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Phenological and molecular studies on the introduced seaweed *Dictyota cyanoloma* (Dictyotales, Phaeophyceae) along the Mediterranean coast of the Iberian Peninsula

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Abstract

Dictyota cyanoloma, a distinctive brown algal species characterized by a blue-iridescent margin, was recently reported as an introduced species in the Mediterranean Sea but little is known about its distribution dynamics, morphological plasticity and genetic structure. In this integrative study, we evaluate its past and present occurrence along the Mediterranean Iberian coast, assess the species' phenology in Palamós (Girona, Spain) and analyze the haplotype diversity by sequencing 49 individuals from nine sampling sites for different chloroplast and mitochondrial DNA regions. Although *D. cyanoloma* currently occurs along all the Mediterranean Iberian coasts (in 19 of 36 localities sampled, mostly in marinas and harbour environments), we were not able to find any herbarium material of this species (at BCN-Phyc and MA) predating the year 1987. In Palamós, *D. cyanoloma* is present all through the year, with a maximum development in winter and a minimum in summer. Fertile specimens are absent during summer (July and August). Sporophytes are dominant from January to June and gametophytes were found only in February, March and June. Information about the antheridia, which has never been described before, is provided. Two chloroplast and three mitochondrial haplotypes were observed, indicating that multiple introductions of *D. cyanoloma* occurred in the study area. Additionally, the genetic structure suggests that spread did not occur through simple advancing wave fronts but by several long-distance dispersal events. Further studies employing microsatellite markers could potentially offer a better resolution to unravel expansion and colonisation dynamics of *D. cyanoloma* in the Mediterranean Sea.

Keywords: Dictyota, Iberian coast, introduced species, phenology, phylogeography.

Introduction

The Mediterranean Sea is considered to be one of the most anthropogenically affected water masses in the world (Coll *et al.*, 2012). As an example, nearly 1000 alien marine species have been reported to date (Zenetos *et al.*, 2010; Hoffman, 2014). Non-indigenous marine species are introduced into new areas primarily as a result of accidental transport via culture of exotic shellfish or shipping (by fouling or ballast tank emptying) (Boudouresque & Verlaque, 2002; Ribera Siguan, 2003; Streftaris *et al.*, 2005; Zenetos *et al.*, 2006; Galil, 2009). In this context, more than 115 introduced benthic algae and seagrass taxa have been found in the Mediterranean Sea (Zenetos *et al.*, 2010; Mineur *et al.*, 2015; Verlaque *et al.*, 2015).

The genus *Dictyota* J.V.Lamour (Dictyotales, Phaeophyceae) was established by Lamouroux (1809) and 91 specific and infraspecific taxa are currently accepted (Guiry & Guiry, 2016). Six species were recognized as indigenous in the Mediterranean Sea (Tronholm *et al.*, 2010; Cormaci *et al.*, 2012), including the recently described *Dictyota cyanoloma* Tronholm, De Clerck, Gómez Garreta & Rull Lluch in Tronholm *et al.* (2010). This species was first reported on the Mediterranean Iberian coast by Rull Lluch *et al.* (2007) as *Dictyota ciliolata* Sonder ex Kützing, on the basis of several specimens collected at Barcelona, Sitges, Palamós, and Sancti Petri. Later, Tronholm *et al.* (2010) demonstrated that despite morphological similarity, the Iberian specimens belonged to a new species which they described as *D. cyanoloma.*

The type of *D. cyanoloma* was a specimen from Palamós harbour (Girona, Spain), and new records of *D. cyanoloma* from the Canary Islands, Morocco, Madeira, Açores, Southern Portugal, Aegean coast, Adriatic Sea, French Mediterranean, Great Brittany and Spanish North Atlantic coasts were soon reported (Taskin, 2013; Bárbara *et al.*, 2014; Santolaria, 2014; Steen *et al.*, 2016). Steen *et al.* (2016), based on phylogenetic affinities, haplotype networks and global distributions of *Dictyota* species, concluded that the species is non-native in the Mediterranean and North Atlantic regions and indicated Australia as its native region. Steen *et al.* (2016) also found herbarium records of *D. cyanoloma* from the Adriatic Sea dating as early as 1935, questioning whether the species remained unreported despite being widespread in the Mediterranean or whether it only spread recently from the Adriatic Sea.

