrium tamarense*[33] with permissibility of two mismatches – one deletion and one insertion. Second, for each pattern hit, 300 nt of both 5′ and 3′ flanking sequences were extracted from corresponding ESTs as candidate sequences. Third, secondary structures were generated with the MFOLD 3.5 software,[34,35] and miRcheck (Bartel Laboratory, Whitehead Institute for Medical Research, Cambridge, MA, USA) was used to judge the hairpin structures.[6] Finally, these raw hairpin candidates were queried in the nonredundant protein sequences database by using BLASTX (National Library of Medicine, Bethesda, MD, USA) with a cutoff E-value of 10^{-6}. Protein coding sequences were removed, and noncoding sequences were kept for further evaluation.

Three parameters, minimum fold energies (MFEs), minimal free energy indices (MFEIs), and G+C content were employed to distinguish miRNAs from other types of coding and noncoding RNAs. MFEs of all candidate miRNA precursors were generated by the MFOLD 3.5 program, and the MFEIs were calculated by the following equation:

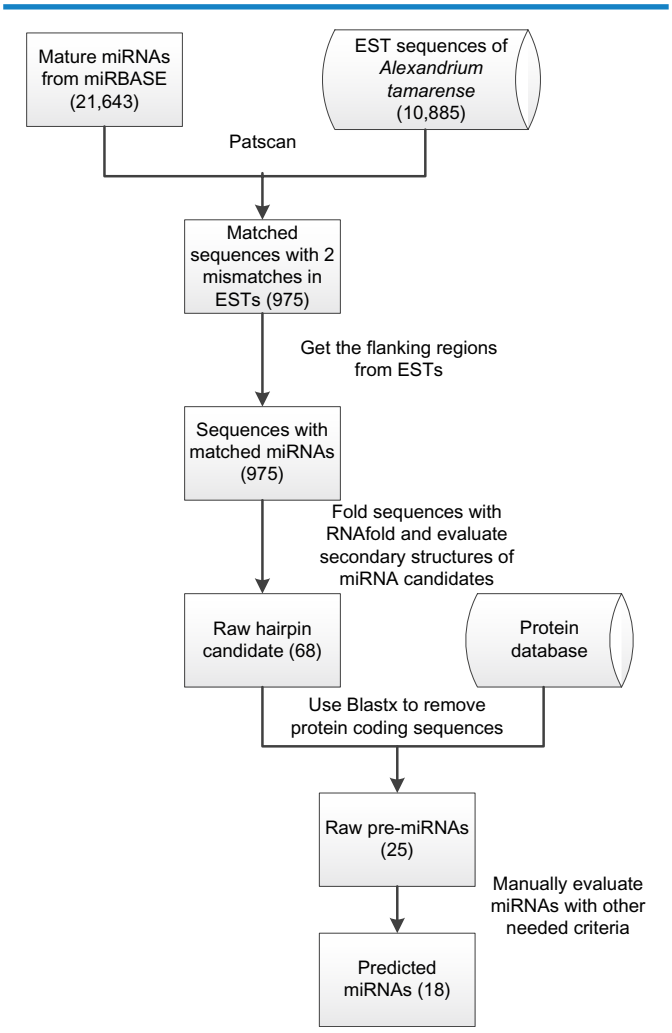

Figure 1. Schematic representation of the search procedures used to predict the miRNA candidates. The numbers in the parentheses indicate the sequence numbers for each step.

Table 1. List of miRNAs predicted from ESTs of *Alexandrium tamarense*.

