Myrionecta rubra population genetic diversity and its cryptophyte chloroplast specificity in recurrent red tides in the Columbia River estuary

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ABSTRACT: For several decades, annually recurring blooms of the photosynthetic ciliate *Myrionecta rubra* have been observed in the Columbia River estuary in late summer. In an effort to understand the dynamics of these blooms, we investigated the genetic variability of *M. rubra* and its cryptophyte plastids within 3 large estuarine blooms formed in consecutive years (2007 to 2009), and conducted a broader spatial survey along the coasts of Oregon and Washington. Analysis of the '18S-28S' sequences specific for Mesodiniidae uncovered at least 5 variants of *M. rubra* within the Columbia River coastal margin in spring and summer, but only one of these *M. rubra* variants was implicated in estuary bloom formation. Using a multigene approach, we show that the bloom-forming variant of *M. rubra* appears to harbor the same cryptophyte chloroplast in recurring blooms. Analyses of chloroplast 16S rRNA, cryptophyte RuBisCO and Photosystem II D2 genes together suggest that the plastid is derived from *Teleaulax amphioxeia*. Free-living cells of this species and of other cryptophytes were practically absent from the bloom patches in the estuary main channels based on 18S rDNA sequence analyses. The respectively low and high proportions of *T. amphioxeia* nuclei and chloroplast signals found in the *M. rubra* bloom of the Columbia River estuary in successive years supports the notion of an association (either endosymbiosis or kleptoplastidy) between *T. amphioxeia* and the bloom-forming *M. rubra* variant, with loss of cryptophyte nuclei. The genetic variability of *M. rubra* uncovered here is relevant to the controversy in the literature regarding the cryptophyte/*M. rubra* association.

KEY WORDS: *Myrionecta rubra* · Red tide · Cryptophyte · Diversity · Columbia River estuary

INTRODUCTION

Myrionecta rubra (Lohman, 1908) Jankowski, 1976, formerly *Mesodinium rubrum* (Lohman, 1908), is a neritic, planktonic ciliate distributed throughout the globe in marine and brackish waters and is well-known for generating expansive, non-toxic blooms in estuaries, fjords, and upwelling areas of the coastal ocean (Lind-holm 1985). The intense red color of the blooms results from high cell-density surface or sub-surface aggregations of *M. rubra* cells (>10^4 cells ml^{-1}, Taylor et al. 1971), each containing several ingested phycoerythrin-rich chloroplasts that originate from cryptophyte algae.

Some disagreement exists in the literature concerning the exact nature of this ciliate/cryptophyte association, with some data suggesting kleptoplastidy

(Gustafson et al. 2000, Johnson & Stoecker 2005, Johnson et al. 2007), while other findings point to endosymbiosis (Hansen & Fenchel 2006). Johnson et al. (2007) have demonstrated that, in culture, *Myrionecta rubra* ingests whole cryptophyte algae and retains their organelles along with the cryptophyte nuclei, which remain transcriptionally active. The prey nucleus of these *M. rubra* cultures could be retained for up to 30 d, during which it services numerous chloroplasts acquired from multiple cryptophyte algae. In contradiction to this transient association between ciliate and cryptophytes, Hansen & Fenchel (2006) presented electron microscopic and physiological evidence from studies of their *M. rubra* culture that suggested permanent endosymbiosis. Johnson et al. (2006 p. 1244) have hypothesized that *M. rubra* 'may be in transition of permanently acquiring plastids'. Regardless of the exact nature of this association, *M. rubra* is considered a mixotroph, having both heterotrophic and a very active photoautotrophic lifestyle. Notably, the highest primary production rates and chlorophyll *a* concentrations ever recorded in aquatic systems have been measured in a *M. rubra* bloom (Smith & Barber 1979).

Myrionecta rubra is also the fastest observed 'jumping' ciliate, with recorded velocities as high as 1.2 cm s^{-1} (Fenchel & Hansen 2006). Vertical migrations of *M. rubra* down to 40 m have been measured in the water column of the Peruvian upwelling zone (Smith & Barber 1979). Its blooms are characterized by a horizontally patchy distribution (patches >340 km^2 have been observed, Rychert 2004) resulting from phototactic aggregation at the surface or sub-surface during daytime (Lindholm 1985, Dale 1987). In the dark, *M. rubra* cells tend to disperse (Dale 1987, Fenchel & Hansen 2006), but irradiance alone does not seem to drive diel migration (Passow 1991, Crawford & Lindholm 1997).

For several decades massive red tides of the photosynthetic ciliate *Myrionecta rubra* have been observed in the Columbia River estuary every summer or early fall. It is unclear what conditions trigger *M. rubra* bloom formation. Hypotheses explaining bloom formation include the combined effect of high light intensity, low turbulence, and perhaps elevated concentrations of dissolved organic compounds produced by preceding diatom blooms or released during rainfall events (Crawford et al. 1997). Anecdotal evidence suggests that *M. rubra* blooms are usually first observed in Ilwaco harbor in Baker Bay on the Washington bank of the Columbia River estuary (see Fig. 1) before later appearing in the estuary main channels (G. C. Roegner and several local fishermen pers. comm.). Hence, to better characterize the populations of *M. rubra* both as the bloom develops in Baker Bay and when it is well-established in the estuary main channels, we explored

the genetic diversity of *M. rubra* populations on water samples collected not only in the estuary, but throughout the Columbia River coastal margin, including sites within and outside of red water patches in the estuary main channels in summer and fall of 2007, 2008 and 2009, in Baker and Willapa bays, and in the Washington and Oregon coastal zones in spring and summer of 2009. In particular, we asked (1) does a specific strain of *M. rubra* form the red water blooms and (2) which cryptophyte chloroplast constitutes bloom-forming *M. rubra*'s plastid?

MATERIALS AND METHODS

Study area. The Columbia River is the second largest river in North America, with an average annual freshwater discharge of 2×10^{11} m^3 and a drainage basin of 670 000 km^2 that includes 7 US states and 2 Canadian provinces (Frey et al. 1984, Sullivan et al. 2001). It is also a critical conduit for shipping, commerce, and a vital source of power generation. The Columbia, therefore, has substantial economic influence on the Pacific Northwest region. The Columbia River estuary (Fig. 1) is a turbid and often highly stratified system (with degrees of stratification varying with tidal cycles and seasons), and is narrow relative to its length. The estuary has a few shallow, wide-mouthed bays, lacks important tributary rivers, and has 2 main channels. The south channel is dredged to enable shipping activities up to Portland (Oregon), while the north channel is dredged moderately and shoals near the freshwater-brackish water interface. Circulation patterns differ between the channels; the north channel is generally flood dominant while the south channel conveys most of the ebb flow. Tides are typical of the eastern North Pacific coast, with diurnal and semi-diurnal frequencies, resulting daily in 2 high-water and 2 low-water periods (Jay 1984). Water flushing time in the estuary is short, varying from 0.5 to 5 d (Neal 1972). Baker Bay is located on the Washington bank of the Columbia River estuary, whilst Willapa Bay is situated north of the Columbia River estuary with a direct link to the Pacific Ocean but none to the Columbia River estuary (Fig. 1). The Washington and Oregon coasts are strongly influenced by the Columbia River, with a northward directed plume during downwelling periods (southerly winds, occurring more often during winter, spring and fall) and a southward directed plume during upwelling periods (northerly winds, occurring more often during summer).

