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Origin of cryptophyte plastids in *Dinophysis* from Galician waters: results from field and culture experiments

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ABSTRACT: Photosynthetic species of the dinoflagellate genus *Dinophysis* retain cryptophyte plastids from the *Teleaulax/Plagioselmis/Geminigera* group via their ciliate prey *Mesodinium rubrum*, but other cryptophyte and algal sources have occasionally been found. Identifying the specific prey of ciliates fed upon by mixotrophic *Dinophysis* species is a requisite to improve predictive capabilities of their bloom formation. Here we examined the origin of *Dinophysis* plastids from Galician waters and their transfer in cross-feeding experiments in the laboratory. Plastid 23S rDNA sequences were obtained from 60 *Dinophysis* specimens from the Galician Rías Baixas and shelf waters. Most sequences in *Dinophysis* cells were identical to *Teleaulax amphioxeia*. Galician shelf samples also yielded *T. amphioxeia*-type sequences, although one of these was closer to a freshwater cryptophyte, and a few others were related with other taxa (diatoms, red algae and proteobacteria). *Mesodinium* cf. *major,* an alternative prey to *M. rubrum*, was identified. Cross-feeding tests in the laboratory showed that *T. amphioxeia*, *T. minuta*, *T. gracilis,* and *Plagioselmis prolonga* sustained growth of *M. rubrum*. *D. acuminata* cultivated on a *M. rubrum*–*T. amphioxeia* system was transferred to *M. rubrum* fed upon *T. minuta*, *T. gracilis* and *P. prolonga*. After >2 mo of acclimation, *T. amphioxeia* plastid 23S rDNA and *psbA* gene sequences from *D. acuminata* were replaced by those of secondary cryptophytes. Here we confirm 2 cryptophytes, *T. minuta* and *P. prolonga*, as suitable prey for *M. rubrum*. Nevertheless, field and laboratory results show that, at least for *D. acuminata*, *T. amphioxeia* represents the main source of plastids.

KEY WORDS: *Dinophysis* · Kleptoplastids · Cryptophytes · *Mesodinium* · 23S rDNA · *psbA* gene

INTRODUCTION

Dinoflagellate species of the genus *Dinophysis* produce diarrheic shellfish poisoning (DSP) toxins (okadaic acid, dinophysistoxins) and pectenotoxins and have deleterious effects on shellfish industries worldwide (Reguera et al. 2014).

Following the pioneer work of Park et al. (2006) with *Dinophysis acuminata,* cultures of mixotrophic species of *Dinophysis* have been established up to now using the ciliate *Mesodinium rubrum* fed with cryptophytes, mainly from the genus *Teleaulax* (Park et al. 2006, Nishitani et al. 2008, Garcia-Cuetos et al. 2010, Kim et al. 2012b, Raho et al. 2014) and to a lesser extent with *Geminigera cryophila* (Park et al. 2008, Hackett et al. 2009, Garcia-Cuetos et al. 2010, Nishitani et al. 2010, Fux et al. 2011). Most mixotrophic *Dinophysis* species have been demonstrated to contain plastids of cryptophyte origin (Takishita et al. 2002, Hackett et al. 2003, Janson & Graneli 2003,

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Janson 2004, Nagai et al. 2008, Park et al. 2008, Garcia-Cuetos et al. 2010, Nishitani et al. 2010), except for warm water species, such as *D. mitra* and *D. miles,* which were found to contain plastids from haptophytes and cyanobacteria, the latter presumed to be ectosymbionts (Koike et al. 2005, Qiu et al. 2011).

Observations from laboratory experiments with *D. caudata* (Park et al. 2010) and from *Dinophysis* spp. cells isolated from field populations (Nishitani et al. 2010) revealed the ability of *Dinophysis* species to simultaneously maintain plastids from different cryptophyte species. A more recent survey in South Korean waters showed that *Dinophysis* spp. contained plastids from multiple algal groups, including raphidophytes and prasinophytes (Kim et al. 2012a).

The active uptake of plastids from cryptophytes and the fate of plastids from different sources in *Dinophysis* have been described using feeding/starvation and cross-feeding experiments (Park et al. 2010, Minnhagen et al. 2011, Raho et al. 2014). These studies revealed that *Dinophysis* is unable to replicate its 'second hand' plastids and they become progressively diluted in the growing population (Minnhagen et al. 2011). Nevertheless, *Dinophysis* cultures can survive up to 3 mo in the absence of prey (Park et al. 2008).

In addition, *Dinophysis* may retain plastids from several cryptophyte prey simultaneously, and cross-feeding experiments showed different turnover times for different kinds of plastids (Park et al. 2010). Experiments of this kind with *D. caudata* cultures showed that this species maintained the original *Teleaulax amphioxeia* plastids, but was able to incorporate new plastids from *T. acuta* that were lost earlier during starvation (Park et al. 2010). The replacement of *T. amphioxeia* plastids by those of *T. gracilis* in *D. acuta* cultures has been recently reported (Raho et al. 2014), suggesting that the original kleptoplastids can be totally lost.

The existence of permanent plastids in *Dinophysis* now appears unlikely, and accumulated evidence points to the periodical acquisition of new organelles by *Dinophysis* as a requisite to maintain its phototrophic capacity. Kleptoplastids are ultrastructurally different in *Dinophysis* and *Mesodinium* (Garcia-Cuetos et al. 2010), but this discrepancy has been explained by the gradual modification of these plastids after being ingested by *Dinophysis* cells (Kim et al. 2012b).

Different cryptophytes have been tested for their suitability as food for *Mesodinium* (Park et al. 2007, Myung et al. 2011, Hansen et al. 2012, Raho et al. 2014). Park et al. (2007) found that the best results with Korean strains were obtained when using species belonging to the PTG (*Plagioselmis*/*Teleaulax*/*Geminigera*) clade. Likewise, Hansen et al. (2012) found that cultures of Danish *Mesodinium rubrum* strains thrived just with cryptophytes of the genus *Teleaulax* (*T. acuta* and *T. amphioxeia*). Furthermore, *psbA* sequences from *Hemiselmis* sp. and *Falcomonas* sp. plastids could not be retrieved in cross-feeding experiments with Spanish strains of *M. rubrum* (Raho et al. 2014).

