A phylogeographical study of the toxic benthic dinoflagellate genus *Ostreopsis* Schmidt

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ABSTRACT

Aim *Ostreopsis* is a benthic and epiphytic dinoflagellate producing potent toxins widespread in tropical and warm temperate coastal areas world-wide. We tested the hypothesis that as it is benthic, it would show distinct biogeographical patterns in comparison with planktonic species. Here, we analyse sequence variability in ribosomal DNA markers to provide the first phylogeographical study of this toxic benthic dinoflagellate.

Location Mediterranean Sea, Atlantic Ocean, Pacific Ocean.

Methods Ribosomal DNA sequence data from partial nuclear LSU (D1/D2 domains) and 5.8S genes and non-coding internal transcribed spacer (ITS) regions were obtained from 82 isolates of *Ostreopsis* species, collected at 26 localities throughout the world. Molecular sequence data were analysed using maximum parsimony, maximum likelihood and Bayesian methods for phylogenetic inference. A statistical parsimony network was obtained based on concatenated LSU and 5.8S rDNA–ITS region sequences of the Mediterranean/Atlantic *Ostreopsis* cf. *ovata* isolates to infer haplotype distribution over their geographical range. Light epifluorescence microscopy analyses were performed on cultured and field *Ostreopsis* material for taxonomic identification, while laboratory experiments for encystment induction were carried out on selected *O.* cf. *ovata* isolates. Toxin assays of *Ostreopsis* species isolates were carried out using the haemolytic-based method.

Results Analyses based on single and concatenated ribosomal genes gave substantially similar results. The rDNA phylogeny revealed different clades corresponding to different species within the genus *Ostreopsis*. In the species *O.* cf. *ovata*, different genetic lineages were correlated with macrogeographical distribution. A network of haplotypes inferred from the Atlantic and Mediterranean isolates of *O.* cf. *ovata* revealed that these two areas might host a single panmictic population. The Atlantic/Mediterranean population of *O.* cf. *ovata* was differentiated considerably from the Indo-Pacific populations. Other species of *Ostreopsis* were found, but they turned out to be restricted to just one of the two main warm-water oceanic basins, the Mediterranean/Atlantic and the Indo-Pacific.

Main conclusions *Ostreopsis* cf. *ovata* was found to be widely dispersed throughout the coastal areas of tropical and some warm temperate seas. In the Atlantic/Mediterranean region it may constitute a panmictic population that is highly distinct from Indo-Pacific populations. *Ostreopsis* cf. *siamensis* was found

only in the Mediterranean Sea, and strains identified as *Ostreopsis lenticularis* and *Ostreopsis labens* were found only in the Indo-Pacific region.

Keywords

Atlantic Ocean, benthic dinoflagellate, distribution, Mediterranean Sea, *Ostreopsis*, Pacific Ocean, phylogeography, ribosomal genes.

INTRODUCTION

Protistan biogeography (sensu Caron, 2009) has been a highly controversial topic in recent years. Finlay (2002) suggested that species < 1 mm in size are probably dispersed throughout the world and only larger species have restricted geographical distributions, making the existence of endemic microbial species questionable (Finlay & Fenchel, 2004). Most microbial species are characterized by large population sizes and high dispersal potential, preventing isolation and allopatric speciation. Based on the morphological species concept, it has been suggested that dinoflagellate species have a widespread distribution, modified by latitude, and occur in both hemispheres (Taylor et al., 2007). The lack of geographical barriers and the mixing of waters due to wind, waves and currents should also facilitate the dispersal of marine plankton (Palumbi, 2003; Smayda, 2007).

Nevertheless, genetic studies have reported several examples of spatial differentiation in marine phytoplankton that contradict this assumption (Ryneckson et al., 2006; Medlin, 2007). As the result of major geological events, fragmentation of the gene pool within a species or species complex might have occurred in distinct oceanic regions, giving rise to genetically distinct endemic oceanic populations (Scholin et al., 1995; John et al., 2003). The toxic planktonic dinoflagellate *Alexandrium tamarens* 'species complex', which includes several clades with distinct geographical distributions based on ribosomal DNA analyses, is one of the world's most studied toxic microbial eukaryotes (Scholin et al., 1994; Lilly et al., 2007; Penna et al., 2008). Benthic microalgal species are expected to have a more varied pattern of geographical