Sample acquisition. Sampling locations in the Columbia River coastal margin are indicated in Fig. 1. Eulerian time series were carried out in August 2007 in the estuary on board the RV 'Barnes' using either a
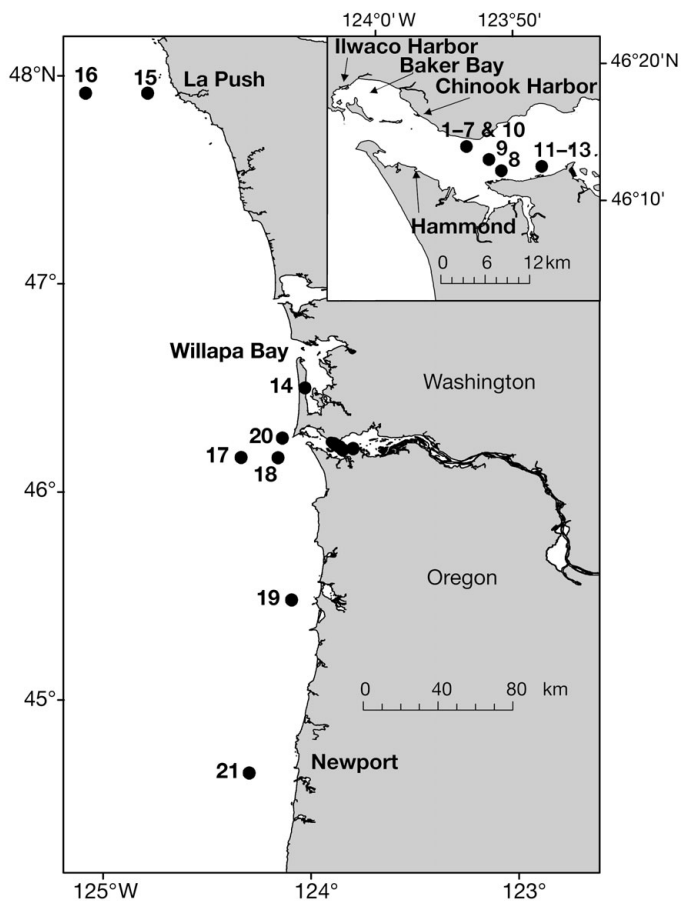
Fig. 1. Columbia River coastal margin showing sites where water samples (1–21) were collected in 2007–2009. The inset, focused on the Columbia River estuary, shows the location of Ilwaco and Chinook harbors in Baker Bay, and of Hammond in the south channel of the estuary

bucket for water collection at the surface (samples 5 and 7), or a high-volume low-pressure pump at 1 to 2 m depth (samples 1–4 and 6) and at the bottom (samples 11–13). Surface water was also collected with a bucket in the estuary main channels in October 2008 (samples 8–9) and September 2009 (sample 10 and at Hammond), as well as in Baker Bay at Chinook and Ilwaco harbors and in Willapa Bay (sample 14) in 2009. In May and September 2009 surface coastal water at 2 to 3 m depth (samples 15–21) was collected on board the RV 'New Horizon' using the ship's 10 l Niskin bottle instrumented rosette. Surface water samples for *Myrionecta rubra* partial '18S-28S' DNA region single-cell PCR (SC-PCR) analyses were collected during the 2009 September bloom period in Ilwaco harbor and during the pre-bloom period in April 2010 on the Oregon coast on the Strawberry Hill line (44° 15.1' N, 123° 15.13' W). Salinity was measured with the ship's Seabird CTD meter or a refractometer.

Nucleic acid extraction. For all DNA analyses, 0.6 to 4 l of water were collected and immediately filtered through 0.2 µm pore size Sterivex filters (PES, ESTAR, Millipore) using a peristaltic pump. Water was forced out of the Sterivex using a plastic syringe and 2 ml of fixative (RNAlater, Ambion) was added. The filters were stored at −80°C aboard the ship, transferred to the onshore laboratory on dry ice, and then stored at −80°C until processing. To extract total nucleic acid, Sterivex filters were first cut into small pieces and the cells contained in the RNAlater solution were collected by gentle centrifugation. The pieces of filter and the cells from the RNAlater solution were transferred to a polycarbonate tube containing 2 ml of extraction buffer (LETS Buffer: 100 mM LiCl, 10 mM EDTA, 10 mM Tris [pH 7.8], 1% SDS), 1 ml Zirconia beads (RiboPure-Bacteria Kit, Ambion) and 2 ml of phenol/chloroform/isoamyl alcohol (PCI) (25:24:1). This was followed by vortexing for 2 min, resting on ice for 3 min, and vortexing again for 2 min. The sample mixtures were centrifuged at $2320 \times g$ for 10 min at 4°C. The aqueous phase was then transferred to a Corex tube, to which 2 ml of PCI was added. The tubes were then shaken, centrifuged at $9279 \times g$ for 10 min at 4°C and the aqueous phase was transferred to a new 15 ml polypropylene tube. An equal volume of 100% ethanol was added to the aqueous phase and this extract-ethanol mixture was applied to a cleaning column (RiboPure-Bacteria Kit, Ambion) according to the manufacturer's instructions. Samples underwent the above extraction twice and the extracts were combined. Nucleic acid quality was visually inspected by agarose gel electrophoresis and concentrations were determined using a Nanodrop spectrophotometer (ND-1000). The samples were stored at −80°C until further processing.

Eukaryotic 18S rRNA gene PCR. Eukaryotic DNA encoding 18S rRNA was amplified on a Bio-Rad DYAD PCR thermocycler using the eukaryote-specific primers EukA (5'-AAC CTG GTT GAT CCT GCC AGT-3') and EukB (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') (Diez et al. 2001). The reaction mixture (25 µl) contained 2.5 µl 10 × *Taq* polymerase Buffer, 0.2 mM deoxynucleoside triphosphate (dNTP), 0.3 µM of each primer, 0.625 U *Taq* Polymerase, 1.5 to 10 ng of template DNA and water. Reactions were run according to the following PCR protocol: initial denaturation of 94°C for 3 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 3 min; and one final extension at 72°C for 5 min. For each sample, PCRs were run in triplicates; products were combined, visualized on an agarose gel and filtered with an UltraClean PCR Clean-Up Kit (MoBio) in accordance with the manufacturer instructions. PCR products were stored at −20°C.

Chloroplast 16S rRNA genes PCR. Chloroplast DNA encoding 16S rRNA was amplified using the universal bacterial primers 907f (5'- AAA CTC AAA GGA ATT GAC GGG -3') (Santegoeds et al. 1998) and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane 1991) encompassing the variable regions V6 to V9. The reaction mixture was the same as above except that 3 to 15 ng of template and 0.2 µM of each primer were used. Reactions were run according to the following PCR protocol: initial denaturation of 94°C for 4 min; 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 90 s; and one final extension at 72°C for 7 min. For each sample, PCRs were run in triplicate; products were combined and further processed and stored as described above.