Identifying the specific prey of ciliates fed upon by mixotrophic *Dinophysis* species is a requisite to improve predictive capabilities of their bloom formation and to obtain optimal growth rates in laboratory cultures. With this aim we explored the plastidic diversity in field populations of *Dinophysis* from Galician waters and plastids transference from different cryptophytes to *D. acuminata* in culture by means of partial sequencing of plastidic 23S rDNA and *psb*A genes. In our laboratory experiments, 4 different cryptophytes species (*T. minuta* CR8EHU, *T. amphioxeia* CR2EHU, *T. gracilis* CR6EHU and *Plagioselmis prolonga* CR10EHU) sustained positive growth of *Mesodinium* (AND-0711), and their plastids were sequestered by *D. acuminata* cells as demonstrated by their 23S rDNA and *psb*A sequences. Results from 23S rDNA sequences from field specimens of *Dinophysis* showed that most sequences belonged to cryptophytes within the *Teleaulax*/*Plagioselmis* genera. Almost all of them were the *T. amphioxeia*/*P. prolonga* type, confirming that these species are the main source of kleptoplastids for *Dinophysis* via its ciliate prey, while a few of them were closely related to *T. minuta*. The amplification of several 23S rDNA sequences in *Dinophysis* specimens from the Galician shelf belonging to other taxa different to cryptophytes is also discussed.

MATERIALS AND METHODS

Field sampling

Dinophysis cells were isolated from surface samples collected during weekly cruises (March 2010 to May 2012) from the Galician Monitoring Programme on board RV 'J. M. Navaz', at Stns P2 (42° 21.40' N, 8° 46.42' W), and PA (42° 23.14' N, 8° 55.36' W) from Ría de Pontevedra and Stns E15 (42° 13.3' N, 8° 47.7' W) and D13A (42° 8.5' N, 8° 57.5' W) from Ría de Vigo (Galician Rías Baixas, NW Spain). Additionally, during the DINVER (*DINophysis* INViERno = winter *Dinophysis*) cruise on board RV 'Ramón Margalef' in

February 2013, samples were obtained at 20 m depth from the shelf adjacent to the rías (Stn ST8; 41° 52.89′ N, 9° 12.22′ W) and from Ría de Pontevedra (Stn ST28; 42° 22.24′ N, 8° 45.66′ W) (Fig. 1). Taxonomic identification of the isolated *Dinophysis* cells (alive or from Lugol's fixed samples in the case of DINVER cruise) was done under a light microscope prior to further molecular analyses (Table 1).

Culture experiments

The following *Dinophysis* strains were isolated from the Galician Rías: *D. acuminata* (VGO1063) from Stn B1, Ría de Vigo (42° 8.22′ N, 8° 51.36′ W); *D. caudata* (VGO1064), from Stn P2 (42° 21.40′ N, 8° 46.42′ W). *D. sacculus* (VGO1132) was isolated from a sample of opportunity collected in the Galician Northern Rías (location not shown). *Mesodinium* cf. *major* was isolated from Stn B1. The ciliate *Mesodinium rubrum* (AND-A0711) and the cryptophyte *Teleaulax amphioxeia* (AND-A0710) were isolated in 2007 from samples collected off Huelva (SW Spain) in the course of weekly samplings of the Andalusian Monitoring Programme. The cryptophyte strains *Falcomonas* sp. (CRY1V) and *Hemiselmis* sp. (CRY6V) were isolated from Stn P2 and all other cryptophytes from the outer Nervión-Ibaizabal river estuary, Bay of Biscay (43° 20′ N, 3° 01-06′ W), in northern Spain, and from Tunisia (*Teleaulax* cf. *merimbula* Cr59EHU) (Table 2).

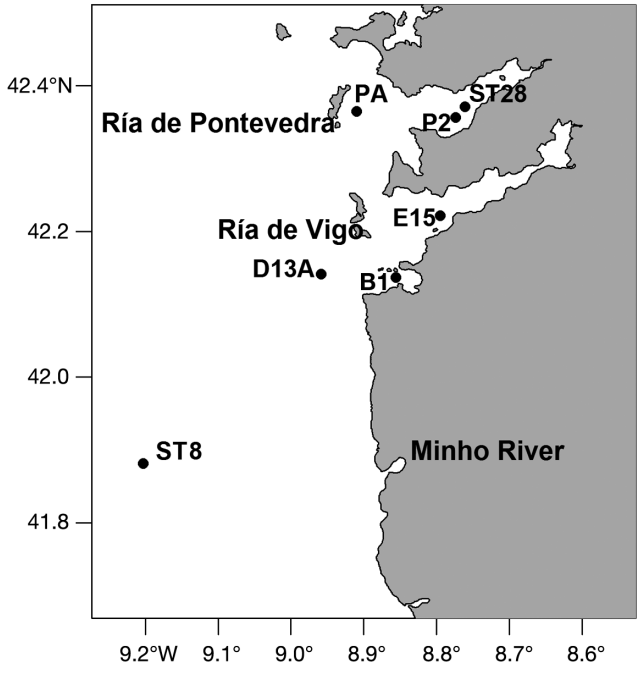

Fig. 1. Location of stations where samples were collected in the Galician Rías, Spain, and from the adjacent oceanic shelf

Feeding experiments

M. rubrum (AND-0711) fed with *T. amphioxeia* was grown with diluted L1-Si medium (1/20) at 15°C, 32 psu, and a 12:12 h light/dark cycle with 150 µmol photons m^2 s^{-1} irradiance. Well-fed *M. rubrum* was starved for 2 mo after confirming the absence of cryptophytes under the light microscope. Ciliate cultures were divided in four 100 ml aliquots and grown with different cryptophyte prey: *T. gracilis*, *T. amphioxeia* again, *T. minuta*, and *P. prolonga*. Five months later *Dinophysis* cells isolated from Ría de Pontevedra, Stn P2, were fed with the different *M. rubrum*/cryptophyte combinations mentioned above. Specific growth rates (µ) were calculated from cell counts as $\mu = (\ln N_2 - \ln N_1 / t_2 - t_1)$, where N_1 and N_2 denote cell numbers (cell m^{-1}) recorded at time t_1 and t_2 (days), respectively. Samples included in the molecular analyses are detailed in Table 2.

DNA extraction, PCR amplification and sequencing

Single *Dinophysis* cells were picked manually with a capillary pipette under a Zeiss Invertoscop D microscope, washed in 3 drops of sterile distilled water, transferred to 200 µl PCR tubes and kept at −20°C for 24 h before direct amplification. Species identification was based on morphological characteristics observed by light microscopy. One ml cultures of the cryptophyte species, *M. rubrum* (AND-0711) and *M.* cf. *major* were centrifuged for 5 min at 12 000 *g* in a benchtop Eppendorf centrifuge, pellets rinsed in milliQ water, centrifuged again and DNA extracted using Chelex® 100 (Bio-Rad) following the extraction procedure of Richlen & Barber (2005).