distribution than planktonic species, as they are less vagile and are usually attached to non-motile substrates such as sand, rocks, corals or seaweed, while planktonic species are easily dispersed by currents. The dispersal dynamics of benthic protists throughout large basins may depend on passive transport of vegetative and/or resting cysts on floating and drifting objects, such as plastics or wood (Barnes, 2002; Masó et al., 2003), or in the ballast water of ships (Hallegraeff, 1993). Benthic protists include a number of dinoflagellate genera, such as the toxic genus *Ostreopsis*, which contains various morphological species that produce potent toxins (Yasumoto et al., 1987; Taniyama et al., 2003). These have been receiving increased attention recently from researchers and public authorities because their proliferation has been associated with human poisoning by toxic aerosols along the Mediterranean coasts (Ciminiello et al., 2008). The genus was first identified in tropical areas (Schmidt, 1901; Fukuyo, 1981; Faust, 1999). Later studies showed *Ostreopsis* species to be present also in the warm temperate seawaters of both hemispheres, including the Mediterranean Sea (Taylor, 1979; Vila et al., 2001; Aligizaki & Nikolaidis, 2006; Mangialajo et al., 2007), Japan (Nakajima et al., 1981) and New Zealand (Rhodes et al., 2002).

To date, resolving the taxonomy of *Ostreopsis* species based only on morphology has been difficult due to the morphological variability of both field material and cultured specimens (Penna et al., 2005). Further, none of the original isolates from tropical areas from which species of *Ostreopsis* species were described has yet been sequenced for the genotype assignment (see also Pin et al., 2001). Given that for many dinoflagellate genera there is a discrepancy between morphology-based taxonomy and genetic studies (Scholin et al., 1994; Penna et al., 2005), the species names applied to strains of different genetic lineages must be treated with caution. With respect to the Mediterranean Sea, molecular phylogenetic and morphological studies show that the *Ostreopsis* species isolates analysed group into two distinct species, *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis*, and that within each species all individuals share the same 5.8S rDNA sequence (Penna et al., 2005).

Tropical areas were once connected by a circum-equatorial current that was interrupted initially by the closure of the Tethys Sea, and finally by the formation of the Isthmus of Panama about 3 Ma. These processes led to the separation of the Tropical Indo-Pacific from the Atlantic, disrupting gene flow within many species (Knowlton et al., 1993; Avise, 2009). This isolation and the subsequent environmental changes (Haug & Tiedemann, 1998; Lunt et al., 2008) controlled the development of global species richness and caused vicariance in many groups of species, which today display patterns of genetic differentiation related to geography, as is clearly demonstrated in tropical coastal macroalgae (Kooistra et al., 1992). This is not the case for cold-water areas, connected by the Antarctic Circumpolar Current in the Southern Hemisphere and by the Arctic Ocean in the Northern Hemisphere, where there is substantial gene flow in many cold-water planktonic species (Griffiths et al., 2009).

In this study we analysed phylogeographical structure based on ribosomal genes within and among numerous *Ostreopsis* isolates of different species from various geographical regions, including new geographical locations with respect to our previous study (Penna et al., 2005). Our aim was to investigate whether the genus *Ostreopsis* displays contrasting biogeographical patterns in tropical and warm temperate areas. In particular, we examined several isolates of *O. cf. ovata* from warm-water regions to infer phylogeographical patterns within this morphospecies.

MATERIALS AND METHODS

Study area, strain isolation, culture and encystment conditions

Ostreopsis isolates were collected from several locations in the Mediterranean Sea, the western and eastern Atlantic Ocean, and the Pacific Ocean (Fig. 1), from net samples or as epiphytic microflora on selected red and brown algae (Rhodophyceae and Phaeophyceae). Cell isolation and strain-culturing conditions were as previously described by Penna et al. (2005). The strains used in this study are listed in Appendix S1 in the Supporting Information. Encystment was induced in selected cultures of *Ostreopsis* species through

inoculation in fresh F/2-Si medium (Guillard, 1975) depleted of nitrate (Anderson et al., 1985). The *Ostreopsis* species isolates were kept at cell concentrations of about 500 cells mL⁻¹ in sterile, polystyrene tissue-culture plates under the experimental conditions indicated above, with the photoperiod adjusted to a 12:12 h light:dark regime. A crossing experiment was also conducted between the intercross *O. cf. ovata* CNR-A1 and *O. cf. ovata* CNR-D1 (250 + 250 cells mL⁻¹), to investigate possible heterothallism in *O. cf. ovata*.