Cryptophyte ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) gene PCR. Cryptophyte RuBisCO gene (*rbcL*) DNA was amplified using the L1R (5'-TCA GCT GTA TCW GTA GAA GC-3') and L2F (5'-AGG AGG AAW AYA TGT CTC AAT CCG-3') primer set and following the protocol described in Hoef-Emden et al. (2005). For each sample, PCRs were run in duplicates; the products were combined, further processed and stored as described above.

Cryptophyte Photosystem II D2 protein gene PCR. Cryptophyte Photosystem II (PSII) D2 protein gene (*psbA*) DNA was amplified using the following primers: pbsA1r (5'-GTA ACC GTG TGC AGC TAC GA-3') and pbsA5f (5'-TCT GCA TCA ATC GGT ATC CA-3'). The reaction mixture (25 µl) contained 2.5 µl DyNAzyme Buffer, 2 µl dNTP (2.5 mM), 0.75 µl of each primer (10 µM), 0.25 U DyNAzyme polymerase (Finnzymes, Woburn, MA, USA), 1.5–7.5 ng of template DNA and water. Reactions were run according to the following PCR protocol: initial denaturation of 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 2 min, extension at 72°C for 90 s; and one final extension at 72°C for 7 min. For each sample, PCRs were run in duplicate; products were combined and further processed and stored as described above.

***Myrionecta rubra* partial '18S-28S' rDNA region PCR.** *M. rubra* partial '18S-28S' rDNA region was amplified using the same reaction mixture and thermocycle as for Cryptophyte PSII D2 protein gene amplification except that the *M. rubra* specific 18S and 28S rDNA primers (oMR18S1412f: 5'-GTC TCG TAA GCA CAA GTC AAC ATC TT-3' and oMR28S236r: 5'-CAA CAA ATA CTA CTC AGT CTG TTT ACG CTT-3') were employed. These primers were designed based on the Mesodiniidae sequences submitted to the National Center for Biotechnology Information (NCBI) by Marande et al. (2009). The PCR fragment amplified contains the 3' end of Mesodiniidae 18S (from nucleotide position 1412), the internal transcribed

spacer (ITS) and the 5' end of the 28S gene to nucleotide position 236. These primers are specific for *M. rubra*, since a single band corresponding to the predicted size was always visualized on an agarose gel, and when PCR products were cloned and sequenced the 5' end of all sequences was always homologous to the *M. rubra* 18S rDNA partial sequences deposited in the NCBI database. For each sample, PCRs were run in duplicate; products were combined, further processed and stored as described above.

Cloning and Sequencing. All PCR products were ligated into a TOPO vector (pCR 2.1, Invitrogen) and used to transform One Shot Top 10 chemically competent *E. coli* cells (Invitrogen), following the manufacturer's instructions, except that transformants were incubated at 37°C for 1 h without shaking prior to plating. Transformed cells were incubated overnight at 37°C on Luria-Bertani plates containing 0.05 mg ml^{-1} of Ampicillin and 0.04 mg ml^{-1} of bromo-chloro-indolyl-galactopyranoside (X-gal).

For both 16S and 18S rDNA analyses, 192 positive (white) colonies were picked, inoculated into 2 ml 96-deep-well plates (ThermoFisher-Matrix) containing 500 µl of 2 × yeast extract/tryptone media and ampicillin (0.05 mg ml^{-1}), and incubated overnight with shaking at 50 rpm at 37°C. Following the incubation, 150 µl of solution were transferred to 400 µl 96-well microtiter plates to which 30 µl of 50% glycerol was added. Plates were stored at –80°C until they were sent to the Genome Sequencing Center at Washington University (St. Louis, MO, USA) for sequencing using the above mentioned primer sets following the Big Dye protocol.

For the cryptophyte RuBisCO gene, cryptophyte PSII D2 gene and *Myrionecta rubra* partial '18S-28S' rDNA gene analyses, positive colonies were picked, inoculated into glass tubes containing 2 ml of 2× yeast extract/tryptone media and ampicillin (0.05 mg ml^{-1}), and incubated overnight with shaking at 50 rpm at 37°C. Miniprep (Qiagen) was performed following the manufacturer's instructions and sequencing was carried out at the Oregon Health and Science University Primate Center (Beaverton, OR, USA) using the M13f and M13r primers and following the Big Dye protocol.

***Myrionecta rubra* partial '18S-28S' DNA region SC-PCR.** Single-cell PCR was performed using a modified rendition of a protocol reported previously (Auinger et al. 2008). Individual *M. rubra* cells, that had been preserved in 1% Lugol's iodine solution and kept at room temperature, were isolated using an inverted microscope and an elongated glass Pasteur pipette. These individual *M. rubra* cells were then rinsed twice into a drop of 0.1 mM EDTA, before being transferred together with 13.5 µl of the rinsing solution into a PCR tube containing 1.5 µl of 12.3 mM $Na_2S_2O_3$. After an

initial incubation for 30 min at room temperature, single *M. rubra* cells were extracted as follows: addition of 1 µl of 1:100 dilution of Viscozyme (Sigma), incubation at 37°C for 2 h, addition of 1 µl of 100 µg ml^{-1} Proteinase K solution (Promega) and 10 µl mineral oil to each tube and a further incubation at 55°C for 50 min. PCR tubes were then subjected to 2 cycles of heating at 95°C for 10 min to deactivate proteinase K and immediately freezing at –80°C for 10 min. Half of this single cell nucleic acid extract was then subjected to *M. rubra* partial '18S-28S' rDNA region PCR as mentioned above, except that the PCR volume was reduced to 12.5 µl and 35 cycles were performed. PCR products were visualized on an agarose gel, filtered with an UltraClean PCR Clean-Up Kit (MoBio) in accordance with the manufacturer instructions, and cloned and sequenced as indicated above for the *M. rubra* partial '18S-28S' rDNA gene analysis of water samples.

Sequence analysis. For each 16S rDNA clone, the forward (907f) and reverse (1492r) sequence reads were combined by aligning them using the Smith-Waterman algorithm (Smith & Waterman 1981) implemented in the EMBOSS (European Molecular Biology Open Software Suite) package, using strict gap parameters (gap opening = 50, gap extension = 5.0). Clones were identified for which the Smith-Waterman alignment covered at least 300 bases with at least 97% identity, and a single combined sequence was generated in which positions of non-identity in the 2 reads were replaced with 'N'. These sequences were submitted to ScalaBLAST (Oehmen & Nieplocha 2006), searching the NCBI non-redundant nucleotide database for homologous sequences. Clones that had database hits with at least 300 bases aligned and with an expectation value ≤1 × 10^{-50} were retained for further analysis.

For the 18S rDNA analysis, since sequencing reads from the 2 primers used (EukA: position ~1–20 and EukB: position ~1780–1800) did not overlap, the 2 sequences were submitted separately to ScalaBLAST, searching the NCBI non-redundant nucleotide database for homologous sequences. These results were used to screen for poor sequence reads and those reads consisting of only vector sequences. The remaining results were limited to significant hits (at least 300 bases aligned, an expectation value ≤1 × 10^{-80}, and percent identity ≥97%) and evaluated to determine if the database hits for the 2 sequence reads (from EukA and EukB) of a given clone were consistent. Only those clones with consistent ScalaBLAST hits were used.