In this study we aim to provide a deeper insight into the dynamics and genetic diversity showed by local populations of *D. cyanoloma* on the Mediterranean coast of the Iberian Peninsula. We therefore conducted: (i) a screening of the principal Spanish herbaria looking for historical records of this species; (ii) a large scale survey of the most important harbours from the Mediterranean Iberian coast to describe the current distribution of *D. cyanoloma* in the region where the species was first reported; (iii) a one year detailed phenology study of one population from Palamós harbour (Girona, Spain) to characterise the sporophytic and gametophytic reproduction; and (iv) a phylogeographic study in order to assess the variability of *D. cyanoloma* across the Mediterranean Iberian coast.

Materials and Methods

Herbaria screening and distribution survey

Looking for evidence of historical records of *D. cyanoloma* in Spain, we screened the BCN-Phyc herbarium (Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona) and the MA herbarium (Real Jardín Botánico de Madrid - CSIC Herbarium), which hold the most important collection data of seaweeds from the Mediterranean coast of the Iberian Peninsula. Specifically, we surveyed all the material of *Dictyota* species (mainly *D. dichotoma* (Hudson) Lamouroux) that could have been misidentified as *D. cyanoloma*.

To characterise the current distribution of *D. cyanoloma* along the Mediterranean coasts of the Iberian Peninsula, and taking into account that virtually all its citations were from harbours, the most important fishing, leisure and commercial harbours throughout the area were visited. In total, 36 harbours were explored from Llançà (Girona) to Algeciras (Cádiz) mainly during winter and spring of 2013 and 2014 (Table 1). The presence or absence of *D. cyanoloma* in each harbour was assessed, collecting some specimens from those harbours in which the species was present. All samples were preserved in 4% formalin in seawater and / or pressed on herbarium sheets and deposited in the BCN-Phyc herbarium (Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona, Spain).

Phenology and morphological-anatomical study

The type locality of *D. cyanoloma* (Palamós harbour) was selected to carry out the study. In this harbour, the seaweed grows on rocks in a shallow area (0.5 m depth) with low hydrodynamism. Monthly sampling was carried out from December 2012 to December 2013. Each month two samples were collected by scraping off all vegetation

from two squares of $10 \times 10 \text{ cm} (100 \text{ cm}^2)$ using a chisel and a hammer. For each specimen of D. cyanoloma, the thallus length, the number of dichotomies from the base to the apex and the width of the third dichotomy were measured as comparative morphological features. The reproductive stage was also assessed by recording the presence/absence of reproductive structures. For each quadrat, the total abundance of D. cyanoloma was quantified. The abundance of specimens of each reproductive type (sterile, fertile sporophytes and fertile male and female gametophytes) was also quantified measuring the cover of the horizontal surface in cm² after spreading the algal thalli in a laboratory tray (Ballesteros, 1986). For the anatomical study of the male specimens, transverse sections were made by hand with a razor blade. Photographs were taken with an AxioCam ERc 5s (Carl Zeiss) digital camera coupled with either a Nikon Eclipse 50i microscope or a Zeiss Stemi 2000-C stereomicroscope. Field photographs were taken with a Nikon Coolpix 4500.

Sampling for DNA extraction and marker selection

Specimens from nine localities (harbour environments) were sampled along the Mediterranean coast of the Iberian Peninsula (Table 1). From each sampling site, five to seven individuals were collected and preserved in silica gel.

Total genomic DNA was extracted following the CTAB method (Doyle & Doyle, 1987) with modifications (Soltis et al., 1991; Cullins, 1992). Following preliminary polymorphism test of several nuclear, mitochondrial and chloroplast markers, the regions cox1, nad6-nad11 and atp9-orf11 of the mtDNA and the region rbcL-rbcS of the cpDNA were chosen for further sequencing of multiple individuals per sampling site (Table S1). Nucleotide sequencing was carried out at the Centres Científics i Tecnològics of the Universitat de Barcelona on an ABI PRISM 3700 DNA analyser (PE Biosystems, Foster City, CA, USA). The sequences were edited with Chromas LITE v2.01 (Technelysium Pty, Tewantin, Australia) and manually aligned in Bioedit (Hall, 1999). See Table S2 of the supporting information for Genbank accession numbers.