Morphological analysis by microscopy

Field samples containing *Ostreopsis* species and cultured specimens were analysed under light microscopy after staining with Fluorescent Brightener 28 (Sigma, St Louis, MO, USA), following Fritz & Triemer's (1985) technique, as indicated in Penna et al. (2005).

DNA extraction, amplification and sequencing

Genomic DNA was extracted from 10 mL cultures in logarithmic growth phase using the DNeasy Plant Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of 5.8S–ITS and LSU ribosomal genes has been described in Penna et al. (2008) and Fraga et al. (2008). The cloning of amplified PCR fragments and sequencing were carried out as in Penna et al. (2008). Alignments were made using ClustalX2 (Larkin et al., 2007) with default settings, and subsequently rechecked by eye and edited manually; unalignable regions were excluded before the phylogenetic analyses. The sequences were deposited in EMBL (European Molecular Biology Laboratory) and are listed in Appendix S1.

Molecular data analyses

Modeltest ver. 3.6 (Posada & Crandall, 1998) was used to determine the evolutionary model that best fitted the data according to Akaike's information criterion; the two-phase invariable model (TIM) with gamma distribution for among-site rate variation was selected for the LSU rDNA; while the two-phase

variable model (TVM) substitution model with gamma distribution for among-site rate variation was used for the 5.8S–ITS rDNA. Maximum parsimony (MP) analyses were performed using heuristic searches with tree bisection–reconnection branch-swapping. Branches were collapsed if their minimum length was 0; ambiguities and gaps were treated as missing data. The robustness of the MP tree was determined by bootstrapping with 1000 pseudoreplicates. Analyses of the concatenated sequences were performed with the same settings, the only exception being the use of a general time reversible (GTR) substitution model with among-site rate variation. These analyses were conducted with PAUP* ver. 4.0b10 (Swofford, 2002). Maximum likelihood (ML) analyses were run with RAxML (Randomized Axelerated Maximum Likelihood) software ver. 7.0.4 (Stamatakis et al., 2005), which adopts a GTR substitution model and allows for estimation of several parameters, such as the proportion of invariant sites and alpha values of the gamma distribution for among-site rate variation. ML analyses were conducted on the LSU and 5.8S–ITS rDNA sequences separately and on the concatenated sequence. Bootstrap values were calculated with 1000 pseudoreplicates.

Bayesian analyses were performed using MrBayes ver. 3.1.2 with the following settings: four Markov chains were run for 2,000,000 generations with a sampling frequency of 100 generations. Log-likelihood values for sampled trees were stabilized after almost 200,000 generations. The last 18,000 trees were used to estimate Bayesian posterior probabilities, while the first 2001 were discarded as burn-in. Results from two independent runs were used to construct a majority-rule consensus tree containing the posterior probabilities (Ronquist & Huelsenbeck, 2003). Bayesian inferences (BI) were performed on both separate and concatenated sequences. The sequences of *Coolia monotis* VGO783, FN256432 and AM902747 were used as outgroups for the 5.8S–ITS and LSU ribosomal gene phylogeny.

A statistical parsimony network (Templeton et al., 1992) was obtained based on concatenated LSU and 5.8S–ITS rDNA sequences of the Atlantic/Mediterranean *O. cf. ovata* strains with TCS ver. 1.18 software (Clement et al., 2000). Standard and molecular diversity indices were calculated with

Arlequin ver. 3.0 (Excoffier et al., 2005) and mega 4.1 (Tamura et al., 2007) software. Standard deviations were obtained by 10,000 bootstrap pseudoreplicates.

Patterns of gene flow between Atlantic Ocean and Mediterranean Sea isolates were investigated using two different coalescent-based approaches within a Bayesian framework. A non-equilibrium model, implemented in IM software (Hey & Nielsen, 2004), was used with the following setting: 50 Metropolis-coupled Markov chains were run for 10,000,000 genealogies under a geometrical heating scheme with a burn-in of 1,000,000 and a uniform prior. Results were drawn from three independent runs.