For cryptophyte RuBisCO gene, cryptophyte PSII D2 gene and *Myrionecta rubra* partial '18S-28S' rRNA gene analyses, forward and reverse sequence reads were combined by aligning them using the ClustalW Multiple alignment option in BioEdit software (Ibis Therapeutics) and BLAST was used to search the NCBI non-redundant nucleotide database for homologous sequences. All *M. rubra* partial '18S-28S' rRNA gene sequences were checked for chimeras using the Bellerophon software (Huber et al. 2004). This analysis returned 5 potential chimera sequences that were then omitted from further analysis. Bioedit alignment of the resulting *M. rubra* partial '18S-28S' rRNA gene sequences was used to determine potential *M. rubra* variants according to the following analysis pipeline: (1) all loci where nucleotide difference existed between sequences were highlighted; (2) any locus with a nucleotide substitution, insertion or deletion in a single sequence was ignored; (3) all loci where at least 3 sequences had a similar nucleotide that was different from other sets of sequences were listed; (4) a table listing all loci that had different nucleotide for at least 3 sequences was generated (e.g. Table 2), and (5) variants were determined based on the analysis of the polymorphism patterns provided by this table. To characterize the geographic structure of *M. rubra* population in the Columbia River coastal margin based on genetic variations in their partial '18S-28S' rDNA region, analyses of molecular variance (AMOVA) were preformed and F_{ST} values calculated using Arlequin software (Excoffier et al. 1992). For these analyses, absolute frequencies of *M. rubra* variants in 5 different populations were transformed into relative values and populations were grouped into estuarine or coastal region as follows. Region 1 comprised the estuary main channel and estuary Baker Bay populations; Region 2 comprised Willapa Bay, and coastal spring and coastal summer populations.

Tables S1 & S2 in the supplement, available at www.int-res.com/articles/supp/a062p085_supp.pdf, provide the accession numbers of all sequences obtained during this study that have been submitted to GenBank (Chloroplast 16S rRNA gene sequences: HQ226122–HQ226448; 18S rRNA gene sequences: HQ226449–HQ227836; *Myrionecta rubra* partial '18S-28S' rRNA gene sequences: HQ227837–HQ227990; SC-PCR *M. rubra* partial '18S-28S' rRNA gene sequences: HQ227991–HQ227994; Photosystem II D2 protein gene sequences: HQ227995–HQ228003, and RuBisCO gene sequences: HQ228004–HQ228013).

Light microscopy. For cell counts, water samples (40 ml) collected in 2007 were fixed with formaldehyde (final concentration 4%) at room temperature for 1 h and stored at –20°C for 1 wk until being returned to the laboratory, where the fixed samples were stored at –80°C until analysis. Alternatively, samples collected in 2008 and 2009 were fixed with Lugol's iodine (final concentration 1%) and stored in dark at 4°C. The sam-

ple (25 ml) was settled overnight according to the Uter-möhl method (Utermöhl 1931, 1958). Using an inverted microscope (Apotome, Zeiss), a minimum of 200 *Myrionecta rubra* (in >5 fields of view) and 100 diatom or dinoflagellate cells were counted per sample. Even though *M. rubra* cells fixed with formaldehyde and stored frozen were often damaged, they were still clearly identifiable. Similar cell counts were obtained with water samples collected in 2008 and fixed with Lugol's iodine or formaldehyde.

RESULTS

Genetic diversity of *Myrionecta rubra* populations

A similar ~604 bp sequence was retrieved for the red water samples of 2007, 2008 and 2009 (>99% similarity among 22 red water sequences). The overall structure of this sequence is depicted on Fig. 2. The first 144 bp region of this sequence was homologous to several uncultured eukaryote partial 18S rDNA sequences in the NCBI database and to a few *M. rubra* 18S rDNA partial sequences (e.g. AB364286 and EF195734 with >98.5% similarity), indicating that bp 144 marks the 3′ end of the 18S rDNA region. The 205 bp 3′ region was closely affiliated with only 2 Mesodiniidae 28S rDNA partial sequences submitted to NCBI (98% and 94% similarity to FJ032687 and FJ032689 respectively; Marande et al. 2009) and thus constitutes a highly divergent *M. rubra* 28S rDNA 5′ region. The intermediate 255 bp sequence was closely related to only one Mesodiniidae 18S rDNA partial sequence (FJ032688), which is highly similar to our sequence until bp 399. This high similarity until the beginning of our 28S rDNA region is in contradiction with all other best hits of 18S rDNA partial sequences in NCBI, which in contrast to sequence FJ032688 all point to bp 144 as marking the 3′ end of the 18S rDNA region, hence suggesting that this Mesodiniidae 18S rDNA partial sequence (FJ032688; Marande et al. 2009) was wrongly annotated and in fact contains both *M. rubra* partial 18S rDNA and the Internal Transcribed Spacer (ITS) regions. Therefore, the intermediate 255 bp of our sequence likely constitutes a unique ITS region for *M. rubra*. It is important to note that a 5.8S rDNA region is not clearly apparent in this 604 bp sequence and also that in an initial attempt to uncover *M. rubra* ITS1 sequence using a 5.8S primer we uncovered a 28S rDNA sequence similar to that presented in this manuscript. All this suggests that, similarly to bacteria, the 5.8S rDNA region of *M. rubra* might be fused with the large subunit rRNA. This lack of a separate 5.8S molecule has also been observed in another eukaryote, the fungi microscrosporidia (Vossbrinck & Woese 1986),

and has been hypothesized to represent a resealing of a split RNA molecule (Williams 2004). In summary, our 604 bp sequence contains a *M. rubra* 18S rDNA 3′ partial sequence (bp 1 to 144), a *M. rubra* ITS sequence (bp 145 to 398) and a *M. rubra* 28S rDNA 5′ partial sequence (bp 399 to 604) (Fig. 2). Henceforth, this entire sequence will be referred to as *M. rubra* partial '18S-28S' rDNA sequence. That this sequence does, indeed, originate from *M. rubra* was confirmed by single cell PCR of *M. rubra* cells of water samples fixed in Lugol's iodine (accession numbers HQ227991–HQ227994, Table S2 in the supplement).

