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Molecular Phylogenetics and Evolution 58 (2011) 283–296

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Evolution and diversification within the intertidal brown macroalgae *Fucus spiralis*/*F. vesiculosus* species complex in the North Atlantic

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ARTICLE INFO

Article history:
Received 26 July 2010
Revised 10 November 2010
Accepted 16 November 2010
Available online 25 November 2010

Keywords:
Fucus spiralis
Fucus vesiculosus
Hybridization
Ice ages
Introgression
Phylogeography

ABSTRACT

We examined 733 individuals of *Fucus spiralis* from 21 locations and 1093 *Fucus vesiculosus* individuals from 37 locations throughout their northern hemisphere ranges using nuclear and mitochondrial markers. Three genetic entities of *F. spiralis* were recovered. In northern and sympatric populations, the presence of "*F. spiralis* Low" in the mid-intertidal and "*F. spiralis* High" in the high-intertidal was confirmed and both co-occurred with the sister species *F. vesiculosus*. The third and newly-discovered entity, "*F. spiralis* South", was present mainly in the southern range, where it did not co-occur with *F. vesiculosus*. The South entity diverged early in allopatry, then hybridized with *F. vesiculosus* in sympatry to produce *F. spiralis* Low. Ongoing parallel evolution of *F. spiralis* Low and *F. spiralis* High is most likely due to habitat preference/local selection and maintained by preferentially selfing reproductive strategies. Contemporary populations of *F. spiralis* throughout the North Atlantic stem from a glacial refugium around Brittany involving *F. spiralis* High; *F. spiralis* South was probably unaffected by glacial episodes. Exponential population expansion for *F. vesiculosus* began during the Cromer and/Holstein interglacial period (300,000–200,000 yrs BP). Following the last glacial maximum (30,000–22,000 yrs BP), a single mtDNA haplotype from a glacial refugium in SW Ireland colonized Scandinavia, the Central Atlantic islands, and the W Atlantic.

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1. Introduction

The intertidal macroalgal genus *Fucus* evolved from a North Pacific ancestor (5.5–2.3 Mya) at or just after the opening of the Bering Strait (5.5–5.4 Mya) and has subsequently colonized and radiated throughout the North Atlantic (Coyer et al., 2006a), especially during the past 3–1 Mya (Coyer et al., in press). The dynamic radiation, which continues in contemporary time as evidenced by the speciation of *Fucus radicans* in the upper Baltic Sea within the past 2000–400 yrs (Pereyra et al., 2009), has long challenged our concepts of species and speciation, largely because of environmentally-determined morphologies and extensive hybridization among the component entities.

Two lineages of *Fucus* are recognized, each comprising both dioecious and hermaphroditic species. Lineage 1 consists of *Fucus serratus* and *Fucus distichus sensu lato* and Lineage 2 of *Fucus vesiculosus*, *Fucus spiralis*, *F. radicans*, *Fucus ceranoides*, and *Fucus virsoides* (Serrão et al., 1999; Coyer et al., 2006a). From a morphological standpoint, over 125 intraspecific taxa have been described for the nine most common species (Guiry and Guiry, 2010). From a genetic standpoint, several studies using molecular techniques have documented hybridization between the various species based on experimental evidence (Coyer et al., 2002a) or occurrence of intermediate genotypes in nature (Coyer et al., 2002b, 2006b,c; Wallace et al., 2004; Engel et al., 2005; Mathieson et al., 2006; Billard et al., 2010). Additionally, frequent establishment of successful reproductive hybrids has been noted (Engel et al., 2005; Coyer et al., 2007). Phylogeography of species comprising Lineage 1 is better understood (Hoarau et al., 2007b; Coyer et al., in press), largely because *F. serratus* and *F. distichus* are older taxa and reinforcement has minimized hybridization (Hoarau et al., unpub. data). In contrast, the confusing morphologies, putatively more complex phylogeographic history, and frequent hybridization among the more recently diverged entities in Lineage 2, present major challenges to sampling and phylogeographic analysis (e.g., Neiva et al., 2010).