Toxin analysis

Extraction of a palytoxin-like compound from cells of *Ostreopsis* species was carried out according to the procedure described in Riobó et al. (2006). The toxicity of methanol extracts was proven by haemolytic assay as previously described (Riobó et al., 2008). The palytoxin standard was obtained from Wako Chemicals (Germany). No differences between the chemical structures of the toxins could be detected based on the methods used in this study.

RESULTS

Morphological analyses: motile and resting stages

Light microscopy analyses of natural populations of *Ostreopsis* species showed a wide distribution of *O. cf. ovata* in Mediterranean waters, in contrast with the limited occurrence of *O. cf. siamensis* (Appendix S1). Field material and, in particular, cultured material showed high morphological variability. Cell size and shape were also quite variable and very small cells, probably gametes, appeared together with bigger cells in the same clonal culture. In a previous study (Penna et al., 2005) the two main groups were named *O. ovata* and *O. cf. siamensis*, based on their original descriptions, but there is no guarantee that they correspond to the same genetic species, as no gene sequences are

available for the holotype strains. In this study our isolates have been given the names *O. cf. ovata* and *O. cf. siamensis* (Fig. 2a–c) for the time being, pending a complete revision of the taxonomy of the genus *Ostreopsis* based on morphology and genetics. Analyses of cultured assemblages of *Ostreopsis* species suggest that this genus and other dinoflagellates produce resting stages. Resting stages were found by crossing *O. cf. ovata* strains from different geographical areas, as well as by single clonal cultures of both species. Encysted stages with granular cytoplasmic components, double walls or outer envelopes, were observed in cultures grown in F/2 medium after depletion of inorganic nitrogen (Fig. 2d).

Molecular analyses

The final alignments of *Ostreopsis* species sequences with *C. monotis* VGO783 as outgroup were as follows: 5.8S–ITS was 364 bp in length (C 18.75%, T 34.81%, A 26.39%, G 20.06%) with 288 polymorphic sites and a transition/transversion ratio of 1.4; LSU was 666 bp in length (C 18.04%, T 32.26%, A 28.19%, G 21.51%) with a transition/transversion ratio of 1.2 and 514 polymorphic sites; and the concatenated ITS–5.8S–LSU was 1030 bp in length (C 18.36%, T 33.08%, A 27.61%, G 20.95%) with 800 polymorphic sites and a transition/transversion ratio equal of 1.2.

There were only minor differences between MP, ML and BI analyses, therefore only the ML phylogenetic tree is presented (Fig. 3). The 5.8S–ITS rDNA phylogeny (Fig. 3a) showed that the most basal lineage included two species of *Ostreopsis*: *Ostreopsis lenticularis* and *Ostreopsis labens*. The 5.8S–ITS rDNA phylogeny identified three major clades within the genus *Ostreopsis*: the first comprised *O. labens* and *O. lenticularis*; the second contained all the *O. cf. siamensis* isolates and *Ostreopsis* sp. VGO881; while the third comprised *O. cf. ovata* from the Mediterranean Sea and the Atlantic Ocean and *O. cf. ovata* from the Indo-Pacific. All nine Mediterranean isolates of *O. cf. siamensis* shared the same sequence. This *O. cf. siamensis* cluster formed a sister clade with an isolate of *Ostreopsis* sp. VGO881 from the Canary Islands. The second major lineage in the genus consisted of three well-resolved groups of *O. cf. ovata* from

the Indo-Pacific and the Mediterranean/Atlantic, strongly supported by high bootstrap values (100% for MP and 96% for ML) and posterior probability (0.98). The first group consisted of isolates of *O. cf. ovata* from Malaysia (Malacca Straits) and Indonesia, the four Indonesian *O. cf. ovata* isolates sharing the same sequences. The other two groups included isolates of *O. cf. ovata* from Malaysia (South China Sea) and from the Mediterranean Sea and Atlantic Ocean (Canary Islands, Madeira and Brazil). The latter grouping was supported by high bootstrap (81% for MP and 100% for ML) and posterior probability (1.00) values. The Mediterranean and Atlantic *O. cf. ovata* isolates showed a high degree of sequence homology.