A total of 154 *Myrionecta rubra* partial '18S-28S' rDNA sequences were retrieved from 154 examined clones that originated from 24 samples collected at different times throughout the Columbia River coastal margin. Table 1 provides the details of how many clones were examined per sample (e.g. 9 and 7 clones were sequenced for sample 7 and 15, respectively). Although the above mentioned *M. rubra* partial '18S-28S' rDNA sequence detected in the 2007 to 2009 red water patches was also found throughout the rest of our data set, there was clear genetic diversity amongst the 154 retrieved *M. rubra* partial '18S-28S' rDNA sequences (>93.8% similarity) (Table 1). Fig. 2 shows the mutation sites of 3 *M. rubra* partial '18S-28S' rDNA sequences retrieved from the Columbia River red water patches in 2007 to 2009 and of the Mesodiniidae partial 18S rDNA-ITS sequence deposited in NCBI (FJ032688; Marande et al. 2009) compared to that of a *M. rubra* culture originating from Antarctica (culture CCMP2563 from Bigelow Laboratory for Ocean Sciences, Maine, USA; Gustafson et al. 2000). Five variants of *M. rubra* partial '18S-28S' rDNA region (A-E) were detected, with totals of 8, 19 and 13 sites of mutation within the 18S rDNA, ITS and 28S rDNA regions, respectively (Table 2). Although identity values between individual sequences across variants (93.8 to 99.0%) were sometimes as high as similarity values between sequences of a single variant group (>98.1%) (Table 3), there were clear patterns of polymorphisms that distinguished the different variants as highlighted by the color map of Table 2. For example, some sequences belonging to variants 'B' and 'D' have up to 99% similarity and share identical partial '18S rDNA sequences, but they consistently have 5 different nucleotides in their ITS- partial 28S′ rDNA sequences. So, clearly it is essential to examine the patterns of polymorphism to distinguish *M. rubra* variants based on partial '18S-28S' rDNA sequences and not simply similarity values. In fact, it is highly likely that with more sequence analysis as additional *M. rubra* isolates are characterized, more variants would be uncovered in the Columbia River coastal margin.
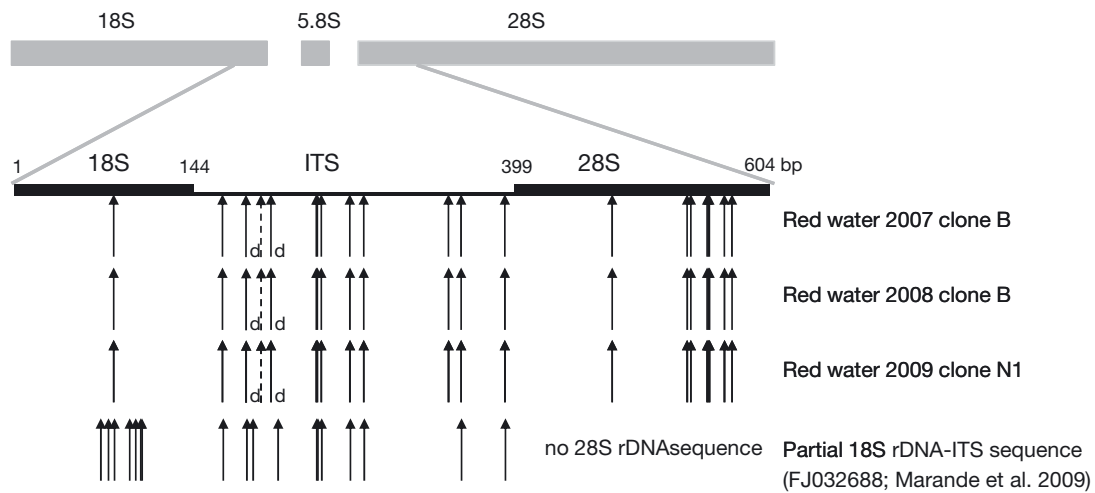
Fig. 2. Sites of sequence polymorphisms (indicated by arrows) of 3 *Myrionecta rubra* partial '18S-28S' rDNA sequences retrieved from the Columbia River estuary red water patches of 2007 to 2009 and of the Mesodiniidae partial 18S rDNA-ITS sequence deposited in NCBI (FJ032688; Marande et al. 2009) originating from environmental DNA collected in the South Atlantic compared to that of a *M. rubra* culture from Antarctica (culture CCMP2563 from Bigelow Laboratory for Ocean Sciences, Maine, USA). Base pair numbering corresponds to the Columbia River red water sequences. d: deletion; dashed arrow: insertion

Table 1. *Myrionecta rubra*. Number of partial '18S-28S' rDNA sequences retrieved for each *M. rubra* variant (A–E) for water collected throughout the Columbia River coastal margin over different time scales or for cells originating from Antarctica and South Atlantic. Salinity values are also listed for most samples. Sampling locations in the Columbia River coastal margin (either sample number in parentheses or sites at Baker Bay and Hammond) are given in Fig. 1. nd: not determined

Location	Season	Sampling depth	Site	Sampling date	Salinity	*M. rubra* variants				
						A	B	C	D	E
Estuary	Main channels Summer '07-'09	Surface	Red Water patch 2007 (#7)	27-Aug-07	9.0		9			
			Red Water patch 2008 (#8)	03-Oct-08	10.0		2			
			Red Water patch 2009 (#10)	07-Sep-09	nd		11			
			Hammond	06-Aug-09	8.0		2			
			Hammond	20-Aug-09	10.0		2			
		Bottom	South Channel (#11)	26-Aug-07	5.9		2			
			South Channel (#12)	26-Aug-07	10.3		2			
			South Channel (#13)	26-Aug-07	13.1		2			
	Baker Bay Spring Summer Fall '09	Surface	Ilwaco harbor	30-Jun-09	nd		10			
			Ilwaco harbor	21-Jul-09	3.4		10			
			Ilwaco harbor	06-Aug-09	15.0		10			
			Baker Bay mud flat[a]	06-Aug-09	13.0		10			
			Chinook harbor	06-Aug-09	16.0		8	1		
			Ilwaco harbor	20-Aug-09	20.0		10			
			Ilwaco harbor	25-Sep-09	15.0		2			
Willapa	Spring '09	Surface	Willapa Bay (#14)	23-Jun-09	28.0		5	1	1	
Coast	Spring '09	Surface	LaPush line (#15)	18-May-09	27.8			2	2	3
			LaPush line (#16)	18-May-09	31.9			4	2	
			Plume (#17)	14-May-09	29.8			2	5	
	Summer '09	Surface	Columbia River line (#18)	01-Sep-09	31.0		1	3	2	
			Cape Meares line (#19)	30-Aug-09	32.4		1	1		5
			Plume (#20)	03-Sep-09	23.6	6	1			
			Newport Hydoline (#21)	29-Aug-09	32.6	2	4	1	1	
Culture			Culture of *M. rubra* (Antarctica)		nd	6				
NCBI			Mesodiniidae (S. Atlantic–FJ032688)		nd		1			

[a]Water collected on mud flat outside Chinook harbor

Table 2. *Myrionecta rubra.* Details of the genetic variations in the partial '18S-28S' rDNA region between the 5 variants of *M. rubra* detected in the Columbia River coastal margin. Nucleotide positions are based on a variant 'A' sequence (*M. rubra* culture clone S1, accession number HQ227837). Base pair differences are visualized by color, with orange, light green, blue, dark green and purple base pairs being similar to variants 'A', 'B', 'C', 'D' and 'E', respectively

	Nuc. position	A	B	C	D	E
18S rDNA partial region	69	T	T	A	T	A
	75	G	G	A	G	A
	80	C	T	T	T	T
	92	A	A	T	A	A
	97	C	C	T	C	C
	101	A	A	G	A	A
	102	G	G	A	G	G
	GAP	GAP	GAP	GAP	GAP	A or G
Internal Transcribed Spacer	167	T	A	A	A	A
	177	A	A	A	A	A or G
	183	T	T	T	A	A
	186	C	T	T	T	T
	190	C	C	C	T	C
	191	G	GAP	A	GAP	A
	192	T	T	T	T	T
	GAP	GAP	G	GAP	G	G
	199	T	T	T	G	G
	206	C	T	T	T	T
	211	T	GAP	A	GAP	A
	242	G	A	A	A	A
	243	T	C	C	C	C
	246	G	A	A	A	A
	269	A	C	C	C	C
	280	G	A	A	A	A
	348	T	C	T	C	C
	358	A	C	C	C	C
	393	C	A	A	A	A
28S rDNA partial region	419	A	A	G	A	A
	469	C	C	T	C	C
	479	C	T	T	T	T
	539	G	T	T	T	T
	542	G	T	G	G	G
	555	T	C	C	C	C
	556	C	T	A	T	A
	557	A	G	A	A	A
	558	C	C	T	C	C
	567	G	G	A	G	A
	569	G	A	T	A	A
	574	G	G	A	G	A
	575	T	C	T	C	C