The dynamic radiation of *Fucus* may stem in part from its predominance in the rocky intertidal biome, which is characterized

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1055-7903/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2010.11.015

by numerous micro-environmental gradients/habitats (both abiotic and biotic) over spatial and temporal scales. Adaptations by intertidal species to the various stressors (e.g., desiccation, temperature, wave exposure, presence/absence of competitors/predators) have provided examples of divergence driven mainly by habitat variation, despite the occurrence of gene flow, such as the intertidal snail *Littorina saxatilis* (reviewed in Johannesson, 2009) and the genetic entities of the *F. spiralis*/*F. vesiculosus* species complex (Zardi et al., submitted for publication). Habitat-driven speciation has also occurred in subtidal *F. vesiculosus*/*F. radicans* (Bergström et al., 2005; Pereyra et al., 2009).

One of the commonly occurring intertidal fucoids, and usually confined to the upper intertidal of rocky shores, is the desiccation-resistant (Davidson and Pearson, 1996), hermaphroditic and mainly self-fertilizing *F. spiralis* (Engel et al., 2005; Perrin et al., 2007). Along eastern Atlantic shores in the northern hemisphere, *F. spiralis* (*sensu lato*) ranges from northern Norway to the Azores and Canary Islands, the latter being the southern-most record for the genus (Lüning, 1990; Haroun et al., 2002). Along western Atlantic shores, *F. spiralis* ranges from Newfoundland (Canada) to southern Maine (USA). In the North Pacific, *F. spiralis* is restricted to the area around the USA/Canadian border and is most likely an introduction (see Coyer et al., 2006a and references therein).

Gamete dispersal in *F. spiralis* is highly limited and recent work in Brittany and Portugal has revealed two genetically distinct entities, one found primarily in the upper shore (=*F. spiralis* High) and the other in the mid-upper region (*F. spiralis* Low) (Billard et al., 2010). Morphological differences exist between the entities and each is subjected to different regimes of desiccation resistance (Billard et al., 2010; Zardi et al., submitted for publication). Although divergence of the habitat-specific ecotypes may have occurred by gradual adaptation of each entity to its respective micro-environment, the possibility of divergence of one entity from the other by hybridization/introgression with a co-occurring species cannot be discounted, especially as inter-specific gene flow is well documented within the genus *Fucus* (Wallace et al., 2004; Billard et al., 2005, 2010; Engel et al., 2005; Coyer et al., 2006b; Mathieson et al., 2006; Perrin et al., 2007).

The dioecious *F. vesiculosus* is a key foundation species in a broad spectrum of habitats ranging from exposed intertidal rocky shores to highly sheltered tidal marshlands. Along eastern Atlantic shores, it inhabits the mid- to high rocky intertidal from northern Norway to Morocco, also extending into the northernmost and brackish regions of the Baltic Sea and in the White Sea into brackish tidal marshlands. Along western Atlantic shores, it is found in similar rocky/marshland habitats ranging from Arctic Canada to North Carolina (USA) and throughout the rocky intertidal of Morocco. References to its occurrence on the Canary Islands and the Azores (Lüning, 1990; Schneider and Searles, 1991; Haroun et al., 2002) are questionable (recently corrected in Algaebase for the Azores).

Superimposed on the North Atlantic intertidal biome, and the phylogenetic/phylogeographic patterns of *Fucus* (as well as many other species), is the profound effect of the last glacial maximum (LGM) from 0.030 to 0.022 Mya (Bradwell et al., 2008). During this period, the presence of ice sheets covering Scandinavia and the British Isles forced several marine and some terrestrial species to the ice-free and more southerly Iberian Peninsula, from which many expanded as the ice receded (Hewitt, 1996; Maggs et al., 2008). However, recent work on a variety of marine benthic invertebrate and algal species has demonstrated the existence of northern ice-free glacial refugia near Brittany (Hurd Deep) and SW Ireland along the eastern Atlantic, and along the Canadian Maritimes in the western Atlantic (reviewed in Maggs et al., 2008; Olsen et al., 2010). Within Lineage 1, *F. serratus* also displayed signatures of glacial refugia in Brittany and SW Ireland (Coyer et al., 2003; Hoarau et al., 2007b) and a glacial refugium for the sister species *F. distichus* may be present off the Canadian Maritimes (Coyer et al., in press).