Analysis of haplotype variation and geographic inference

Molecular diversity indices, such as number of haplotypes (n), number of polymorphic sites (s), haplotype diversity (Hd) and nucleotide diversity (π) for each sampling site were calculated in DnaSP 4.1 (Librado & Rozas, 2009). Additionally, we constructed two haplotype networks - one for the mtDNA dataset and the other for cpDNA dataset - using the software TCS v. 1.21 (Clement *et al.*, 2000). The partitioning of the genetic diversity among and within *D. cyanoloma* localities was evaluated by molecular variance analysis (AMOVA) using ARLE-QUIN v. 3.5 (Excoffier & Lischer, 2010). ARLEQUIN

Code	Province / Harbour	Coordinates		Date of visit / sampling			
Cout	Cirona	Cu	orumates	Date of visit / sampning			
TTA	$\frac{1}{1}$	4000015 74"NI	2º 0'47 12"E	27/00/2013			
DOP	Liança $(1)(8)$	42 22 23.74 IN,	3 9 47.12 E	27/03/2013			
FOR	Poit de la Selva (*)	42°20 29.34 N,	3°12 10.85 E	24/05/2015			
ESC	L Escala (*)	42° / 8.67 N,	3° 8'46.72"E	25/05/2013			
RUS	Roses (*)	42°15′15.04″N,	3°10′51.58″E	12/06/2013			
EST	L'Estartit (*)(§)	42° 3'10.21"N,	3°12′18.63″E	25/03/2013			
PAL		41°50′43.62″N,	3° / 39.39 E	19/12/2012, 16/01/2013, 15/02/2013,			
	$\mathbf{D}_{\mathbf{r}}$			25/05/2012,18/04/2013, 06/05/2013, 16/06/2013, 22/08/2013			
	Palamos $(*)(g)$			20/09/2013, 15/10/2013, 28/11/2013,			
				28/12/2013			
STF	Sant Feliu de Guíxols (*)	41°46'48 60"N	3° 1'57 46"E	16/01/2013			
BLA	Blanes (*)	41°40'31 96"N	2°47'57 55"F	15/02/2013			
	Barcelona	11 10 51.90 14,	2 11 51.55 E				
ADM	Arenys de Mar	11°31'12 18"N	2°33'33 68"E	08/02/2013			
MAT	Mataró (*)	41 34 42.46 N,	2 33 35.08 E	08/04/2013			
PRE	Premià de Mar	41 51 45.47 IN,	2 20 29.03 E	15/01/2013			
FIM	El Magney	41 29 21.02 N,	2 21 30.90 E	16/01/2013			
	El Masnou De delene	41°28 35.06 N,	2°18 44.85 E	16/01/2013			
	Badalona	41°20 5.19 N,	2°14 28.73 E	10/01/2015			
BAK	Daraalana (*)	41°21 47.14 N,	2°10 58.76 E	08/11/2008, 4/12/2008, 25/02/2009, 29/04/2009, 18/05/2009, 26/05/2009			
	Barcelona (*)			03/05/2014			
GAR	Garraf	41º15'2 53"N	1°53'58 14"E	25/02/2013			
SIT	Sitges	41°14'2 00''N	1°40'25.06"E	25/02/2013			
511	Tarragona	41 14 2.90 N,	1 49 25.00 E	25/02/2015			
TAR	Tarragona (*)	41° 6'12 00"N	1°12' <i>44 74</i> ''E	13/02/2013			
LAM	L'Ametlla de Mar	$41 \ 0 \ 13.09 \ N$,	1 13 44.74 L	16/04/2014			
LAM	L'Ampollo	40 32 49.13 N,	0 46 7.40 E	16/04/2014			
SCP	L'Ampona	40 48 38.03 N,	0 42 55.80 E	16/04/2014			
SCK	Sant Carles de la Rapita	40°37'1.69''N,	0°35'48.54"E	10/04/2014			
	Castelló						
VIN ~	Vinaròs	40°27'50.20"N,	0°28'20.98"E	17/04/2014			
PEN	Peñíscola	40°21'25.08"N,	0°24'20.63"E	17/04/2014			
CAS	Castelló de la Plana (*)(§)	39°58'3.66"N,	0° 1'19.02"E	17/04/2014			
	València						
SAG	Sagunt	39°38'50.22''N,	0°12'33.30"O	22/03/2013			
VAL	València (*)	39°26'1.40"N,	0°18'47.17"O	21/03/2013			
GAN	Gandia	38°59'50.43"N,	0° 9'23.62''O	23/03/2013			
	Alicante						
DEN	Dénia	38°50'25.70"N,	0° 6'55.24"E	23/03/2013			
ALI	Alicante	38°19'43.63''N,	0°29'58.47"O	24/03/2013			
SPO	Santa Pola	38°11'23.78"N,	0°33'48.54"O	25/03/2013			
TOR	Torrevieja	37°58'15.99"N,	0°40'56.04"O	25/03/2013			
	Murcia						
CAR	Cartagena (*)(§)	37°35'4.91''N,	0°59'5.68"O	26/03/2013, 25/11/2013, 17/05/2014			
	Almería						
ALM	Almería (*)(§)	36°49'50.35''N,	2°28'1.68"O	16/04/2013, 24/11/2013, 17/05/2014			
MOT	Motril (*)(§)	36°43'26.00''N,	3°31'33.60"'O	17/01/2013			
	Málaga						
MAL	Málaga (*)	36°43'3.15"N,	4°24'47.92''O	17/04/2013, 23/11/2014			
ETP	Estepona (*)(§)	36°24'53.71"N,	5° 9'34.83"O	16/05/2014			
	Cádiz						
ALG	Algeciras (*)(§)	36° 7'2.34''N,	5°26'7.62"O	18/04/2013, 23/11/2013			