Table 3. *Myrionecta rubra.* Percentage similarity between the 154 *M. rubra* partial '18S-28S' rDNA region sequences retrieved in the Columbia River coastal margin. Letters A to E characterize the 5 *M. rubra* variants

	A	B	C	D	E
A	99.3–100				
B	94.8–96.5	98.1–100			
C	93.8–95.0	94.7–96.5	98.8–100		
D	95.2–96.0	97.6–99.0	95.0–96.2	99.3–100	
E	94.2–95.2	96.2–97.6	96.7–97.8	97.2–98.0	99.1–99.8

In the Columbia River estuary, both in the main channels and in Baker Bay, one variant ('B') was predominantly detected (92 times out of a total of 93 examined estuarine clones, i.e. 98.9%), while in Willapa Bay and the Washington and Oregon coastal zones *Myrionecta rubra* populations were more diverse (Table 1). Table 1 also shows that variant 'B' was detected throughout the water column of the estuary since its sequence was not only uncovered in surface water but also in water collected at 1 m from the estuary bottom (samples 11 to 13). To further characterize the geographic structure of *M. rubra* populations in the Columbia River coastal margin, AMOVA was performed in Arlequin (Excoffier et al. 1992) using data from Table 1; estuary populations (estuary main channels and estuary Baker Bay) formed Region 1, and Willapa Bay, coastal spring and coastal summer populations belonged to Region 2. These 2 regions were characterized by different salinities, with a range of 3.4 to 20.0 found for Region 1 (estuary) and 28.0 to 32.6 for Region 2 (coast) (Table1). The percentages of variance were the largest (52.2%, p = 0.2160) between regions, and the smallest between populations within regions (8.9%, p = 0.0019). There was 38.8% of variance within populations (p < 0.0001). Grouping of these 2 regions was confirmed by F-statistic analysis: F_{ST} values calculated between *M. rubra* populations from the estuary main channels and Baker Bay (Region 1) (0.0, p = 0.99), and between the 3 populations of Region 2 (0.1 to 0.3, p < 0.045), were lower than F_{ST} values obtained between populations of different regions (0.4 to 0.8, p = 0.0000 to 0.0360) (Table 4). These F_{ST} values indicate that the *M. rubra* variant frequencies are similar within a given region but widely different between estuary (Region 1) and coast (Region 2).

To verify that different variants of *Myrionecta rubra* are the same species, *M. rubra* partial '18S-28S' DNA region SC-PCR analysis was carried out for *M. rubra* cells collected during the 2009 September bloom period in Ilwaco harbor or during the pre-bloom period in April 2010 on the Oregon coast on the Strawberry Hill line (44° 15.1′ N, 123° 15.13′ W). Morphologically

identical *M. rubra* cells (based on light microscopy observations) from these 2 locations returned *M. rubra* partial '18S-28S' DNA region sequences related to variant 'B' for Ilwaco harbor (accession numbers HQ227992 and HQ227993), but variants 'A' and 'C' for the Strawberry Hill line sample (accession numbers HQ227991 and HQ227994 respectively) (Table S2 in the supplement).

Six *Myrionecta rubra* partial '18S-28S' rDNA sequences, all belonging to variant 'A' were retrieved from a *M. rubra* culture of an isolate from Antarctica (culture CCMP2563 from Bigelow Laboratory), while the Mesodiniidae partial 18S rDNA-ITS sequence deposited in NCBI (FJ032688; Marande et al. 2009) from environmental DNA collected in the South Atlantic was identical to variant 'C' with the exception of 1 nucleotide difference in position 206 (Table 1).

Chloroplast specificity of *Myrionecta rubra* in Columbia River estuary main channels

To assess whether bloom-forming *Myrionecta rubra* populations of different years have similar chloroplasts, we collected surface water in and out of red water patches during the established *M. rubra* bloom period of 2007 and 2008 (samples 1 to 9 in Fig. 1) and performed cell counts using light microscopy as well as sequence analysis of 16S and 18S rDNA clone libraries.

High *Myrionecta rubra* cell numbers (>1000 cells ml^{-1}) were observed in samples 2, 5, 7 and 8, whilst lower numbers (<650 cells ml^{-1}) were found in samples 1, 3, 6 and 9, and none were detected in sample 4 (Fig. 3A). Thus, samples fell into 3 groups: *M. rubra*-

Table 4. *Myrionecta rubra.* Geographic structure of *M. rubra* populations in the Columbia River coastal margin assessed using F_{ST} values between 5 *M. rubra* populations (estuary main channels, Baker Bay, Willapa Bay, coastal spring and coastal summer) determined based on genetic variations in their partial '18S-28S' rDNA region and showing that *M. rubra* variant frequencies are similar within a given region but widely different between estuary (Region 1) and coast (Region 2). Data from Table 1 were transformed into relative values and used to calculate F_{ST} using Arlequin software (Excoffier et al. 1992). *p > 0.05

| | Region 1 | | Region 2 | | |
	Estuary channels	Baker Bay	Willapa Bay	Coastal spring	Coastal summer
Region 1					
Estuary channel	0.0				
Baker Bay	0.0*	0.0			
Region 2					
Willapa Bay	0.4	0.4	0.0		
Coastal spring	0.7	0.8	0.3	0.0	
Coastal summer	0.5	0.6	0.1	0.1	0.0

rich (2, 5, 7 and 8), containing some *M. rubra* (1, 3, 6 and 9), and containing no *M. rubra* (4).

Fig 3B shows that *Myrionecta rubra*-rich surface waters (samples 2, 5, 7 and 8) had high percentages of cryptophyte chloroplasts 16S rDNA sequences (45 to 96%). Cryptophyte chloroplast sequences retrieved from these red water samples were similar in August 2007 and October 2008 (99.1% similarity) and were all most closely affiliated with *Teleaulax amphioxeia* (98.5 to 99.9% similarity). Thus, for the first time, the results indicate that the same cryptophyte chloroplast is utilized by bloom-forming *M. rubra* in successive years.

In contrast, few cryptophyte 18S rDNA sequences (0 to 10%), all closely affiliated with *Teleaulax amphioxeia*, were detected in and out of the red water patches in 2007 and 2008 (Fig. 3C). In fact, except for sample 4 which was characterized by an absence of *Myrionecta rubra* cells, our 18S rDNA clone libraries were dominated by *M. rubra* sequences (more than 38%). This result suggests that free-living cryptophytes are not abundant in surface waters in the estuary main channels during *M. rubra* bloom periods.