For amplification of plastid 23S rDNA universal primers p23Sr_f1 (5′-GGA CAG AAA GAC CCT ATG AA-3′) and 23Sr_r1 (5′-TCA GCC TGT TAT CCC TAG AG-3′) (Sherwood & Presting 2007) were used; for amplification of 18S rDNA (only for *M.* cf. *major*), SSUD01 (5′-ACC TGG TTG ATC CGC CAG-3′) and SSUR01 (5′-TGA TCC TTC YGC AGG TTC AC-3′) (Moon-van der Staay et al. 2000); and for amplification of *psbA* plastidial gene, psbAF (5′-ATG ACT GGT ACT TTA GAA AGA GG-3′) and psbAR2 (5′-TCA TGC ATW ACT TCC ATA CCT-3′) (Hackett et al. 2003). The PCR reactions were performed using

Table 1. List of *Dinophysis* cells isolated in field samples and the plastidial 23S rDNA sequences retrieved. Sampling dates are shown as dd.mm.yy. See Fig. 1 for location of sampling stations. GenBank accession numbers

Sample		Closest match		Sample		Closest match	
Date	Stn	Species	Acc. no.	Date	Stn	Species	Acc. no.
31.05.10	P2	*D. acuminata*	(KP142616)	08.08.11	P2	*D. acuminata*	(KP142611)
31.05.10	P2	*D. caudata*	(KP142641)	08.08.11	P2	*D. acuminata*	(KP142612)
28.06.10	P2	*D. caudata*	(KP142637)	22.08.11	P2	*D. acuminata*	(KP142684)
28.06.10	P2	*D. caudata*	(KP142638)	05.09.11	P2	*D. acuminata*	(KP142602)
28.06.10	P2	*D. tripos*	(KP142639)	05.09.11	P2	*D. acuminata*	(KP142603)
28.06.10	P2	*D. tripos*	(KP142640)	05.09.11	P2	*D. caudata*	(KP142604)
11.10.10	P2	*D. caudata*	(KP142635)	05.09.11	P2	*D. acuminata*	(KP142605)
11.10.10	P2	*D. caudata*	(KP142636)	26.09.11	P2	*D. caudata*	(KP142597)
15.02.11	E15	*D. caudata*	(KP142634)	26.09.11	P2	*D. acuminata*	(KP142598)
16.03.11	P2	*D. acuminata*	(KP142630)	26.09.11	P2	*D. acuminata*	(KP142599)
16.03.11	P2	*D. acuminata*	(KP142631)	26.09.11	P2	*D. acuminata*	(KP142600)
16.03.11	P2	*D. acuminata*	(KP142632)	26.09.11	P2	*D. acuminata*	(KP142601)
16.03.11	P2	*D. acuminata*	(KP142633)	26.09.11	P2	*D. acuminata*	(KP142683)
17.03.11	D13A	*D. caudata*	(KP142627)	10.10.11	P2	*D. acuminata*	(KP142589-91)
17.03.11	D13A	*D. caudata*	(KP142628)	10.10.11	P2	*D. caudata*	(KP142592-93)
17.03.11	D13A	*D. caudata*	(KP142629)	10.10.11	P2	*D. acuminata*	(KP142594-96)
21.03.11	P2	*D. acuminata*	(KP142625)	21.11.11	P2	*D. caudata*	(KP142573-75)
21.03.11	P2	*D. caudata*	(KP142626)	21.11.11	P2	*D. caudata*	(KP142576-78)
09.04.11	PA	*D. caudata*	(KP142615)	21.11.11	P2	*D. caudata*	(KP142579-80)
26.05.11	PA	*D. acuminata*	(KP142614)	21.11.11	P2	*D. caudata*	(KP142581-83)
13.06.11	P2	*D. acuminata*	(KP142608)	21.11.11	P2	*D. caudata*	(KP142584-86)
13.06.11	P2	*D. acuminata*	(KP142609)	21.11.11	P2	*D. acuta*	(KP142587)
13.06.11	P2	*D. acuminata*	(KP142613)	21.11.11	P2	*D. acuta*	(KP142588)
11.07.11	P2	*D. caudata*	(KP142617)	28.05.12	P2	*D. acuminata*	(KP142570)
11.07.11	P2	*D. caudata*	(KP142618)	28.05.12	P2	*D. acuminata*	(KP142571)
11.07.11	P2	*D. acuminata*	(KP142619)	28.05.12	P2	*D. acuminata*	(KP142572)
11.07.11	P2	*D. acuminata*	(KP142620)	07.03.13	ST28	*D. acuminata*	(KP721496-99)
11.07.11	P2	*D. acuminata*	(KP142621)	07.03.13	ST28	*D. acuminata*	(KP721500-01)
11.07.11	P2	*D. acuminata*	(KP142622)	07.03.13	ST28	*D. acuminata*	(KP721502-05)
11.07.11	P2	*D. acuminata*	(KP142623)	07.03.13	ST8	*D. acuta*	(KP826902-05)
11.07.11	P2	*D. acuminata*	(KP142624)	07.03.13	ST8	*D. acuta*	(KP721506)
08.08.11	P2	*D. acuminata*	(KP142606)	07.03.13	ST8	*D. acuminata*	(KP826906-08)
08.08.11	P2	*D. acuminata*	(KP142607)	07.03.13	ST8	*D. fortii*	(KP721507-10)
08.08.11	P2	*D. acuminata*	(KP142610)			(KP826909)	

a thermo-cycler (Eppendorf), following the conditions detailed by the authors previously mentioned. PCR reaction mixtures (25 µl) contained 1 to 3 *Dinophysis* cells each, 1 mM $MgCl_2$, 2.5 µl 10× PCR buffer, 125 nM of each primer, 25 nM dNTPs and 0.65 units Taq DNA polymerase (Bioline). The PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized by SyBR safe DNA gel staining (Invitrogen) and UV transillumination.

The amplified products were purified using an ExoSAP-IT (USB Corporation). Positive PCR products were cloned into pGEM-T Easy Vector System (Promega) and transformed to *Escherichia coli* strain DH5α according to the manufacturer's protocol. Positive white clones were selected for colony PCR and amplified using the pGEM-T specific primers T7 (TAA TAC GAC TCA CTA TAG GG) and SP6 (TAT TTA GGT GAC ACT ATA G). Correct length amplicons were selected for sequencing. Finally, the obtained PCR products were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and an Applied Biosystems ABI 310 automated sequencer.