Table 1. Province, localities, coordinates and date of visit of each sampled site of *Dictyota cyanoloma*. Harbours where *Dictyota cyanoloma* was found are indicated by (*). Populations studied from the point of view of genetic diversity are indicated by (§).

was also used to perform a Mantel test in order to determine whether genetic distance was correlated with geographical distance between sampling sites. Mantel tests were performed between the pairwise fixation index (F_{sT}) matrix and the geographical distance matrix of *D. cyanoloma* locations, estimating the significance level by 100,000 permutations.

Results

Historical data and distribution survey

After surveying all the Spanish Dictyota specimens of the herbaria BCN-Phyc (336 sheets, from 1950 to date) and MA (30 sheets, since the early nineteenth century to date), we did not find any voucher which could correspond to a misidentified collection of D. cyanoloma. In the field surveys, the species was found in 19 of the 36 harbours visited along the entire Mediterranean coast of the Iberian Peninsula from the South (Algeciras, Cádiz) to the North (Llançà, Girona) and occurred in all important commercial harbours except in Alicante (Fig. 1 and Table 1). Individuals of D. cyanoloma were observed growing in infralittoral habitats with low hydrodynamism, on breakwaters or artificial structures, at 0.3-1 m deep, forming well-developed populations in some of the harbours (Algeciras, Almería, Motril, Tarragona, Palamós, L'Estartit) or only as few scattered individuals in the other harbours.

Vegetative and reproductive phenology

Specimens of D. cyanoloma were observed throughout the year. In summer (July, August and September) the individuals had the lowest mean value in thallus length, the minimum observed in August (0.9 ± 0.33 cm). The maximum thallus length was found in winter and early spring (January, February and March), February being the month with the longest thallus length (7.64 \pm 2.03 cm). The width of the third dichotomy, as well as the number of dichotomies from the base to the top, displayed the same tendency: maximum values in January, February and March, February being the month with the widest values $(6.7 \pm 0.9 \text{ mm})$ and maximum number of dichotomies (6.11 ± 1.34) . The minimum values were found in summer, August being the month with the lowest values for width of dichotomies $(1.32 \pm 0.48 \text{ mm})$ and September the month with the lowest values in number of dichotomies (1.89 ± 1.02) (Fig. 2). The total cover of the 10x10 cm quadrats follows the same tendency, maximum cover values in winter (February 256 cm²) and minimum values in summer (August 10 cm²) (Fig. 3).