The LSU rDNA phylogeny (Fig. 3b) showed some differences in tree topology compared with 5.8S–ITS rDNA analysis. The first split from the outgroup was constituted by the isolate *Ostreopsis* sp. VGO881, while the second was constituted by the same strain of *O. lenticularis* that formed the basal divergence in the 5.8S–ITS rDNA phylogeny, supported by high bootstrap (100% for MP and ML) and posterior probability (1.0) values. Furthermore, another two lineages were delineated by LSU phylogeny and supported by high bootstrap and posterior probability values: one group included *O. cf. siamensis* from the Mediterranean Sea; the other consisted of *O. cf. ovata* from the Mediterranean Sea and Atlantic Ocean (Canary Islands, Madeira Island and the Brazilian coast), Malaysia and Indonesia. The Mediterranean and Atlantic *O. cf. ovata* clade also showed high homology within the LSU phylogeny. The phylogenetic tree based on the concatenated ITS–5.8S and LSU rDNA sequences (Fig. 4) comprised 55 different isolates, whereas 78 and 66 isolates were employed for 5.8S–ITS and LSU phylogenetic inference, respectively. The patterns retrieved from concatenated ITS–5.8S–LSU and LSU were very similar with only very slight differences, such as the positioning of *O. cf. ovata* PR04 from Malaysia. While this isolate was placed as sister clade of the Atlantic/Mediterranean cluster in the ITS–LSU phylogeny, in the LSU phylogeny it was more closely related to the *O. cf. ovata* isolates from Indonesia. All the clusters were supported by high bootstrap and posterior probability values.

A network was inferred within the *O. cf. ovata* clade formed by isolates from the Mediterranean and Atlantic based on the 5.8S–ITS and LSU combined sequences (Fig. 5). Nucleotide sequences were aligned to form a 981 bp fragment. A total of 26 haplotypes and 116 polymorphic sites were found, with 96 substitutions (transition/transversion ratio of 0.86) and 36 indels. Within the Mediterranean and Atlantic *O. cf. ovata* clade, the different haplotypes were separated by a maximum of 78 mutational steps from the most frequent one, CBA-C, which was found in eight isolates. Across this spectrum of variation there was no apparent pattern of correlation with the geographical distribution of the isolates: isolates from the Mediterranean Sea and the Atlantic coasts of Spain and Brazil were widespread throughout the network. Some isolates, such as VGO614, CBA-T, CBA-P, OS10BR and OS04BR, were particularly divergent, being separated by as many as 34–78 mutational steps from the most frequent haplotype, CBA-C.

Molecular diversity among isolates (Table 1; Appendices S2 and S3) was assessed by means of the net number of nucleotide differences, based on 5.8S–ITS, LSU and concatenated ITS–5.8S–LSU genes. 5.8S–ITS rDNA sequences showed the species *O. cf. siamensis*, *O. cf. ovata*, *O. lenticularis* and *O. labens* to be highly differentiated, with the greatest divergence between Atlantic/Mediterranean *O. cf. ovata* isolates and Malaysian/Indonesian *O. cf. ovata* isolates. LSU diversity was high among *O. cf. siamensis*, *O. cf. ovata* and *O. lenticularis*, but differentiation was lower among Atlantic/Mediterranean and Indo-Pacific *O. cf. ovata* isolates than for the 5.8S–ITS rDNA sequences.

The two coalescent-based Bayesian methods for inferring gene flow between the Mediterranean Sea and Atlantic Ocean groups of isolates showed migration in both directions, but they could not explain whether the migration rate was higher from the Atlantic Ocean to the Mediterranean Sea or vice versa (data not shown).

Toxin analysis

The haemolytic assay (inhibition of haemolysis in the presence of ouabain, a palytoxin antagonist) indicated unambiguously the presence of palytoxin-like compounds in all the analysed extracts of *Ostreopsis* species.

DISCUSSION

Over the past few decades, molecular-based phylogenetic studies have been used by biogeographers to reconstruct the causal connections between evolutionary processes and the ecological attributes of taxa and biota. The role of molecular genetics is becoming increasingly crucial in shaping the future of integrative biogeography (Riddle et al., 2008).

In this study, phylogenetic analyses of three nuclear molecular markers, LSU, 5.8S and the non-coding ITS regions of the ribosomal multi-gene family, showed that the genus *Ostreopsis* is composed of different genetic lineages corresponding to different species, and that this held even when the sample collection size was increased and included samples from very distant localities.