In contrast, little is known about the phylogeography and colonization/extinction history of species within Lineage 2 (*F. vesiculosus* and *F. spiralis*), along the northern glaciated and the southern-most permanently ice-free areas of the North Atlantic before, during, and after the LGM. In particular, the southern areas may have played an important long-term role in the evolutionary trajectories of Lineage 2 through uninterrupted survival, hybridization, and expansion.

In the present study, we used mtDNA haplotypes to examine *F. spiralis* and *F. vesiculosus* populations throughout their distributional ranges and microsatellite genotypes to verify which genetic entities occur in *F. spiralis* populations from different sites and at contrasting vertical positions on the rocky shore. The study addresses mechanisms underlying divergence and speciation in this species complex. Accordingly, our aims were to: (1) identify phylogeographic trajectories within the *F. spiralis* complex, including the High and Low shore entities, and *F. vesiculosus*, (2) track signatures of hybridization; (3) identify possible refugia for both species; and (4) estimate the effects of the LGM on *F. spiralis* and *F. vesiculosus* relative to other species of *Fucus*, which have been strongly influenced by the LGM and the immediately prior Eemian interglacial period (Coyer et al., 2003, 2006a; Hoarau et al., 2007b).

2. Materials and methods

2.1. Sampling

Fucus spiralis and *F. vesiculosus* were sampled along allopatric (species are never found together in the region), parapatric (species separated by several meters along the vertical shore height) and sympatric (*sensu stricto*, areas where individuals occur intermingled) zones. In sympatric zones, where *F. vesiculosus* and the forms of *F. spiralis* were intermingled together, initial species identification was based on shore location and morphology, specifically the presence of vesicles for *F. vesiculosus* and the branching pattern and shape of the receptacles for *F. spiralis*. Within *F. spiralis*, no selection of morphotypes was used in the field sampling, but identifications were later verified using microsatellite loci for several sites (see below).

2.2. F. spiralis

A total of 733 individuals were collected from 21 locations throughout the distributional range of *F. spiralis* (Table 1). As samples collected specifically for this study were combined with samples collected from a number of previous studies, it was necessary to reconcile four different sampling methods to ensure equivalent comparisons and to separate high and low shore samples to the best of our ability. The collection method at each location is described below and noted in Table 1. All of the mtDNA data and approximately 50% of the microsatellite data are new and all data are analyzed here for the first time in a fully integrated context of the multiple species and their phylogeography.

Method 1 (this study) consisted of random walks along two 50-m transects parallel to shore and collecting individuals of both *F. spiralis* and *F. vesiculosus* at 1-m intervals in five locations. One transect was established along the upper shore (=*F. spiralis* High, parapatric) and the other on the lower shore (=*F. spiralis* Low, sympatric). Method 2 was similar to Method 1, except that samples were collected only from the high shore transect where *F. spiralis* occurred alone (parapatric); no sampling was conducted in the sympatric zone; if no sympatric zone existed (e.g., allopatric range

Table 1
Sampling locations and sample sizes for *F. spiralis* and *F. vesiculosus*. Fsp vs. Fv, intertidal sampling positions: allopatry (A) defined as the two species do not occur together in that site; sympatry (S) defined as they do overlap in the location; parapatry (P) defined as co-occurrence in the given site, but are not overlapping, and unknown (U). Sampling method and original source (see Section 2.2) for *F. spiralis*: 1, this study; 2, this study; 3, Perrin et al. (unpub.); 4, Billard (2007) and Billard et al. (2010). *N*, number of individuals collected. Not sampled/applicable (na).