Phylogenetic analysis

The partial sequences of plastid 23SrDNA (378 bp) and *psbA* (896 bp) were aligned using CLUSTAL X (Thompson et al. 1997) in Bioedit (Hall 1999). Phylogenetic analyses of 23S rDNA were performed using a K2+G model (Kimura-2 parameter model, with gamma shape parameter 0.33) and a general time-reversible (GTR) model for *psbA*. Maximum likelihood (ML) phylogenetic analyses were conducted in PhyML 3.0 (Guindon et al. 2010) on the South

Table 2. List of cryptophyte strains and their plastidial 23S rDNA and *psbA* sequences *Dinophysis* species fed with *Mesodinium rubrum* (the latter fed with cryptophyte microalgae). GenBank accession numbers

Species	Acc. no.
Cryptophyte culture samples 23S rDNA	
Teleaulax amphioxeia AND-A0710	(KP142646)
T. amphioxeia CR2EHU	KP142644)
T. minuta CR8EHU	(KP142642)
T. acuta CR3EHU	(KP142645)
T. gracilis CR6EHU	(KP142643)
T. cf. *merimbula* CR59EHU	(KP721511)
Plagioselmis prolonga CR10EHU	(KP142657)
Plagioselmis sp. CR11EHU	(KP142648)
Hemiselmis sp. CCVIEO CRY6V	KP142649)
Falcomonas sp. CCVIEO CRY1V	(KP142650)
Rhodomona lens	(KP142647)
***Dinophysis* sp. (*psbA*)**	
Mesodinium rubrum AND-A071	(KP142651)
Dinophysis acuminata VGO1064 (+ *Teleaulax amphioxeia* AND-A070)	(KP142662-63)
D. acuminata VGO1064 (+ *T. amphioxeia* AND-A070)	(KP142674-82)
D. caudata VGO1063 (+ *T. minuta* CR8EHU)	(KP142653)
D. acuminata VGO1064 (+ *T. minuta* CR8EHU)	(KP142654-56)
D. acuminata VGO1064 (+ *T. gracilis* CR6EHU)	(KP142664-73)
D. acuminata VGO1064 (+ *Plagioselmis prolonga* CR10EHU)	(KP142658-60)
D. sacculus VGO1132 (+ *P. prolonga* CR10EHU)	(KP142566-69)

of France bioinformatics platform (www.atgc-montpellier.fr/phyml). Bootstrap values were estimated from 1000 replicates. The number of sites without polymorphism was 301 (79.4%) in 23S rDNA and 782 (87.4%) in *psbA* analyses. The phylogenetic relationships were also determined using Bayesian phylogenetic inference, and in this case, the substitution models were obtained by sampling across the entire GTR model space. Bayesian trees were performed with MrBayes v3.2.4 (Huelsenbeck & Ronquist 2001). The phylogenetic analyses involved 2 parallel analyses, each with 4 chains. Starting trees for each chain were selected randomly using the default values for the MrBayes program. The number of generations used in these analyses was 1 000 000. Posterior probabilities were calculated from every 200th tree sampled after log-likelihood stabilization ('burn-in' phase). All final split frequencies were <0.02. Overall topologies by ML and Bayesian inference methods were very similar. The phylogenetic tree was represented using the ML method with bootstrap values from ML and posterior probabilities from the Bayesian inference. Phylogenetic trees were represented using FigTree v1.3.1 software.

RESULTS

Mesodinium spp. and *Dinophysis acuminata* feeding experiments

The suitability of 6 cryptophyte species belonging to the genera *Teleaulax* (*T. gracilis, T. amphioxeia, T. minuta*), *Plagioselmis* (*P. prolonga*), *Hemiselmis* and *Falcomonas* as prey for *M. rubrum* (AND-A0711) was tested. The only cryptophytes that sustained positive growth rates for *M. rubrum* were the 3 *Teleaulax* species (maximum specific growth rates of 0.27, 0.33 and 0.24 d^{-1} with *T. gracilis*, *T. minuta* and *T. amphioxeia*, respectively) and *P. prolonga* (0.29 d^{-1}), as shown in Fig. 2. Cultures of *M. rubrum* fed on *T. gracilis, T. amphioxeia, T. minuta* and *P. prolonga* were given as prey to *D. acuminata* (Table 2). After 2 mo acclimation, *D. acuminata* plastid 23S rDNA and *psb*A sequences were obtained to find out if the original *T. amphioxeia*-like plastids had been replaced by the new plastids via *M. rubrum*. As expected, we found that the *psbA* gene sequences from *D. acuminata* fed with *M. rubrum* matched the sequences of the 4 cryptophytes (*T. amphioxeia, T. minuta, T. gracilis* and *P. prolonga*) with high bootstrap support (Fig. 3). The nucleotide differences between cryptophytes representative of each group are specified in Table 3.

Analyses of plastid 23S rDNA from field samples

The plastid 23S rDNA phylogeny shown in Fig. 4 includes all *Dinophysis*, *Mesodinium* and cryptophyte sequences obtained from field samples and culture experiments (see details in 'Materials and methods').

Firstly, 84 sequences (378 bases) of plastid 23S rDNA were retrieved from *Dinophysis* spp. cells from the Galician Rías. Secondly, a group of cryptophyte sequences from GenBank database and those from cryptophytes isolated from the Nervión-Ibaizabal River estuary and from Galician waters were also included, in addition to *M. rubrum* AND-0711 and its prey *T. amphioxeia* AND-0710 (the prey items used for *Dinophysis* cultures in our laboratory). Thirdly, sequence data generated from culture experiments (described in 'Materials and methods') were used for phylogenetic analysis.