Sterile individuals were found throughout the year except in February, March and April. Sporophytes were found almost all year through excluding July and August. Male gametophytes were observed only in December and February, and female individuals in February and June. Fertile thalli were more abundant than non-fertile thalli from January to June (64-100%) whereas the non-fertile thalli were dominant from July to December (65-100%). In July and August all specimens were sterile.



Fig. 1: Distribution map of *Dictyota cyanoloma* on the Mediterranean coast of the Iberian Peninsula. (ALG) Algeciras, (ETP) Estepona, (MAL) Málaga, (MOT) Motril, (ALM) Almería, (CAR) Cartagena, (VAL) València, (CAS) Castelló de la Plana, (TAR) Tarragona, (BAR) Barcelona, (MAT) Mataró, (BLA) Blanes, (STF) Sant Feliu de Guíxols, (PAL) Palamós, (EST) L' Estartit, (ESC) L'Escala, (ROS) Roses, (POR) Port de la Selva, (LLA) Llançà.



Fig. 2: Average of thallus length (grey colour), width of the third dichotomy (black line) and number of dichotomies (grey line) of *Dictyota cyanoloma* per month.



Fig. 3: Total cover of Dictyota cyanoloma per month.

Sporophytes were found in 10 of the 12 months, being dominant from January until June (45-100%). Dominance of sporophytes over gametophytes and sterile individuals reached a maximum in March and April, all individuals being sporophytes. The percentage of gametophytes (Fig. 4) never reached more than 27%, February being the month with the maximum percentage of male individuals (12.3%) and female individuals (14.8%).

Antheridia were grouped in ellipsoidal sori on both sides of the frond, leaving a sterile zone near the margin (Fig. 5). The length of the sori was (220) 300-640 (901) μ m and (100) 140-320 (360) μ m wide, with (36) 60-180 (247) antheridia per sorus. Sori were surrounded by 3-4 (5) layers of unicellular paraphyses and covered by an indusium. The antheridia were (31) 37-70 (78) μ m high and (10) 14-25 (29) μ m wide. Each antheridium had one stalk cell.

Haplotype diversity

The mitochondrial *cox*1, *nad6-nad*11, *atp9-orf*11 and the chloroplast *rbc*L-*rbc*S DNA regions were se-

quenced for 49 individuals of D. cyanoloma from nine localities along the Mediterranean coast of the Iberian Peninsula, obtaining two alignments of 1848 bp (mtD-NA) and 495 bp (cpDNA). Sequence comparisons revealed nine polymorphic sites for mtDNA regions and one for the cpDNA region, defining three and two haplotypes respectively. The mitochondrial cox1 region revealed one nucleotide polymorphism, nad6-nad11 region revealed two nucleotide polymorphisms and two insertions/deletions, atp9-orf11 showed four nucleotide polymorphisms and finally, the plastid rbcL-rbcS region revealed a single nucleotide polymorphism. Within the mitochondrial dataset, haplotype mt1 occurred in five localities; haplotype mt2 occurred in five others; and haplotype mt3 was found only in two sampling sites (Fig. 6A; Table 2). The frequency of the three mitochondrial haplotypes is not very different among them: mt1 is the most frequent (41%), followed by mt2 (39%) and mt3 (20%). The plastid dataset revealed only two haplotypes (Fig. 6B): cpl being more abundant (61%) and occurring



Fig. 4: Percentage of female gametophytes (patron), male gametophytes (squares), sporophytes (black colour) and sterile individuals (grey colour) of *Dictyota cyanoloma* per month.



Fig. 5: Dictyota cyanoloma. A) Antheridia distribution; B) Surface view of a male sorus; C) Section of a male sorus with paraphyses; D) An empty male sorus showing only the paraphyses.

in seven localities; cp2 revealed as less abundant (39%), being found in five sampling sites.