Interpretation of relative abundances in environmental microbial eukaryotic rRNA gene surveys has to be carried out with caution because of the potential for misinterpretation due to PCR bias and variable gene copy number or amount of cellular DNA among different protist groups. This can be illustrated in our dataset by looking at the diatom distribution based on cell counts, 16S rDNA and 18S rDNA analyses since this group can easily be identified by all 3 approaches. The relative distribution of diatoms vs. *Myrionecta rubra* cell numbers concur well with that obtained with 18S rDNA clone libraries, since both analyses showed a prevalence of *M. rubra* in most samples (1 to 3 and 5 to 9) and a dominance of diatoms in sample 4 (Figs. 3A and C). There was less agreement for diatom relative abundance between chloroplast 16S rDNA and 18S rDNA analyses, however (Figs. 3B and C). For example, in sample 7 diatom sequences made up almost 40% of the chloroplast 16S rDNA clone library but were undetected by 18S rDNA analysis. Nonetheless, the low diatom cell number (32 cell ml^{-1}; Fig. 3A) measured in sample 7 agrees well with the absence of a diatom signal found in the 18S clone libraries, hence suggesting that our chloroplast 16S rDNA analysis may overestimate diatom abundances. One might also speculate that the *M. rubra* nuclear signal might dominate our 18S rDNA clone libraries to such an extent that the observed cryptophyte 18S rRNA gene signal is in fact highly under-represented. No cryptophyte was, however, detected in the 18S rDNA clone library of sample 4 (Fig. 3, Table S3 in the supplement) even though *M. rubra*
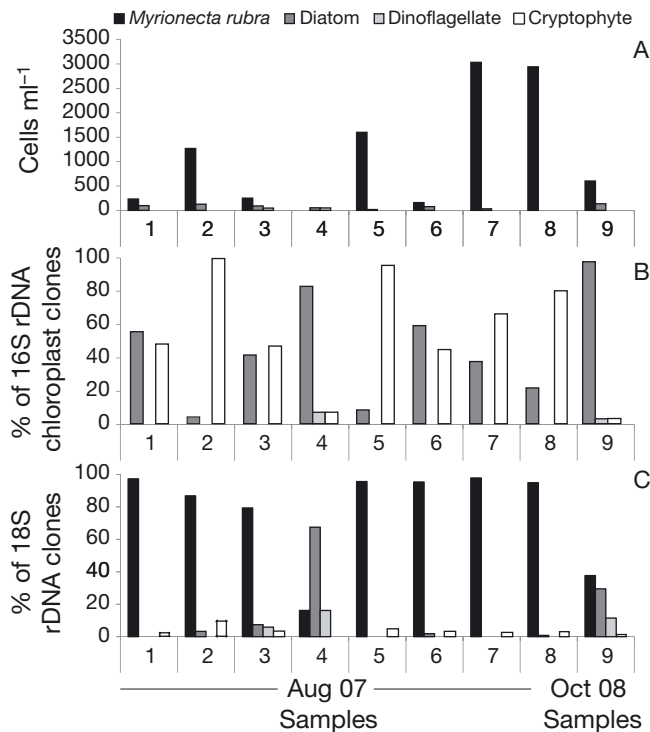
Fig. 3. Diversity of *Myrionecta rubra*, diatoms, dinoflagellates and cryptophytes, based on (A) light microscopic cell counts, (B) the proportion of their chloroplast 16S rRNA gene sequences and (C) their 18S rRNA gene sequences, showing variability between surface water samples collected in the Columbia River estuary over a week at the same location in the north channel in August 2007 (samples 1 to 7) or at the same time but at 2 different locations in south channel in October 2008 (samples 8 and 9). Sampling locations are in Fig. 1

sequences were unlikely to be in overwhelming numbers in the clone library since they only represented 14% of the total. Therefore, the high number of *M. rubra* sequences might not be the reason for the low detection of cryptophyte 18S rRNA gene sequences in our samples. On the other hand, using the same approach, cryptophyte sequences constituted up to 15–20% of 18S rDNA clones libraries generated from Columbia River coastal margin water samples, in which little or no *M. rubra* was detected (P. Kahn pers. comm.). Our molecular genetic approach should thus enable us to detect cryptophyte presence. Taken together, our findings suggest that the low cryptophyte 18S rDNA signal detected during bloom periods in the estuary main channels may indeed be representative of low cryptophyte abundance. This is supported by FlowCAM observations of water samples collected during the *M. rubra* bloom period of September 2009 throughout the water column over a tidal cycle in the estuary north channel at the same location as sample 1 (Fig. 1) and by fluorescence microscopy cell counts of a

red water patch collected in September 2010 in the south channel since both analyses (FlowCAM and fluorescence microscopy) did not reveal the presence of a large number of free-living cryptophytes (data not shown).

Cryptophyte chloroplast specificity in the Columbia River estuary main channels compared to Baker and Willapa bays

To assess the spatial variability in cryptophyte chloroplast specificity of *Myrionecta rubra* assemblages in the Columbia River coastal margin, cryptophyte chloroplast DNA sequences found in the estuary main channels during the bloom periods of 2007, 2008 and 2009 were compared to that of Willapa and Baker bays.

Cryptophyte RuBisCO gene sequences retrieved from the red water samples collected in the estuary main channels during the blooms of 2007 to 2009 (>98.7% similarity) were similar to those collected in Willapa Bay on 23 June 2009 and in Baker Bay at both Ilwaco and Chinook harbors on 06 August 2009 (>94.9% similarity), and were most closely affiliated with *Teleaulax* sp. (AB164408, 94 to 99% similarity). In good agreement, cryptophyte Photosystem II (PSII) D2 gene sequences retrieved from the same red water samples were also similar to those collected in Willapa Bay on 23 June 2009 and Baker Bay at Ilwaco harbor on 06 August 2009 (>98.5% similarity), and were most closely affiliated with *Teleaulax amphioxeia* (AY453068, >99.1% similarity) as well. These results confirm that the *Myrionecta rubra* cells in and outside of the estuary main channels during blooms contain the same cryptophyte chloroplast, that of *T. amphioxeia*.

DISCUSSION

For several decades, annually recurring massive summer blooms of the photosynthetic ciliate, *Myrionecta rubra* have been observed in the Columbia River estuary (Oregon and Washington, USA), a rapidly flushed system where biological production is primarily detritus based. Analysis of the '18S-28S' sequences specific for Mesodiniidae uncovered at least 5 variants of *M. rubra* within the Columbia River coastal margin, which includes the Columbia River estuary, Willapa Bay, and the adjacent continental shelves off Oregon and Washington, (Fig. 2, Tables 1 & 2). The existence of different variants of *M. rubra* was further confirmed by *M. rubra* partial '18S-28S' DNA region SC-PCR analysis since variants 'A', 'B' and

'C' were detected by this approach using morphologically identical (under light microscopy) *M. rubra* cells. Only one of the variants (labeled 'B' in Table 1) was implicated in estuary bloom formation during the summer in all 3 years included in this study. Why this particular strain of *M. rubra* is capable of bloom formation could be explained by its cryptophyte prey preference, or physiological adaptation to the conditions of the late summer estuary. The strain might be a highly proliferative variant, with distinct cell cycle control or which undergoes macronuclear differentiation involving heightened gene amplification, above that found in other variants. Although recent studies have shed light on the mechanisms of macronuclear development in ciliate species (Nowacki et al. 2008, Motl & Chalker 2009), there are currently no reported studies of the mechanisms that operate during macronucleus formation in *M. rubra*. Exploration of the nuclear dualism of *M. rubra* might provide clues to the highly proliferative nature of the bloom-forming variants of *M. rubra*.