All *Ostreopsis* isolates were found to be toxic. Some of the *Ostreopsis* strains examined in the present study were analysed by liquid chromatography–mass spectrometry (LC–MS) in a previous study (Penna et al., 2005), and no differences in their chemical toxin profiles were found. However, there may be differences in the chemical structure of the palytoxin complex produced by *Ostreopsis* (Usami et al., 1995; Tan & Lau, 2000; Lenoir et al., 2004), and it is possible that the differences in the molecular analyses found in this study correspond to chemical differences in the toxin profiles. To test this hypothesis further, chemical assays need to be carried out with all the *Ostreopsis* isolates.

Conserved LSU and 5.8S ribosomal genes and more polymorphic and neutral ITS regions were found to be useful in delineating the phylogenetic position of the *Ostreopsis* species and the preliminary phylogeographical pattern within the species *O. cf. ovata*. A total of 126 ribosomal sequences of strains collected from several new localities, mainly in the Mediterranean Sea, but also world-wide, such as the Brazilian coast in the south-west Atlantic Ocean, the Canary

Islands and Madeira in the north-east Atlantic Ocean, and the north of the Sulawesi Peninsula (Indonesia) and Malaysia in the Indian and east Pacific Oceans, were analysed. Phylogenetic analyses based on individual and concatenated LSU, 5.8S genes and ITS regions were consistent, showing that within the genus *Ostreopsis* four main clades can be identified: (1) a clade represented by a single isolate of *Ostreopsis* sp. VGO881; (2) a clade constituted by *O. lenticularis* and *O. labens*; (3) a clade constituted by *O. cf. siamensis*; and (4) a final clade comprising *O. cf. ovata*.

The two strains in clade (2) were deemed by different authors to belong to two distinct species, *O. lenticularis* and *O. labens*; however, our phylogenetic analyses proved that they clustered together and their identification should be revised. Furthermore, the clade with all nine isolates of *O. cf. siamensis* from the Mediterranean Sea had no isolates from the Indo-Pacific region, where *O. siamensis* was originally described by Schmidt (1901); this raises the possibility that these Mediterranean isolates may represent a different taxonomic unit.

Species of the genus *Ostreopsis* are conspicuous when field samples are observed by appropriate methods, so they can be considered as ‘flagship taxa’ in the sense provided by Tyler (1996) and Foissner (2006), given that they are very showy and difficult for an experienced observer to overlook. In this study, isolated *Ostreopsis* cells were almost always grown successfully in culture and then sequenced; we therefore consider our sampling to be representative of the location and, presumably, this is the case for isolates from other localities with sequences in GenBank.

Within the genus *Ostreopsis*, *O. cf. ovata* isolates were widely dispersed from the western to eastern Atlantic basin, throughout the Mediterranean Sea, in the Indian Ocean and in the eastern Pacific. Phylogenetic analyses placed these *O. cf. ovata* isolates into the Mediterranean/Atlantic clade; the Malacca Strait clade (recovered with only 5.8S–ITS sequences); and the South China Sea and Celebes Sea clades. This genetic differentiation within *O. cf. ovata* can be broadly linked to the geographical origin of the isolates. In particular, for Atlantic/Mediterranean *O. cf. ovata*, the connection between the two warm

tropical sides of the Atlantic Ocean is maintained by the Canary and Equatorial Currents in an east–west direction, and by the Gulf Stream/Azores Currents and North Equatorial Counter Current from west to east (Pingree et al., 1999; Tomczak & Godfrey, 2003). Furthermore, *Ostreopsis* species produce resting stages and these might be able to survive dispersal more successfully than vegetative stages. If a drifting object containing living cells or cysts of *Ostreopsis* species crosses the Atlantic on ocean currents, that population could easily disperse along the whole coast, but material is less likely to reach a small island such as Madeira. This could explain the slight genetic differences in the Madeira *O. cf. ovata* clade compared with the Mediterranean/Canarian/Brazilian *O. cf. ovata* clade. The Canary Islands are relatively close to the African coasts and in the middle of the Canary Current that flows along the north African coast, which may explain the lack of differentiation there.

The Atlantic Ocean and the Mediterranean Sea can be regarded as a single, huge geographical area connected by the Straits of Gibraltar, whereas the Asian isolates originate from two different areas of the Indo-Pacific region: the Indian Ocean, including the Malacca Straits (Malaysia); and the Pacific Ocean, comprising the northern coast of Malaysia in the South China Sea and the Celebes Sea (Indonesia).