MIRNA NAME	EST ID	PREDICTED MIRNA(5'-3')	LENGTH	SIMILAR MIRNAS
ata-miR-546	CK784287.1	AUGGCGCACGGUGUCGGGG	19	mmu-miR-546
ata-miR-1275	CK783557.1	AGCGGGGGGGGGAGGCGUCG	20	hsa-miR-1275
ata-miR-3178	CF947709.1	GGGCGCGGCGGCGGUCGUCC	20	hsa-miR-3178
ata-miR-3181	CF948147.1	UCGCGGACCGCGGCGCCGGCAG	22	hsa-miR-3181
ata-miR-3196	CK785050.1	GGGGCUGGCGGGGGCCGCUGU	21	hsa-miR-3196
ata-miR-3665	CV553918.1	AGGAGGAGGGGGCGGCAGCAG	21	hsa-miR-3665
ata-miR-4266a	CV554227.1	CAGGAGGCCAGGGCCCCGC	19	hsa-miR-4266
ata-miR-4266b	CV554442.1	GCGCUGCGAGGCGAUGGCC	19	hsa-miR-4266
ata-miR-4267	CK783603.1	UGCAGCUGCGCGGCACGGA	19	hsa-miR-4267
ata-miR-4486	CF947090.1	AGGGCUGGGCAGCGCCGGGA	20	hsa-miR-4486
ata-miR-4488	CK785476.1	AGGCGCCGGGGCCCGGCGCGC	21	hsa-miR-4486
ata-miR-4492a	CK783952.1	CCUUGGGCUGGGUGCGGCCG	20	hsa-miR-4492
ata-miR-4492b	CK783763.1	CCUUGGGCUGGGUGCGGCCG	20	hsa-miR-4492
ata-miR-4492c	CF948508.1	CCUUGGGCUGGGUGCGGCCG	20	hsa-miR-4492
ata-miR-4492d	CF947702.1	GGGGCUCGGGCCGCGCCUGC	20	hsa-miR-4492
ata-miR-4492e	CF947520.1	CCCUGGCUGCGGCCCGCGCC	20	hsa-miR-4492
ata-miR-4508a	CK785922.1	GCGGGGCUCCCCGCGCGGGG	20	hsa-miR-4508
ata-miR-4508b	CF947749.1	CCAGCCGGGCGCGCCGCGCG	20	hsa-miR-4508

$$\mathrm{MFEI} = 100 \times \frac{\mathrm{MFE}}{\mathrm{Length}} \mathrm{of} \frac{\mathrm{RNA}}{\mathrm{G+C}} \% \quad (1)$$

Candidate miRNAs were considered as potential miRNA only if they fit the following criteria: 1) an RNA sequence could fold into an appropriate stem–loop hairpin secondary structure; 2) a mature miRNA sequence site was present in one arm of the hairpin structure; 3) a mature miRNA sequence had less than six mismatches with the opposite miRNA* sequence (miRNA* refers to the small RNA processed from the hairpin arm opposite the mature miRNA) in the other arm; 4) no loops or breaks were found in miRNA* sequences; and 5) the predicted secondary structures had higher MFEIs and negative MFEs.

Target prediction for identified miRNAs. psRNA Target[36] was used to screen potential target genes to predict the possible function of identified potential miRNAs. The transcriptomes from two sequenced diatom species, *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, were used as libraries for the target search. Parameters were set as follows: maximum expectation = 3; target accessibility = 20. Subsequently, miRNA-target duplexes were checked manually.

Results and Discussion

Identification of putative miRNAs from *Alexandrium tamarense*. In this study, a strategy using a combination of software and bioinformatics methods based on homology searching and secondary structure evaluation was employed to screen for potential miRNAs from *Alexandrium tamarense*. A total of 18 miRNAs were identified from 10,885 *Alexandrium tamarense* ESTs (Table 1). These predicted miRNAs ranged in size from 19 nt to 21 nt, which is consistent with that of most metazoan miRNAs. As the sequences surrounding miRNAs can form hairpin structures that distinguish them from other RNAs,[37] the secondary structures of all the putative pre-miRNAs were used for miRNA evaluation (Supplementary File 1). The location of hairpin structures in the precursor sequences of all predicted miRNAs varied greatly, with more than half of the hairpin structures located at the 5′ end of their precursor sequences, and the remainder were located at the 3′ end (Supplementary File 1).