Analyses of chloroplast 16S rRNA, cryptophyte RuBisCo and PSII D2 genes suggest that each year the bloom-forming variant of *Myrionecta rubra* contains the same chloroplast, that of the cryptophyte *Teleaulax amphioxeia* (Fig 3B). Furthermore, this specific association (*T. amphioxeia* chloroplast/bloom-forming variant of *M. rubra*) is sustained both as the bloom develops in Ilwaco harbor and when it is well-established in the estuary main channels. Existing cultures of *M. rubra* are able to ingest several different prey cryptophyte species with an apparent preference for cryptophytes belonging to Clade 2 (e.g. *T. amphioxeia*, *T. acuta* and *Geminigera cryophila*) (Park et al. 2007). Nevertheless, in accordance with our findings, Nishitani et al. (2010), using SC-PCR, detected nucleomorph sequences belonging to *T. amphioxeia* in all of the 114 *M. rubra* cells that they had collected in the coastal waters off Japan. Only 2 additional sequences (closely related to *T. acuta* and *G. cryophila*) were uncovered in 2 of the 114 *M. rubra* clones. The predominance of *T. amphioxeia* chloroplast sequences in *M. rubra* from both the Columbia River estuary and the coast of Japan suggests that in temperate regions either (1) *T. amphioxeia* chloroplasts might be the preferred cryptophyte prey or (2) *M. rubra* cells harbor a permanent *T. amphioxeia*-like endosymbiont. The only *M. rubra* culture of polar origin (McMurdo Sound, Antarctica; culture CCMP2563 from Bigelow Laboratory) for which we have partial '18S-28S' rDNA region sequences belonged to variant 'A' (Table 1); it shared only 94.8 to 96.3% similarity with our Columbia River bloom-forming variant 'B', and was fed polar cryptophyte *T. acuta*. Although it is presently unknown whether *T. acuta* is the favored cryptophyte prey of variant 'A' in natural environments, it is conceivable

that genotypically distinct *M. rubra* variants with relatively large '18S-28S' rDNA sequence differences contain/ingest different cryptophyte plastids.

In contrast to the chloroplast 16S rDNA data, comparatively few 18S rRNA gene sequences were related to *Teleaulax amphioxeia* (2 to 10%) (Fig 3C), suggesting that cryptophyte nuclei were largely absent from these bloom-forming *Myrionecta rubra* cells. This disagrees with findings from Johnson et al. (2007) who showed that in cultures of an Antarctic *M. rubra*, cryptophyte nuclei remained transcriptionally active, and from Hansen & Fenchel (2006) who, using transmission electron microscopy, documented the presence of cryptophyte nuclei in cultures of *M. rubra* cells from waters off Denmark. Preferential retention of cryptophyte chloroplasts over nuclei has, however, been suggested by Gustafson et al. (2000), who showed that *M. rubra* cells from the Antarctic culture that had not been fed for 14 d lacked cryptophyte nuclei but still possessed chlorophyll *a* after 17 additional days of starvation. Similarly, in the same *Myrionecta rubra* culture a regular decrease in prey nuclei per cell was observed during 10 wk of starvation (Johnson & Stoecker 2005). The respectively low and high proportions of *T. amphioxeia* nuclei and chloroplasts signals found in the *M. rubra* bloom of the Columbia River estuary in successive years seem to support the notion of an association (either endosymbiosis or kleptoplastidy) between *T. amphioxeia* chloroplasts and the bloom-forming *M. rubra* variant, with loss of cryptophyte nuclei from *M. rubra* in the main estuary channels during established bloom periods.

Given that our 18S rRNA clone libraries did not uncover cryptophyte sequences other than those related to *Teleaulax amphioxeia*, the small proportion of *T. amphioxeia* 18S rRNA gene sequences detected also indicates that free-living cryptophytes were not present in high abundance in the Columbia River estuary main channels in either 2007 or 2008 during the established bloom periods. This is supported by Flow-CAM observations of water samples collected during the *Myrionecta rubra* bloom period of September 2009 throughout the water column over a tidal cycle in the estuary north channel at the same location as sample 1 (Fig. 1) and by fluorescence microscopy cell counts of a red water patch collected in September 2010 in the south channel since neither analysis revealed the presence of a large number of free-living cryptophyte (data not shown). This implies either that *T. amphioxeia* may be a permanent endosymbiont or, in view of the absence of cryptophyte nuclei mentioned above, that cryptophyte chloroplasts are acquired elsewhere. Observations by Johnson et al. (2007) suggest that the *M. rubra* cells in their culture, grown at low temperature, can retain their cryptophyte chloroplasts for up to

ca. 30 d. Furthermore, Smith & Hansen (2007) have shown that *M. rubra* cells incubated at 15°C can not only persist during extended periods of starvation, but even perform 3 to 4 cell divisions after prey has been depleted. It seems probable that if *M. rubra* cells acquire cryptophyte prey, they do so in the estuary, but not within the main channels. In fact, free-living cryptophytes were detected by light microscopy in water collected in Baker Bay at Ilwaco harbor on 21 July 2009 (data not shown). Future studies would need to establish if Baker Bay is the main estuarine location where *M. rubra* ingests cryptophytes. It is essential to note that these observations do not lend more support for karyoklepty over endosymbiosis because cryptophyte ingestion remains a long-term requirement for optimum growth and photosynthesis for all *M. rubra* cells (Gustafson et al. 2000, Johnson & Stoecker 2005), even in *M. rubra* cultures that showed evidence of endosymbiosis whereby feeding likely provides essential growth factors (Hansen & Fenchel 2006).

Future studies should investigate the genetic diversity of free-living cryptophytes in the Columbia River coastal margin and especially focus on determining whether inherent characteristics of the bloom-forming *Myrionecta rubra* variant explain why this variant alone generates blooms in the Columbia River estuary or if it is the nature of the association between *Teleaulax. amphioxeia* chloroplast and the 'B' *M. rubra* variant that drives proliferation and bloom formation. In this context it is noteworthy that the only two other *M. rubra* partial '18S-28S' rDNA sequences that did not originate from the Columbia River coastal margin, but from Antarctica (culture CCMP2563 from Bigelow Laboratory) and the South Atlantic (NCBI Mesodiniidae sequence FJ032688; partial '18S rDNA-ITS only; Marande et al. 2009), were identical to variants 'A' and 'C', respectively (Table 1). Furthermore, the only two Mesodiniidae 28S rDNA partial sequences from the South Atlantic that are submitted to NCBI (FJ032687 and FJ032689; Marande et al. 2009) are also different. FJ032689 is identical to the partial 28S rDNA sequences of variant 'C', while FJ032687 is more similar to the partial 28S rDNA sequences of variant 'D' (with the exception of 1 nucleotide difference in position 479) (Table 2). Clearly the genetic diversity in *M. rubra* populations is likely not limited to the Columbia River coastal margin. Therefore, the controversy in the literature regarding the exact nature of the cryptophyte chloroplast/*M. rubra* association (endosymbiosis vs. kleptoplastidy) might be at least in part attributed to the genetic variability of *M. rubra* uncovered in this study.

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