Within *O. cf. ovata*, a clear genetic distinction was observed between the Atlantic/Mediterranean and the Indo-Pacific isolates. One possible explanation of this scenario could be the formation of the Isthmus of Panama about 3 Ma, which would have separated *Ostreopsis* species and populations into two tropical-to-temperate geographical regions: the Atlantic, including the Caribbean and Mediterranean Seas; and the Indo-Pacific. This separation caused an increase in surface salinity in the Caribbean, enhanced the flow of the Gulf Stream, and possibly intensified Northern Hemisphere glaciations (Haug & Tiedemann, 1998). These changes in oceanic circulation triggered by temperature and salinity suggest that ecological differentiation of *Ostreopsis* species is likely to have occurred on both sides of the Isthmus of Panama, allowing geographical and subsequent genetic differentiation of the populations

of *O. cf. ovata*. The Atlantic/Mediterranean, Indian and Pacific Ocean *O. cf. ovata* clades can safely be considered as distinct populations, which probably evolved separately over a long period. Greater genetic divergences were observed between morphospecies, although more isolates from each morphotype are required for better analysis of their evolutionary relationships.

The results of the statistical parsimony analysis of the Atlantic/Mediterranean *O. cf. ovata* based on the concatenated ITS–5.8S–LSU sequences showed at least three main haplotype groups. However, this grouping has no clear correspondence with the geographical origin of the different isolates. Each cluster, in fact, comprises isolates deriving from different and distant geographical areas, such as the western and eastern coasts of the Atlantic Ocean. These three groups of *O. cf. ovata* might be considered part of a unique, large, panmictic population with no clear geographical barriers inhibiting gene flow. The connection between the Atlantic Ocean and the Mediterranean Sea through complex current systems (Millot, 2005) could be the driving force behind shaping the current phylogeographical pattern of *O. cf. ovata* in this area. These current systems favour high dispersal rates even over large distances (Patarnello et al., 2007), and this would explain the close relationships between the Atlantic and Mediterranean isolates.

Apart from *Ostreopsis*, other phytoplankton groups show geographical patterns world-wide. Genetic studies have shown that a greater number of genetically distinct geographical clades are present within the same morphotype species, with identical or only slightly different morphologies, such as the *Alexandrium tamarense* ‘species complex’ (John et al., 2003; Lilly et al., 2007). Group I of this species complex appears to be widely dispersed at high, cold latitudes of the North and South Atlantic and Pacific oceans, while Group II and Group IV seem to be restricted to the warm, temperate seas of the Mediterranean and Asia; in particular, Group II is confined to the Mediterranean Sea, as is also the case for *O. cf. siamensis*. Furthermore, in marine diatoms, *Skeletonema* includes different species with distinct biogeography (Kooistra et al., 2008); *Skeletonema japonicum* occurs in cool temperate coastal regions, *Skeletonema grethae* is found only on the Atlantic coast of the USA, and *Skeletonema ardens*

has a tropical distribution similar to that of *O. labens* and *O. lenticularis*. Even the tiny planktonic warm-water prokaryote *Prochlorococcus* has been observed to have phylogeographical structure: due to their large populations and short generation times *Prochlorococcus* cells evolve more quickly than ocean currents can mix them, which results in locally distinct microdiversity (Martiny et al., 2009). On the other hand, Cermeño & Falkowski (2009) argued on the basis of the morphological species concept that marine planktonic diatoms are dispersed world-wide.

There are significant geographical barriers between the tropical Atlantic and the tropical Pacific constituted by continents or cold-water currents. Thus, the most studied free-living microbial eukaryotes (i.e. within picoplankton groups) can exhibit spatial genetic diversity, as demonstrated by different studies (Massana et al., 2002; Guillou et al., 2004; Viprey et al., 2008). Molecular data are essential in delineating microbial biogeography, as has long been recognized for macroorganisms (Avice & Aquadro, 1982).

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Biosketch

Antonella Penna and her co-authors study taxonomic, phylogenetic and phylogeographical questions concerning marine phytoplankton and microphytobenthos, with a particular focus on toxin-producing species. The first author is also one of the members of Bentox-net, an Italian network for the study of *Ostreopsis* species and other potentially toxic benthic microalgae (<http://www.bentoxnet.it>).