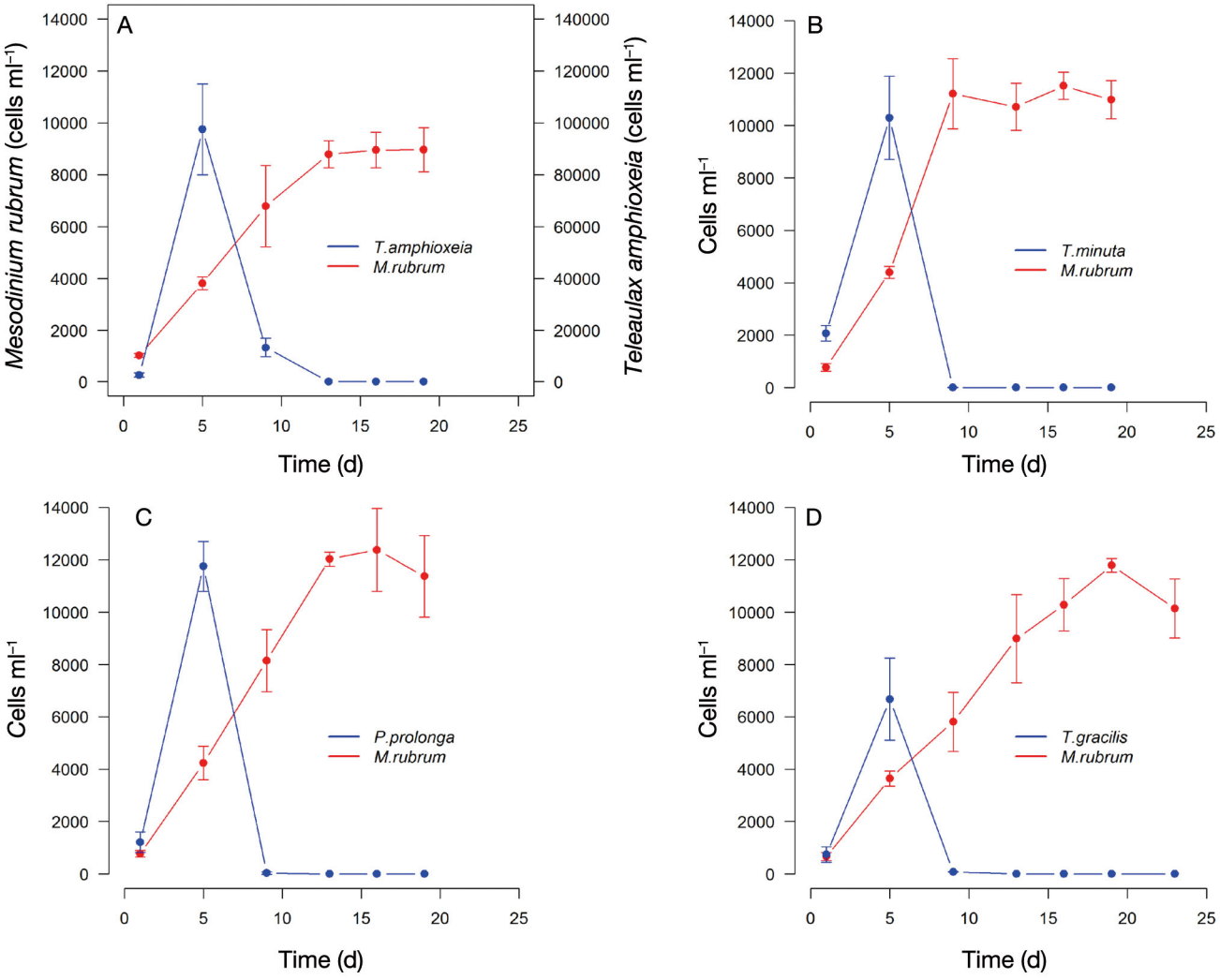

Fig. 2. Growth curves (cells ml^{-1}) of *Mesodinium rubrum* and its prey species in feeding experiments with different cryptophyte preys: (A) *Teleaulax amphioxeia*; (B) *T. minuta*; (C) *Plagioselmis prolonga*; (D) *T. gracilis*. Error bars are ±SD

The molecular phylogeny based on this alignment and inferred from the ML method, yielded the tree topology shown in Fig. 4. The results revealed 4 *Teleaulax* groups. A major *Teleaulax* group included 84 sequences of most *Dinophysis* spp. samples collected in Ría de Vigo and Ría de Pontevedra. Eight of these sequences were identical to *T. amphioxeia* AND-0710 and *P. prolonga* CR10EHU, one differed in 3 nucleotides and 75 differed in the same single nucleotide. We termed this a '*T. amphioxeia* group' (Group 1). A second '*T. minuta* group' (Group 2) included: *T. minuta*, *Dinophysis* cultures grown upon *M. rubrum* fed with *T. minuta*, 3 clones of *D. acuminata* grown with *M. rubrum* fed with *T. amphioxeia* and 3 *D. caudata* from field samples. Unfortunately, the molecular information used to construct the phylogeny did not allow resolving the polytomies observed in the *T. amphioxeia* and *T. minuta* groups.

A third group included *T. gracilis* and *D. acuminata* cultured with *M. rubrum* and *T. gracilis*. Finally, *T. acuta* and *Plagioselmis* sp. formed a fourth group. The nucleotide differences between cryptophytes representative for each of the enumerated *Teleaulax* groups are specified in Table 4.

Some 23S rDNA sequences not corresponding to cryptophytes were found in 3 *Dinophysis* cells (*D. acuta*, *D. acuminata* and *D. fortii*) isolated from a shelf station (Stn ST8) visited during the DINVER cruise. Amplicons from a single *D. acuta* cell yielded 4 different plastid types: (1) 98% match with an uncultured eukaryote (clone CG1, GenBank acc. no.

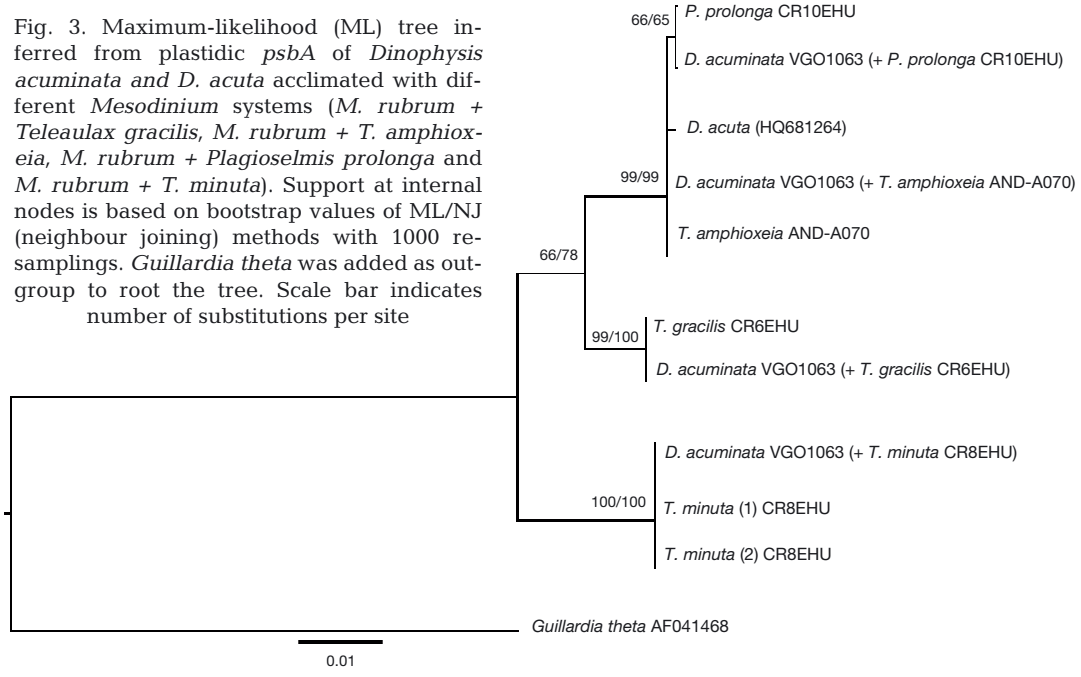

Fig. 3. Maximum-likelihood (ML) tree inferred from plastidic *psbA* of *Dinophysis acuminata and D. acuta* acclimated with different *Mesodinium* systems (*M. rubrum + Teleaulax gracilis, M. rubrum + T. amphioxeia, M. rubrum + Plagioselmis prolonga* and *M. rubrum + T. minuta*). Support at internal nodes is based on bootstrap values of ML/NJ (neighbour joining) methods with 1000 resamplings. *Guillardia theta* was added as outgroup to root the tree. Scale bar indicates number of substitutions per site