Region/location	Code	Map ID (Figs. 4 and 5)	Fsp/Fv	*N F. spiralis*	Fsp sampling method	*N F. vesiculosus*
Pacific						
San Juan Is, WA, USA	SJI	1	A	48	3	na
Western Atlantic						
Appledore Is, MA, USA	APP	2	P	18	2	31
Woods Hole, MA, USA	WHO	3	na	na	na	29
Inverness, NS, CAN	INV	4	na	na	na	27
St. Lunaire, NL, CAN	STL	5	na	na	na	22
Greenland						
Tasiluk	TAS	6	na	na	na	30
Iceland						
Grindavik	GRI	7	P	22	2	na
Patreksfjörður	PAT	8	na	na	na	31
Sandgerði	SDG	9	na	na	na	31
Blönduós	BLO	10	na	na	na	32
Breiðdalsvik	BRE	11	na	na	na	31
Norway						
Tromsø	TRM	12	P	20	2	25
Trondheim	TRD	13	na	na	na	30
Kirkenes	KIR	14	na	na	na	32
Faeroes						
Suduroy	SUD	15	na	na	na	32
Shetland						
Hamma Voe	HVO	16	P	21	2	na
Flora Tang	FTA	17	na	na	na	32
East Voe of Quaff	EVQ	18	na	na	na	32
Scotland						
Oban	OBA	19	P	23	2	32
Aberlady Bay	ABB	20	na	na	na	32
Northern Ireland						
Giants Causeway	GIC	21	na	na	na	32
England						
New Brighton	NBR	22	S + P	47	1	na
Praa Sands	PRA	23	na	na	na	24
Sennen Cove	SEN	24	na	na	na	24
Ireland						
Labasheeda	LAB	25	P	24	2	31
Spanish Point	SPP	26	na	na	na	32
Seafield	SEA	27	na	na	na	28
Galway	GAL	28	na	na	na	26
Kattegat						
Göteborg, SWE	GOT	29	P	24	2	32
Egmont, DEN	EGM	30	na	na	na	32
Tjärnö, SWE	TJA	31	na	na	na	32
Hovs Hallar, SWE	HOH	32	na	na	na	32
Baltic						
Askö, SWE	ASK	33	na	na	na	11
Normandy, FRA						
Cap Gris Nez	CGN	34	U	24	3	31
Sotteville	SOT	35	na	na	na	31
Brittany, FRA						
Port Lazo	PLA	36	S + P	47	1	na
Santec	SAN	37	S + P	35	4	na
Perharidy	PER	38	A	24	2	32
Menez-Ham	MEH	39	na	na	na	29
Aber Wrach	ABW	40	na	na	na	27
Spain						
Santoña, Cantabria	STA	41	S + P	24s + 24P	1	na
Castello, Asturias	CAS	42	A	16	2	na
Canary Islands						
El Medano, Tenerife	TEN	43	A	23	2	na
El Burrero, Gran Canaria	GCA	44	A	18	2	na
Portugal						
Viana do Castelo	VIA	45	P	40	4	32
Albufeira (S)	ALB	46	A	24	2	na

(*continued on next page*)

Table 1 (*continued*)

Region/location	Code	Map ID (Figs. 4 and 5)	Fsp/Fv	N F. spiralis	Fsp sampling method	N F. vesiculosus
Aveiro	AVE	47	na	na	na	32
Ayamonte	AYA	48	na	na	na	32
Azores (A)						
São Miguel Island	AZO	49	A	23	3	na
Morocco						
Oualidia	MOR	50	A	48	3	na

in the South) the transect was taken in the middle of the *F. spiralis* zone (this study). Method 3 utilized samples from two locations per site, separated by 100–200 m (see Engel et al., 2005) whose taxon identity had been previously confirmed by microsatellite genotyping (Perrin et al., unpub. data, Engel et al., 2005 for Gris Nez). In Method 4, we selected individuals previously determined by microsatellite genotyping (in order to avoid hybrids) as *F. spiralis* High, *F. spiralis* Low (or as *F. vesiculosus*) that had been collected along a vertical transect on the shore or with random coordinates (a subset of individuals from Billard et al. (2010) and also from Billard (2007) for Viana).

2.3. F. vesiculosus

Samples were collected from 1093 individuals among 37 locations covering the distributional range (Table 1). From each location, 24–32 individuals were collected at 1-m intervals along a transect line parallel to shore. Additional samples of *F. vesiculosus* from France (parapatric and see Method 4 above) were used in the analyses aimed at discriminating among genetic entities within *F. spiralis*.