Author contributions: A.P., S.F. and C.V. conceived the ideas and wrote the manuscript; S.F. and M.G.G. performed the morphological analyses; C.B., S.C. and A.P. conducted the molecular analyses; P.R. did the toxin analyses; A.P. and C.V. analysed the molecular data.

Figure 1 Geographical distribution of the genus *Ostreopsis* (Mollweide's map projection). Open circles represent localities where the species were recorded based only on morphology. Filled circles indicate samples sequenced in this study or by other authors.

Figure 2 *Ostreopsis* cf. *siamensis* and *Ostreopsis* cf. *ovata* from cultures. Light microscopy (LM)–phase contrast: (a) epithelial view of *O.* cf. *siamensis* CNR-T5 from Italy, Mediterranean Sea; (b) hypothecal view of *O.* cf. *siamensis* CNR-T5. LM–epifluorescence: (c) epithelial view of *O.* cf. *ovata* CBA-6 from Indonesia, Pacific Ocean. LM–phase contrast: (d) *O.* cf. *ovata* cyst stage obtained by crossing isolate CNR-A1 with isolate CNR-D1. Scale bar, 10 μ m.

Figure 3 (a) Maximum likelihood (ML) phylogenetic tree of the genus *Ostreopsis* based on the ITS region and 5.8S gene sequences; (b) ML phylogenetic tree of the genus *Ostreopsis* based on the D1–D2 domains of the LSU gene sequences. Numbers on the major nodes represent, from left to right, maximum parsimony (MP) (1000 pseudoreplicates), ML (1000 pseudoreplicates) bootstrap values and Bayesian posterior probability values. Only bootstrap values > 50% are shown. Asterisks at the major nodes mark discrepancies between the different methods. The trees were rooted using *Coolia monotis* VGO783 (FN256432 and AM902747) as outgroups for ITS–5.8S and LSU sequences

Figure 4 Maximum likelihood (ML) phylogenetic tree of the genus *Ostreopsis* based on the ITS–5.8S–LSU gene concatenated sequences. Numbers on the major nodes represent, from left to right, maximum parsimony (MP) (1000 pseudoreplicates), ML (1000 pseudoreplicates) bootstrap values and Bayesian posterior probability values. Only bootstrap values > 50% are shown. The tree was rooted using *Coolia monotis* VGO783 (FN256432 and AM902747) concatenated sequences as outgroups for the ITS–5.8S and LSU genes.

Figure 5 Statistical parsimony network of haplotypes of *Ostreopsis* cf. *ovata* species isolates based on concatenated ITS region, 5.8S and LSU ribosomal gene sequences. The sizes of the circles are proportional to the number of

isolates found to have that haplotype. Small closed circles indicate missing haplotypes.

Table 1 Molecular diversity indexes in the 5.8S–ITS rDNA gene among and within isolates of *Ostreopsis* species from the Mediterranean Sea/Atlantic coasts (Med./Atlantic) and Indo-Pacific areas.

Figure 1

Figure 2

Table 1

Species	Ostreopsis cf. siamensis	Ostreopsis cf. ovata (Med./Atlantic)	Ostreopsis cf. ovata (Malaysia)	Ostreopsis cf. ovata (Indonesia)	Ostreopsis lenticularis	Ostreopsis labens
Ostreopsis cf. siamensis (n = 9)	0					
Ostreopsis cf. ovata (Med./Atlantic) (n = 47)	32.8 ± 2.44	0.61 ± 0.11				
Ostreopsis cf. ovata (Malaysia) (n = 12)	31.64 ± 2.32	7.82 ± 1.35	3.39 ± 0.57			
Ostreopsis cf. ovata (Indonesia) (n = 4)	30.7 ± 2.48	10.39 ± 1.55	6.84 ± 1.32	0		
Ostreopsis lenticularis (n = 1)	47.37 ± 2.77	44.88 ± 2.62	40.51 ± 2.49	41.49 ± 2.59	n.a.	
Ostreopsis labens (n = 1)	47.06 ± 2.78	44.88 ± 2.62	40.74 ± 2.49	41.49 ± 2.59	0.93 ± 0.53	n.a.

Data are mean pairwise nucleotide differences within isolates (on the diagonal), and net pairwise differences between isolates (below diagonal) expressed as percentages. SD calculated on 10,000 bootstrap replicates.

n.a., not applicable.