Genbank accession numbers of the haplotype and nucleotide diversities of the nine *D. cyanoloma* locations for both plastid and mitochondrial datasets are shown in Table 2. Six sampling sites (Llançà, L'Estartit, Palamós, Castelló de la Plana, Cartagena and Almería) failed to reveal any haplotype diversity, having one fixed haplotype for both regions (mtDNA and cpDNA). In contrast, two haplotypes were found in both regions (mtDNA and cpDNA) for the other three sampling sites (Motril, Estepona & Algeciras). Haplotype diversity within localities varied from Hs=0



Fig. 6: Distribution of the haplotypes of *D. cyanoloma* sampled from the Mediterranean coast of the Iberian Peninsula, and the respective haplotype composition (pie charts) per sampling site. The different colours in the pie charts represent different haplotypes. LLA, Llançà; EST, L'Estartit; PAL, Palamós; CAS, Castelló de la Plana; CAR, Cartagena, ALM, Almería, MOT, Motril; ETP, Estepona; ALG, Algeciras. A) Mitochondrial haplotypes. B) Chloroplast haplotypes.

to Hs=0.4 (mean Hs=0.1259) for both mitochondrial and chloroplast regions. Overall haplotype diversity for mtD-NA is Hd= 0.655 and Hd=0.485 for cpDNA. The AMOVA analyses grouping sequences by sampling sites are shown in Table 3. On average, 76.81% of the total variation was attributable to differentiation among sampling sites for cp-

DNA and 85.23% for mtDNA region, while the rest corresponds to differences within sampling sites (23.19% for cpDNA and 14.77% for mtDNA). The Mantel test showed that geographical and genetic distances between localities were not correlated (r=0.1104, P=0.733 for cpDNA and r=0.168, P=0.878 for mtDNA).

Table 2. Diversity measures for populations of *Dictyota cyanoloma*. Number of individuals (n) and haplotypes (h), values of haplotype diversity (Hd) and nucleotide diversity (π) for each location sampled.

		Mitochondrial			Chloroplast			
	n	h	Hd	π	n	h	Hd	π
Llançà	6	mt3	0	0	6	cp1	0	0
L'Estartit	5	mt1	0	0	5	cp1	0	0
Palamós	7	mt2	0	0	7	cp2	0	0
Castelló de la Plana	5	mt1	0	0	5	cp1	0	0
Cartagena	6	mt2	0	0	6	cp2	0	0
Almería	4	mt3	0	0	4	cp1	0	0
Motril	5	mt1/mt2	0.3333	0.00072	5	cp1/cp2	0.3333	0.00067
Estepona	5	mt1/mt2	0.4	0.00086	5	cp1/cp2	0.4	0.00081
Algeciras	5	mt1/mt2	0.4	0.00086	5	cp1/cp2	0.4	0.00081
Total	49	3	0.655	0.0016	49	2	0.485	0.00098

Table 3. AMOVA results grouped by sampling sites according to the two regions (mitochondrial and chloroplast).

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	F _{ST}	Р
	Among populations	8	78.650	1.755	85.23		< 0.0001
Mitochondrial	Within populations	40	12.167	0.304	14.77		< 0.0001
	Total	48	90.816	2.059		0.852	
	Among populations	8	9.313	0.199	76.81		< 0.0001
Chloroplast	Within populations	41	2.467	0.060	23.19		< 0.0001
	Total	49	11.780	0.259		0.768	

Discussion

Distribution

After surveying all material assigned to different Dictyota species from BCN-Phyc and MA herbaria, we were not able to observe any misidentified historical collection of *Dictyota cyanoloma* prior to the first specimens collected at Palamós in 1987 (Rull Lluch et al., 2007) and preserved at BCN-Phyc herbarium. Moreover, even though it is not usual to sample in harbours, some previous studies on benthic communities along the Mediterranean coast of the Iberian Peninsula had included harbour habitats (Ballesteros et al., 1984; Ballesteros, 1989) but in no case had the authors referred to an unidentified Dic*tyota* species. In 1988, an extensive study on the algal communities from the harbour of Blanes - where D. cyanoloma has currently been found (Table 1) - did not report the occurrence of any Dictvota species (Rodríguez-Prieto et al., 1997). These results contrast with the data from the Croatian coast, where some herbarium records (since 1935) of D. cyanoloma have been found (Steen et al., 2016) suggesting that the colonization of the Spanish coast could have occurred later. Alternatively, the presence of this species along the Mediterranean coast of the Iberian Peninsula could have been overlooked since older times, not expanding its range or frequency until recently.