2.4. DNA extraction

All samples of both species were individually stored in silica crystals until DNA extraction. A subset of 24 *F. spiralis* individuals from each site within a location was analyzed, with the exception of Method 3 samples (from Perrin et al., unpub.) for which all 48 individuals were used in most cases. DNA was isolated from 5 to 10 mg of dried tissue either with NucleoSpin® 96 Plant kit (Macherey–Nagel), DNeasy™ 96 Plant kit (QIAGEN), or a CTAB method as described in Hoarau et al. (2007a) and modified by Coyer et al. (2009). For PCR reactions, samples extracted with both kits were diluted 1:100 and those by CTAB extractions 1:10.

2.5. Microsatellite loci

For locations in sympatry, where multiple genetic entities were known or suspected to occur in sympatry, microsatellite loci were used to identify High–Low population structure within *F. spiralis* and hybridization with *F. vesiculosus*, thereby conclusively separating High and Low *F. spiralis* prior to the phylogeographic analysis.

A total of 91 individuals of *F. spiralis* Low (hereafter Fsp-Low), *F. spiralis* High (hereafter Fsp-High) and *F. vesiculosus* from Santec and *F. spiralis* from Perharidy were amplified at 12 EST-SSR derived loci from Coyer et al. (2009) (F9, 12, 17, 21, 34, 36, 42, 49, 58, 59, 60, 72) and at a single diagnostic microsatellite locus (L20) (Engel et al., 2003). All PCR reactions (10 μL) contained 1× HotMaster *Taq* polymerase buffer (5Prime) with 2.5 mM of $MgCl_2$, 1 mM of each dNTP, 0.2 μM of each primer, 0.05U HotMaster *Taq* Polymerase and 1 μL of diluted DNA. Amplifications were carried out either on a MyCycler Personal Thermal Cycler (Bio-Rad) or a Veriti™ Thermal Cycler (Applied Biosystems) using the following profile: initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C for 20 s, annealing temperature (T_a, Table 1) for 10 s, 65 °C for 35 s; and a final extension at 65 °C for 10 min. In order to facilitate cost-efficiency while increasing discrimination sensitivity among population clusters, five (of the 12) microsatellite loci (F21, 34, 36, 42, 49) were selected to maximize F_{ST} values and allelic frequencies (data not shown). Using these loci, 264 *F. spiralis* individuals were genotyped from seven additional locations (New Brighton, Port Lazo, Santoña, Viana do Castelo, Albufeira, Morocco, Tenerife; see Table 1) across the distributional range of *F. spiralis*. PCR products were separated an ABI 3730 Sequencer (Applied Biosystems) and visualized with GeneMapper Software Version 3.7 (Applied Biosytems).

2.6. Mitochondrial DNA

The most polymorphic region (*c.* 210 BP) of a longer intergenic spacer (600–700 BP) (Coyer et al., 2006a–c) was amplified in both *F. spiralis* and *F. vesiculosus* with the primers FvSSCPf (5′-CCCGTACTAATCCCCATCAGAAGTA-3′) and FvSSCPr (5′-GGCTTCTTGATGATT AAAGTCTCAT-3′) labeled with FAM and HEX, respectively. The smaller fragment was chosen to facilitate SSCP analysis (see below).

PCR reactions (10 μL) for *F. spiralis* contained 1 μL of diluted DNA, 1× HotMaster Buffer with 1.5 mM $MgCl_2$ (5Prime), 0.2 mM of each dNTP, 0.15 μM of each labeled primer and 0.045U HotMaster *Taq* DNA polymerase (5Prime). PCRs were performed either on a MyCycler Personal Thermal Cycler (Bio-Rad) or a Veriti™ Thermal Cycler (Applied Biosystems). Amplification conditions were: initial denaturation step of 2 min at 94 °C, then 40 cycles of 94 °C for 10 s, 63 °C for 5 s, 65 °C for 40 s, and a final extension at 65 °C for 10 min. GenBank Accession numbers for the 16 IGS haplotypes are: HM583762–HM583764 for haplotypes *c1–c3* (shared with *F. vesiculosus*) and HM583764–HM583777 for haplotypes *s1–s13*.