Presently, a total of 99 miRNAs have been identified from several algal lineages, including diatoms, brown algae, red algae, and green algae.[20–23,38] The components of RNAi machinery have also been identified in some algal species,[24] suggesting the existence of an miRNA system in algae. However, the miRNA system does appear to be present in all algal groups. For example, homologs of all the key miRNA biogenesis components in the genome of *Aureococcus anophagefferens* (pelagophyte) are lacking,[24] indicating that the miRNA system may have been lost independently in certain species or lineages. Information regarding the miRNA machinery in dinoflagellates is still limited because they lack a fully sequenced genome. In the present study, miRNAs and their precursors were predicted directly from EST sequences, with results providing evidence for the presence of miRNA systems in dinoflagellates. Considering the large genome size

of *Alexandrium tamarense*, it can be assumed that the number of miRNAs may be underestimated in this species. To decisively identify the complete miRNA systems in dinoflagellates, more robust approaches (such as high-throughput small RNA sequencing) are required in order to identify potential additional novel miRNAs.

Characteristics of the identified putative miRNAs. The 18 putative miRNAs were classified into 12 families on the basis of sequence similarities. There were five members in the miR-4492 family, two members in the miR-4508 and miR-4266 families, and only one member in the nine other families (Table 1). It has been reported that dinoflagellates experienced tertiary endosymbiosis events during their evolution, as their nuclear genomes are the largest among those of eukaryotes and their genes are always arranged in tandem arrays.[25] Gene duplication is considered to be a major source of emergence of novel miRNA genes.[39,40] Considering the complexity of the evolutionary history of dinoflagellates, it can be deduced that their multimember miRNA families could have arisen through gene duplication events.

The sequences of miRNAs predicted in *Alexandrium tamarense* were similar to those of certain human or mouse miRNAs, although the seed miRNAs used for homologous searches were taken from both plant and animal species (Table 1). This finding can be explained by observations from previous studies that have found that most diatom miRNAs share similarities with those of animals.[21] Because diatoms have a mosaic genome that contain genes from animals, plants, and bacteria, it is speculated that these animal-like miRNAs may have been introduced in diatoms via gene transformation and endosymbiosis during evolution.[41] Like the genome of diatoms, the genome of dinoflagellates has also been dramatically influenced by small- and large-scale lateral transfer through serial endosymbiosis.[25] Thus, we deduced that the 18 putative miRNAs of *Alexandrium tamarense* may have originated through lateral or horizontal gene transfer during the early stages of its evolution.

The length of *Alexandrium tamarense* pre-miRNA varied from 57 nt to 200 nt, which was consistent with that of many eukaryote organisms. However, the A+U content of predicted miRNA precursors ranged from 25% to 32%, which was lower than that of most known miRNA precursors (Table 2). Given that the ESTs of *Alexandrium tamarense* showed high GC-content,[29,42] the present result also suggests a high GC-content in miRNA genes. MFEs and MFEIs are two important criteria for distinguishing miRNAs from other types of coding and noncoding RNAs. In previous reports, the sequences of miRNA precursors exhibited significantly higher MFEs and MFEIs than those of other noncoding RNAs or mRNAs.[8–10] The potential miRNAs identified in *Alexandrium tamarense* also showed both higher MFEs (10.59–64.00) and MFEIs (0.23–0.37) (Table 2), which is in agreement with the previous in silico miRNA prediction results.[7,12,15]

Table 2. Characteristics of miRNA precursors of *Alexandrium tamarense*.