Dictyota cyanoloma currently shows a wide distribution along the Iberian Mediterranean coast, being present in more than half of the surveyed harbours from the French border to Gibraltar. Certainly, some environmental characteristics of harbours such as water and sediment pollution, structures providing artificial substrates, altered temperature regime and marine traffic have been associated with increased introduction incidence (Carlton, 1996; Glasby et al., 2007; Schaffelke & Hewitt, 2007). However, despite that the first records of D. cyanoloma in the Mediterranean Sea corresponded to anthropogenic environments, the species has also been recently reported in more natural habitats (Steen et al., 2016). The occurrence of this seaweed outside harbour environments on the Iberian Mediterranean coast needs to be studied more thoroughly.

Phenology

It is assumed that sexual reproduction may enhance the success of exotic species, since recombination of genetic material allows more rapid adaptive evolution (Sakai *et al.*, 2001; Lambrinos, 2004). The presence of all life stages of *D. cyanoloma* in the Mediterranean region implies that the species can reproduce sexually outside its native range. The Palamós population persists throughout the year, and shows a clear annual vegetative and reproductive cycle. In summer the population's cover is sparse; the individuals are small and sterile, with a reduced number of dichotomies. In autumn the thallus length starts to increase, the individuals are taller and mainly sterile, but a few fertile sporophytes also occur in this period of the year. In winter the population shows its greatest development; the specimens reach their maximum size and their greatest number of dichotomies; the population's cover also reaches its higher values. In this period almost all specimens are fertile sporophytes, although some fertile male and female gametophytes also occur in February. Through spring, the average size of the specimens, mainly fertile sporophytes, progressively decreases until disappearing at the beginning of summer, when new young individuals start to appear. Therefore, in Palamós, *D. cyanoloma* occurs all year round, but it is particularly abundant and fertile during the winter-spring period.

No previous data exist about the phenology of D. cyanoloma. Concerning other Dictyota species, most phenological studies refer to the seasonal variation. In this respect, the phenology of Iberian D. cvanoloma is similar to other Dictyota species occurring in the same geographical area, such as D. dichotoma, Dictyota spiralis Montagne and Dictyota fasciola J.Agardh. All these species occur throughout the year and they are always fertile, except D. fasciola which in autumn is exclusively sterile (Polo, 1978; Ribera Siguan, 1978; Rodríguez-Prieto & Polo, 1986). The seasonal phenology of D. cyanoloma - with a maximum development in winter and a minimum in summer – also matches other *Dictvota* species occurring in Valencian and Basque Countries (Barceló, 1987; Casares, 1987), even though no gametophytes were found in these areas. In Málaga (Conde, 1981) and Argentina (Gauna et al., 2013), D. dichotoma occurs throughout the year, while it is lacking in October in the Canary Islands (Tronholm et al., 2008) and from April to August in Mallorca (Gómez Garreta, 1982). Regarding the period where *Dictvota* species reach their greatest size, in Mallorca the tallest specimens of both D. fasciola and D. spiralis occur in spring, and in the Canary Islands the tallest D. dichotoma occurs in early summer, unlike D. cyanoloma, which has its maximum development in winter. In the Palamós population of D. cyanoloma both maximum abundance and tallest specimens occur during the same period of the year (winter). This also happens in D. dichotoma from Argentina (Gauna et al., 2013), although in this area the higher values occur in summer. In contrast, in the D. dichotoma population from the Canary Islands, maximum abundance and thallus length do not coincide at the same time (Tronholm et al., 2008).