Table 3. Number of different bases in the partial *psbA* alignment between cryptophytes species included in the cross-feeding experiments

	Tam	*Pp*	*Tm*	*Tg*
Teleaulax amphioxea (*Tam*)	–	1	29	15
Plagioselmis prolonga (*Pp*)		–	30	16
T. minuta (*Tm*)			–	26
T. gracilis (*Tg*)				–

Table 4. Number of different bases in the partial 23S rDNA alignment between various cryptophytes species representative of the 4 *Teleaulax* groups defined in our study and *Plagioselmis prolonga*

	Tm	*Pp*	*Tm*	*Tg*	*Tac*
T. amphioxea (*Tam*)	–	–	7	9	12
P. prolonga (*Pp*)		–	7	9	12
T. minuta (*Tm*)			–	12	17
T. gracilis (*Tg*)				–	11
T. acuta (*Tac*)					–

GU317429) of diatom origin (Gao et al. 2011), (2) 98% similarity (393/400 bases) with a cryptophyte closely related with *Plagioselmis nannoplanctica* (GenBank acc. no. JX470950) and (3) 82% match with red algal (florideophyceae) sequences. In addition, 2 sequences from bacterial origin (proteobacteria) were found in the aforementioned *D. acuta* and in a *D. fortii* cell. In the latter, 4 sequences of cryptophyte origin (*T. amphioxeia* Group 1) were also found.

In the *D. acuminata* cell from Stn ST8, 3 cloned sequences showed 98% similarity with uncultured eukaryote clones (CH2 and CA12, GenBank acc. nos. GU317440 and GU317379) of diatom origin (Gao et al. 2011). A summary of the plastid types found in the present study is shown in Table 5.

Mesodinium cf. *major*

Mesodinium ciliates with morphological features (larger size, medusa-like forms, bright red coloured cells) matching that of *M. major* and different from *M.* cf. *rubrum* from southern Spain were often found in the rías during our field sampling. We were able to maintain several thousands of cells of *M.* cf. *major* for up to 3 mo in 6-well plates and to amplify their 18S rDNA (GenBank acc. no. KP721512). The results obtained showed a single nucleotide substitution (within an alignment of 1495 base length) in comparison with the equivalent sequence from a Danish strain of *M. major* (GenBank acc. no. JN412737; García-Cuetos et al. (2012) and 3 different bases regarding 2 *M. rubrum* strains from Japan (GenBank acc. nos. AB364286 and EF195734; Park et al. 2007, Nishitani et al. 2008). We confirmed under the light microscope that *D. tripos* ingested *M.* cf. *major.* However, we did not succeed in establishing long-lived cultures of *M.* cf. *major* with any cryptophyte prey tested (*T. amphioxeia*, *T. gracilis*, *T. minuta* or *P. prolonga*).

DISCUSSION

Plastid transfer and acquisition

Available studies on *M. rubrum* showed that this ciliate species only harbours plastids from cryptophytes belonging to the PTG clade and exhibits different growth rates depending on the cryptophyte prey (Park et al. 2007, Myung et al. 2011, Hansen et al. 2012). Although different cryptophyte strains were tested previously, to date only 4 unambiguously identified species, *Teleaulax amphioxeia*, *T. acuta*, *T. gracilis* and *Geminigera cryophila*, have been shown to support *Mesodinium* growth in laboratory experiments (Hackett et al. 2009, Hansen et al. 2012, Raho et al. 2014). Results were negative when using other genera. Hansen et al. (2012) found no evidence of plastid replacement when *M. rubrum* fed on *Hemiselmis tepida* and preyed cells (often partially digested) were located in food vacuoles. In our study, *M. rubrum* strain AND-0711 did not survive after a few weeks when fed with either *Hemiselmis* sp. or *Falcomonas* sp. isolated from the Galician Rías.