PRE-MIRNA NAME	LENGTH	A + U(%)	PN[A]	MFES[B]	MFEIS[C]
pre-pre-ata-miR-546	66	32	5′	12.33	0.27
pre-pre-ata-miR-1275	79	28	3′	20.86	0.37
pre-pre-ata-miR-3178	57	28	5′	10.59	0.26
pre-pre-ata-miR-3181	59	27	5′	11.24	0.26
pre-pre-ata-miR-3196	83	29	5′	24.54	0.42
pre-pre-ata-miR-3665	89	28	5′	22.40	0.35
pre-pre-ata-miR-4266a	200	26	5′	48.56	0.33
pre-pre-ata-miR-4266b	105	30	3′	20.59	0.28
pre-pre-ata-miR-4267	125	32	5′	23.04	0.27
pre-pre-ata-miR-4486	211	31	3′	49.88	0.34
pre-pre-ata-miR-4488	206	25	5′	64.00	0.41
pre-pre-ata-miR-4492a	109	29	3′	17.96	0.23
pre-pre-ata-miR-4492b	109	29	3′	17.96	0.23
pre-pre-ata-miR-4492c	109	28	3′	17.96	0.23
pre-pre-ata-miR-4492d	155	28	5′	27.16	0.24
pre-pre-ata-miR-4492e	175	27	3′	39.29	0.31
pre-pre-ata-miR-4508a	165	25	5′	40.14	0.32
pre-pre-ata-miR-4508b	178	25	3′	44.76	0.34

Note: [a]Position of miRNA at pre-miRNA, [b]Minimum free energy, [c]Minimum free energy indexes

Notably, none of the predicted miRNAs from *Alexandrium tamarense* showed sequence or structural similarities with the other 99 known miRNAs from different algal species.[20–23,38] Interestingly, after searching the current miRNA database, the twelve human and mouse miRNAs corresponding to miRNAs identified in *Alexandrium tamarense* did not have homologs in most other organisms either (data not shown). Therefore, these 18 miRNAs may be ancient, as they were not identified in other lineages; it is possible that these miRNAs could have been generated via horizontal gene transfer at the early stages of evolution. However, there are contradictory opinions regarding the evolutionary origins of protist miRNAs. The discovery of animal and plant miRNAs in some protists suggests that they have been inherited from the last common ancestor of eukaryotes.[43] However, a recent report suggested that miRNAs may have evolved independently within eukaryotes through exaptation of their shared inherited RNA interference machinery.[44] Current findings indicate that the miRNAs in these lower eukaryotes lineages were complex, and more data are urgently required to better understand their evolution.

Targets of the identified miRNAs and their putative roles. The functional importance of an miRNA is ultimately defined by its target genes and the regulation it exerts on the expression of these genes. In order to determine the probable roles of the putative miRNAs in the present study, target genes were predicted with the psRNATarget software

Table 3. Predicted targets for newly identified miRNAs in *Alexandrium tamarense*.

TARGETED SPECIES	MIRNA NAME	TARGET TRANSCRIPT NO.	PREDICTED FUNCTION
Thalassiosira pseudonana	ata-miR-1275	24762	
		1878	
		9892	Zn-finger protein
	ata-miR-3196	10738	
	ata-miR-3665	24125	
		12022	
		2919	
		41694	
		8106	
		8429	
		21043	Crystallin
		10608	
		24499	
		2435	
	ata-miR-4492a	36716	Pyridine nucleotide-disulphide oxidoreductase
	ata-miR-4492b	36716	Pyridine nucleotide-disulphide oxidoreductase
	ata-miR-4492c	36716	Pyridine nucleotide-disulphide oxidoreductase
	ata-miR-4492d	25476	Ovarian tumour, otubain
Phaeodactylum tricornutum	ata-miR-1275	46173	Basic-leucine zipper (bZIP) transcription factor
	ata-miR-3178	21538	Ribonucleoprotein complex
		26714	Naringenin-chalcone synthase
	ata-miR-3181	15125	
	ata-miR-3665	34146	Ribosomal protein
		37299	
		46530	
		48105	
		51283	Pistil-specific extensin-like protein
	ata-miR-4488	49168	Acterial extracellular solute-binding protein
	ata-miR-4492a	38548	
		46052	
		48581	
		50367	
	ata-miR-4492b	38548	
		46052	
		48581	
		50367	
	ata-miR-4492c	38548	
		46052	
		48581	
		50367	
	ata-miR-4492d	1637	
	ata-miR-4508a	33257	