In our study, the proportion between fertile and sterile specimens of *D. cyanoloma* follows the same pattern of temporal variation observed in cover and size of specimens. In summer and autumn sterile specimens dominate over fertile specimens and in winter and spring fertile individuals dominate over sterile individuals. Sporophytes always dominate over gametophytes when the latter exist. Very few studies take into account the temporal variation of the different life-cycle generations proportion, as well as the fertile and sterile specimen proportion in the genus *Dictyota*. In *D. dichotoma* from the Canary Islands and Argentina, unlike *D. cyanoloma*, the fertile specimens dominate over sterile ones throughout the year. However, while in the Canary Islands the sporophytes always dominate over the gametophytes, in Argentina gametophytes are the dominant life stage in all months of the year (Tronholm *et al.*, 2008; Gauna *et al.*, 2013).

Our phenological observations suggest that *D. cyanoloma* on the Mediterranean coast of the Iberian Peninsula may benefit from increased growth rates during winter, when native species of *Dictyota* sharing similar niches [e.g. *D. dichotoma* var. *intricata* (C.Agardh) Greville] show lower abundance and a smaller size (Benedetti-Cecchi & Cinelli, 1993). Similarly, the phenology of *D. fasciola* and *D. spiralis* in Mallorca (Gómez Garreta, 1982) and that of *D. dichotoma* in the Canary Islands (Tronholm *et al.*, 2008) could support this idea. This hypothesis should be further tested by studying the community ecology of the habitat (harbour platforms) over the whole year in this geographical area.

Haplotype diversity

Our indicative results revealed noticeable molecular polymorphism within and among sampling sites of D. cyanoloma from the Mediterranean Iberian Peninsula coast. Interestingly, mtDNA regions nad6-nad11 and atp9-orf11 as adapted from Steen et al. (2016) proved four times more variable than cox1, a typical barcoding marker between seaweed species (e.g. Leliaert et al., 2014 and references therein). The plastid RubisCo spacer (rbcL-rbcS) has been already used in several phylogenetic studies (e.g. Siemer et al., 1998; Yoon et al., 2001; Lee et al., 2011), showing significant variability at the interspecific level. However, this marker is significantly less variable than cox1 at intra-specific level among different red seaweed species (Robba et al., 2006). In D. cyanoloma, we have observed the same amount of variability in rbcL-rbcS spacer as in cox1. The results confirm the potential usefulness of these mitochondrial and plastid regions at the intra-specific level in brown algae as previously suggested by Engel et al., (2008).

The five haplotypes observed for both mitochondrial and chloroplast markers suggest that several thalli or propagules (either in multiple colonization events or in a single one carrying several different genotypes) have been responsible for the introduction of this species along the Mediterranean coast of the Iberian Peninsula. The distributional pattern of the haplotypes show that six of the nine studied sampling sites of *D. cyanoloma* present only one haplotype per population and only three localities (Algeciras, Estepona and Motril) show two haplotypes. In accordance with this low haplotype diversity within the sampling sites (mean Hs=0.1259), AMOVA results show that *D. cyanoloma* from the Mediterranean Iberian Peninsula coast presents significantly more genetic dif-

ferentiation among sampling sites than within sampling sites. Additionally, spatial autocorrelation analysis indicates that the haplotypic structure of D. cyanoloma in this region does not correlate to geography. Being particularly evident in the northern distribution area, nearby localities do not share the same haplotype but a different one according to both mitochondrial and plastid DNA markers (Fig. 6). This pattern of genetic structure also suggests that geographic spread did not occur through a smoothly expanding range with simple advancing wave fronts [the "wave of advance" model (Fisher, 1937)], but by hopscotch jumps with long-distance dispersal typical of human transport (Lawson Handley et al., 2011). Moreover, the three sampling sites that show a greater number of haplotypes are the southern ones, located near to the Strait of Gibraltar, a place of intensive maritime traffic, whereas the northern ones are more uniform.

Considering our limited sampling data, these evidences could reflect the role of marine navigation as a dispersal vector of this species in the area. In this context, extending this fine-scale sampling to include more samples per locality, over longer time periods and also expanding the study to include the entire reported range of *D. cyanoloma*, would provide more information to clarify the colonization process of the species.

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