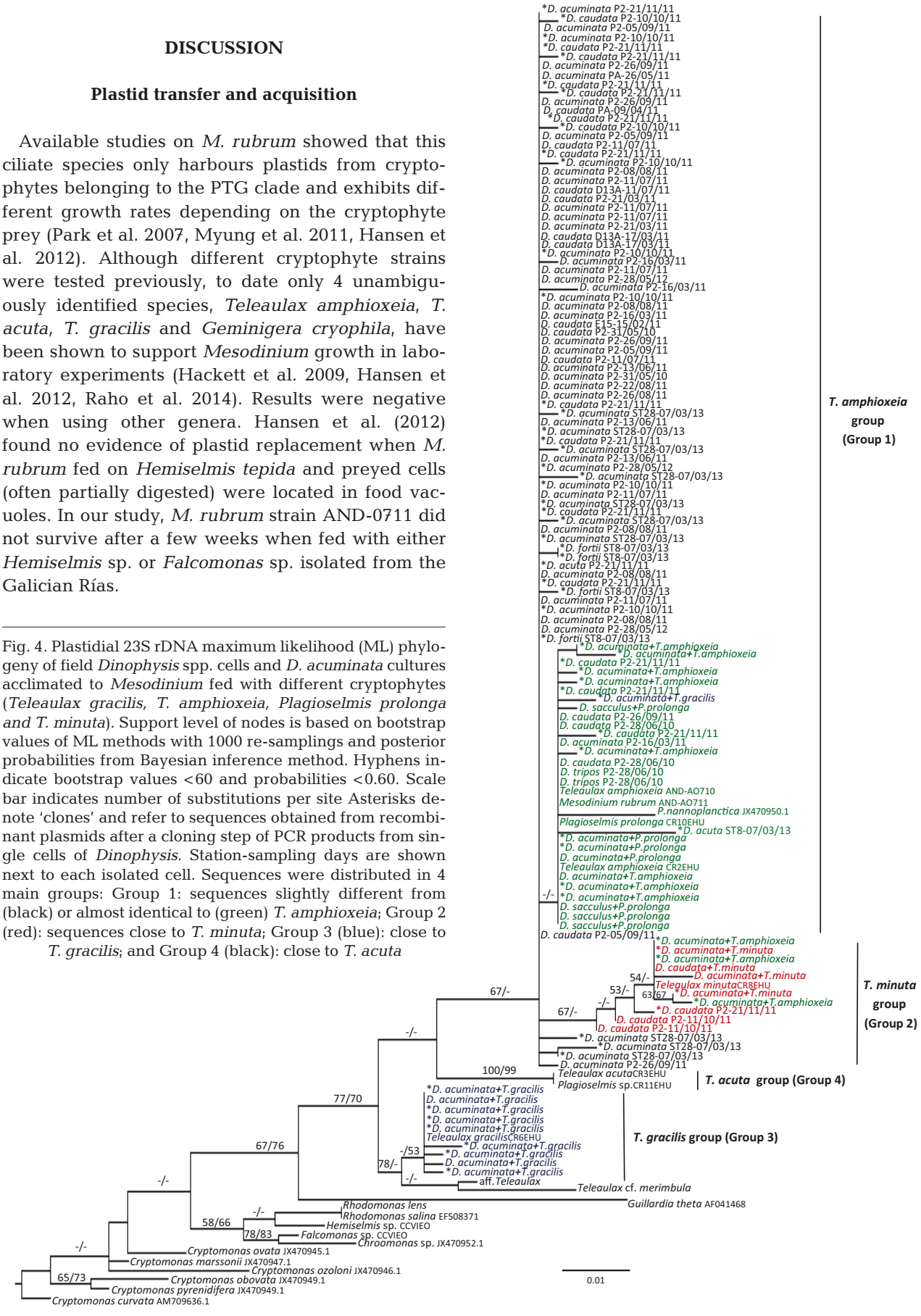

Fig. 4. Plastidial 23S rDNA maximum likelihood (ML) phylogeny of field *Dinophysis* spp. cells and *D. acuminata* cultures acclimated to *Mesodinium* fed with different cryptophytes (*Teleaulax gracilis, T. amphioxeia, Plagioselmis prolonga and T. minuta*). Support level of nodes is based on bootstrap values of ML methods with 1000 re-samplings and posterior probabilities from Bayesian inference method. Hyphens indicate bootstrap values <60 and probabilities <0.60. Scale bar indicates number of substitutions per site Asterisks denote 'clones' and refer to sequences obtained from recombinant plasmids after a cloning step of PCR products from single cells of *Dinophysis*. Station-sampling days are shown next to each isolated cell. Sequences were distributed in 4 main groups: Group 1: sequences slightly different from (black) or almost identical to (green) *T. amphioxeia*; Group 2 (red): sequences close to *T. minuta*; Group 3 (blue): close to *T. gracilis*; and Group 4 (black): close to *T. acuta*

Table 5. List of *Dinophysis* cell clones and plastid types found within each *Dinophysis* clone. Sampling dates shown as dd.mm.yy

Species and source (Stn, date)	No. of clones	Plastid types						
		Teleaulax amphioxeia	*T. minuta*	*T. gracilis*	*Plagioselmis nannoplanctica*	Florideophyceae	Proteobacteria	Diatoms
Field sampling								
D. acuminata (P2, 10.10.11)	6	6	–	–	–	–	–	–
D. caudata (P2, 10.10.11)	2	2	–	–	–	–	–	–
D. caudata (P2, 21.11.11)	15	14	1	–	–	–	–	–
D. acuta (P2, 21.11.11)	1	1	–	–	–	–	–	–
D. caudata (P2, 11.10.11)	–[a]	–	1	–	–	–	–	–
D. caudata (P2, 11.10.11)	–[a]	–	1	–	–	–	–	–
D. acuminata (ST28, 07.03.13)	4	4	–	–	–	–	–	–
D. acuminata (ST28, 07.03.13)	2	2	–	–	–	–	–	–
D. acuminata (ST28, 07.03.13)	4	4	–	–	–	–	–	–
D. acuta (ST8, 07.03.13)	4	–	–	–	1	1	1	1
D. acuminata (ST8 , 07.03.13)	3	–	–	–	–	–	–	3
D. fortii (ST8, 07.03.13)	5	4	–	–	–	–	1	–
Feeding experiment								
D. acuminata (+ *T. amphioxeia*)	10	7	3	–	–	–	–	–
D. acuminata (+ *P. prolonga*)	2	2	–	–	–	–	–	–
D. acuminata (+ *T. gracilis*)	8	1	–	7	–	–	–	–
D. acuminata (+ *T. minuta*)	2	–	2	–	–	–	–	–

[a]These sequences arose from direct sequencing, without cloning step

Plastid dynamics within *Dinophysis* cells have been studied by several authors using the *psbA* gene that encodes the PSII reaction centre protein D1 (Park et al. 2010, Minnhagen et al. 2011, Raho et al. 2014). *psbA* is considered a highly conserved gene (Morden & Sherwood 2002) and a useful marker to trace the identity of plastids in the cryptophyte-*Mesodinium*-*Dinophysis* food chain. In the present study we found 2 new cryptophyte prey, *T. minuta* and *Plagioselmis prolonga*, suitable for sustained growth of *M. rubrum* AND-0711, in addition to other known prey (*T. gracilis* and *T. amphioxeia*). Similar results were found by Raho et al. (2014) after cross-feeding experiments with *D. acuta* and the same *M. rubrum* fed upon *T. amphioxeia* and *T. gracilis*. Our finding of *T. minuta*-like plastidal sequences in *Dinophysis* isolated from the Rías Baixas was corroborated after feeding *Mesodinium* on the same species in laboratory cultures. The feeding on *P. prolonga* should not be interpreted as the finding of a new genus suitable as prey for *Mesodinium*. Although not formally synonymized, *Teleaulax* and *Plagioselmis* can be interpreted as alternative morphotypes (campylomorph and cryptomorph) of the same taxon (Laza-Martínez et al. 2012). Moreover, the *P. prolonga* strain used in this study showed sequences nearly identical to *T. amphioxeia*, suggesting it could represent its cryptomorph morphotype.