(Table 3). As the genome information of dinoflagellates is still unavailable, the transcriptomic information from two diatom species (*T. pseudonana* and *P. tricornutum*) was chosen as alternative target libraries. Eighteen genes were targeted by seven miRNAs in *T. pseudonana* and 24 target genes of ten miRNAs were observed in *P. tricornutum* (Table 3). Notably, some miRNAs targeted more than one transcript. For example, ata-miR-3665 had ten target genes in *T. pseudonana* and five in *P. tricornutum*. The possible functions of these targets were further analyzed on the basis of their annotation information. The genomes of *T. pseudonana* and *P. tricornutum* have not been fully annotated, and the functions of any protein-coding genes are still unknown. Therefore, only the function of twelve genes was reliably described (Table 3). For miRNA regulation, translational repression and mRNA cleavage are major silencing mechanisms. Based on the predictions, the silencing mechanism was 'mRNA cleavage' in nearly three-fourths of the cases (Supplementary File 2).

It has been reported that most of the miRNA target genes encoding transcription factors, signal transduction factors, and metabolic transporters are involved in the regulation of growth and development.[15,45] Similar functional biases were also observed in this study. For example, ata-miR-1275 targeted a zinc finger gene in *T. pseudonana* and basic-leucine zipper (bZIP) genes in *P. tricornutum* (Table 3). Since zinc finger and bZIP genes encode important transcription factors involved in numerous cellular processes, it is inferred that ata-miR-1275 might be involved in cellular regulations in *Alexandrium tamarense*. Some miRNAs were targeted to housekeeping genes, such as crystalline (target of ata-miR-3665 in *T. pseudonana*), ribonucleoprotein complex (target of ata-miR-3178 in *P. tricornutum*), and ribosomal protein (target of ata-miR-3665 in *P. tricornutum*). Moreover, there were other targeted genes with more diverse functions (for example, pyridine nucleotide–disulfide oxidoreductase—a target of ata-miR-4492 in *T. pseudonana*—and naringenin–chalcone synthase—a target of ata-miR-3178 in *P. tricornutum*). *Alexandrium tamarense* is a unique protist that causes HABs and paralytic shellfish poisoning. The impact of HABs on marine ecosystems and on the seafood industry is substantial. Therefore, understanding the functions of miRNAs and their targets will help to decipher the basic biology and toxin production mechanism for this species.

Conclusion

Our preliminary results based on in silico analysis not only demonstrate the existence of an miRNA system in dinoflagellates, but they also provide clues for understanding the function and evolution of these miRNAs in algal lineages.

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Author Contributions

Conceived and designed the experiments: DG, LQ, LS. Analyzed the data: DG, LQ, ZH, QZ, JW. Wrote the first draft of the manuscript: DG, QG. Contributed to the writing of the manuscript: DG, LQ, QG, LS. Agree with manuscript results and conclusions: DG, LQ, ZH, QZ, JW, QG, LS. Jointly developed the structure and arguments for the paper: DG, LQ, ZH, QZ, JW, QG, LS. Made critical revisions and approved final version: DG, LQ, ZH, QZ, JW, QG, LS. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

Supplementary Materials

Supplementary File 1. Stem–loop structures for pre-miRNAs.

Supplementary File 2. Detailed information of all the predicted miRNAs. Two sheets are in the EXCEL file containing the information of predicted miRNAs and the predicted targets, respectively. Sheet 1 lists all the information of the putative miRNAs including their length, accession number, mature position, hairpin position, orientation, hairpin sequence, and structures. Sheet 2 lists all the miRNA-targeted gene information generated by psRNATarget, including their target, expectation, UPE, target position, alignment, and inhibition style.

Supplementary File 3. Statistical characteristics of *Alexandrium tamarense* ESTs.

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