Origin of plastids in field samples of *Dinophysis*

Sequences of *Dinophysis* plastids from a few scattered field samples have become available in the last 10 yr (Takahashi et al. 2005, Minnhagen & Janson 2006, Nishitani et al. 2010, Kim et al. 2012a, Stern et al. 2014), and in most cases the dominance of *Teleaulax*-like plastids was confirmed. A more comprehensive study in Japanese coastal waters (Nishitani et al. 2010) found a certain degree of polymorphism in *Dinophysis* spp. plastids, but all of them were ascribed to cryptophytes. According to these authors, *T. amphioxeia* was the main plastid source, but 37 plastidic 16S rDNA gene sequences in *D. acuminata* and *D. norvegica* belonged to an unknown cryptophyte closer to *G. cryophila* and *T. acuta*. Similar results were reported from the Baltic Sea and the North Sea (Minnhagen & Janson 2006), where *T. amphioxeia* was the dominant source, while *G. cryophila* was only detected in samples from the Greenland Sea. *Teleaulax*-like plastids were reported from Scottish samples, but also a *Rhodomonas*/*Storeatula* sequence in a single cell of *D.* cf. *acuta* (Stern et al. 2014). The most striking findings came from the Bay of Biscay, where E. Nézan (unpubl. data) confirmed the presence of small dinoflagellates (i.e. *Scrippsiella* spp.) inside *D. acuminata* specimens (Reguera et al. 2010), and from South Korean waters where, in addition to the dominance of *T. amphioxeia* and *T. acuta*-type plastids, several sequences from raphidophytes (*Heterosigma akashiwo*) and prasinophytes (*Pyramimonas* sp.) were retrieved (Kim et al. 2012a). These results from Korean waters suggested that alternative prey to the classical *Mesodinium*-cryptophyte chain could be exploited by *Dinophysis*.

In our study, sequences of 23S rDNA from *Dinophysis* field samples showed plastids identical (or almost) to those of *T. amphioxeia*/*P. prolonga* and *T. minuta*. Thus, our molecular results confirmed the dominance of *Teleaulax* plastids in field populations of *Dinophysis* spp. Nevertheless, the inability to resolve polytomies found in the major clade grouping most plastid sequences from field specimens (Fig. 4) precluded any conclusions about the relationships between the 2 groups of sequences including those of *T. amphioxeia* and *T. minuta* (Fig. 4). Taking this into account, it can be concluded that within the *Teleaulax* clade, most plastid sequences were closely related to *T. amphioxeia*, and minor portions to *T. minuta* and *T. gracilis*.. These results also suggest that several *Teleaulax* species sustain growth of *Mesodinium* and *Dinophysis* in the Rías Baixas, as confirmed in laboratory experiments in this and former studies.

Regarding other *Teleaulax* species, *T. merimbula* has never been reported from European waters, whereas *T. gracilis* and *T. acuta* are probably common (Hill 1991, 1992, Laza-Martínez et al. 2012 and references therein). On the northeast Iberian coast, close to the present study area, these species have been observed to be common and usually abundant (Laza-Martínez et al. 2012, authors' unpubl. obs.). Moreover, in a recent survey performed in 6 European coastal sites, *T. gracilis* was detected in 12 out of 13 analysed samples (Massana et al. 2015). In spite of the fact that both *T. gracilis* and *T. acuta* are known to sustain growth of *Mesodimium*, their sequences were not detected in *Dinophysis* from Galician waters.

In addition, as also found by other authors (Hackett et al. 2003, Kim et al. 2012a), we detected plastid sequences different from those of cryptophytes in 3 *Dinophysis* cells from Galician shelf waters (GenBank acc. nos. KP826902 to KP826909). The florideophyceae-like sequence found in *D. acuta* cells from the Galician shelf (Stn ST8) had insufficient similarity with the ones available in GenBank database (82%) to draw firm conclusions regarding its origin. Nevertheless, it corroborates a previous report about plastid-encoded *psb*A and 18S rDNA of florideophyceae origin in *Dinophysis* cells from the northwest Atlantic (Hackett et al. 2003). Other sequences in *Dinophysis* in the Galician shelf matched those from eukaryotes resembling diatoms (96 to 99% similarity to *Thalassiosira pseudonana*) obtained in a former study on deep-water samples from the northeast Pacific (Gao et al. 2011).

Moreover, one of the *D. acuta* cells from the oceanic Stn ST8 that contained both florideophycean and diatom sequences also revealed a different cryptophyte sequence (close to a freshwater cryptophyte, *Plagioselmis nannoplanctica*), suggesting that other *Teleaulax*/*Plagioselmis* sources different from those commonly retrieved from the Rías Baixas are also possible.

In the light of the results described above, the possibility remains that a more intensive sampling could have led us to find other algal groups as plastid sources for *Dinophysis* in the Rías Baixas. Our findings support the possibility that *Dinophysis* can feed on alternative prey to *M. rubrum*, as suggested by previous results that proposed different ciliate genera such as *Laboea* and *Strombidium* as possible vectors for *Dinophysis* plastids (Kim et al. 2012a). The different plastid types eventually found would likely reflect the mixotrophic behaviour and recently ingested prey in food vacuoles, rather than tempo-

rary plastids. In this sense, our study confirmed that one of such alternative prey is *M.* cf. *major*, which appeared frequently in our field samples and could be a relevant resource for *Dinophysis* populations in the region. Other possible prey from the genus *Mesodinium* could be 2 recently described benthic species (*M. chamaeleon* and *M. coatsi* in Moestrup et al. 2012 and Nam et al. 2015, respectively). *M. chamaeleon* appears to graze on flagellates including cryptomonads and harbours green or red plastids temporarily in its food vacuoles (Moestrup et al. 2012). *M. coatsi* is able to feed upon cryptophytes from the genus *Chroomonas* and *D. caudata* feeds on such ciliate species but does not retain these second-hand *Chroomonas* plastids (Kim et al. 2015). In this regard, it is already known that the few nuclear-encoded functional photosynthetic genes identified in *D. acuminata* (Wisecaver & Hackett 2010) are derived from multiple algal sources, not just cryptophytes. *Dinophysis* reflects the complex evolutionary history of the dinoflagellate lineage. However, a possible role of the phagocytotic feeding behavior in the lateral gene transfer from other algal groups has been also hypothesized to explain the mosaic pattern of photosynthetic genes in other mixotrophic algae, such as the chloroarachniophyte *Bigelowiella natans* (Archibald et al. 2003).

In conclusion, it appears that *Dinophysis* from Galician coastal waters maintains primarily a *T. amphioxeia*-type plastid. Other plastid sources appeared uncommon but their occurrence suggests alternative prey and unknown food chains for *Dinophysis*. Considering that *T. amphioxeia*-type plastids were predominant in our field samples, it can be expected that *M.* cf. *major*, a common ciliate in Galician waters, harbours a cryptophyte prey very similar to *T. amphioxeia*. Identification of suitable cryptophyte preys for *M.* cf. *major* in the Galician Rías and characterization of *Mesodinium* species in the region is a priority in our ongoing studies.

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