PCR reactions (10 μL) for *F. vesiculosus* contained 0.5 μL of the DNA template, 1× Hotmaster *Taq* polymerase buffer (5Prime), 0.2 mM of each dNTP, 0.15 μM of each primer (Biolegio), and 0.5U HotMaster *Taq* polymerase (5Prime). Amplification conditions were: initial denaturation step of 2 min at 94 °C, then 40 cycles of 94 °C for 10 s, 65 °C for 40 s, 65 °C for 40 s, and a final extension at 65 °C for 10 min. GenBank Accession numbers for the 17 IGS haplotypes are: HM583778–HM583780 for haplotypes *c1–c3* (shared with *F. spiralis*) and HM583781–HM583794 for haplotypes *v1–v14*.

2.7. Single-strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) (Orita et al., 1989) was used in both species to detect sequence variation in the mtDNA-IGS. We used capillary array electrophoresis SSCP (CAE-SSCP) (Larsen et al., 2007) where capillary electrophoresis replaces the traditional polyacrylamide gel. PCR products were cleaned with Sephadex G50 filtration (Sigma–Aldrich) and amplification products were separated on an ABI3130XL genetic analyzer (Applied Biosystems) using a non-denaturing polymer (5% CAP/ 10% glycerol) (Applied Biosystems) at 24 °C.

PCR products were diluted 1:200 after purification with Sephadex® G-50 (Sigma–Aldrich) and then denatured. SSCP gels were run in non-denaturing polymer with GeneScan®-350 ROX™ Size

Standard (Applied Biosystems) on an ABI 3130 Sequencer (Applied Biosystems) at 24 °C. SSCP gels were read using GeneMapper Software Version 3.7 (Applied Biosytems). All distinct haplotypes (i.e. different peak sizes derived from different electrophoretic mobilities) were sequenced in at least in two individuals.

2.8. Direct sequencing

Direct sequencing of DNA samples utilized the primers and conditions described above, but using a 20 µL PCR volume and non-labeled primers. PCR products were purified with ExoSAP-IT® (USB Corporation) enzyme and cycled-sequenced with reactions consisting of 1 µL of BDT-mix, 1 µL of primer (5 µM), 5 µL Sequence Reaction Buffer, and 6 µL of MiliQ water. Amplifications utilized a Veriti™ Thermal Cycler (Applied Biosystems) for 35 cycles of: 15 s at 96 °C, 50 °C for 1 s, 60 °C for 4 min with a soft ramp of 0.3 °C/s. Samples were purified with Sephadex® G-50 (Sigma–Aldrich), denatured in formamide, and visualized on an ABI 3730 Sequencer (Applied Biosystems). Sequences were aligned using Variant Reporter Software v1.0 (Applied Biosystems) and by eye using Bioedit v. 7.0.1.

2.9. Data analysis

2.9.1. Microsatellite loci

Population structure and classification of *F. spiralis* individuals as hybrids across the intertidal gradient were analyzed using Structure v. 2.2 (Pritchard et al., 2000), which uses a Bayesian approach to assign individuals to groups (=*K*) based on minimizing Hardy–Weinberg and Linkage disequilibria among their genotypes. The number of expected populations was tested using $K = 2$, 3, 4 and 5; each with 10 iterations. The initial expectation was to recover three groups: Fsp-Low, Fsp-High and *F. vesiculosus*. Two sets of multilocus data were utilized in the analysis: the 91 individuals amplified at 12 microsatellite loci in both *F. spiralis* and *F. vesiculosus* individuals; and the 264 *F. spiralis* individuals amplified with the subset of five loci. For both data sets, a burn-in period of 2×10^5 repetitions and a run length of 10^6 steps were used. To test the performance (convergence) of the program, the analysis was replicated five times under identical parameterizations, but with different random number seeds.

Diversity measures were calculated for each genetic entity per location as identified above using STRUCTURE within *F. spiralis*. Allelic richness (*A*), corrected for sample size was calculated using HP-Rare v. 1.1 (Kalinowski, 2005). Nei's gene diversity (H_{exp}) (Nei, 1978), as well as estimators of F_{ST} and F_{IS} (Wright, 1969) as θ and f (Weir and Cockerham, 1984) were calculated with Genetix v.4.03 (Belkhir et al., 2001).

2.9.2. mtDNA-IGS

Haplotype (h) (Nei, 1987) and nucleotide (π) (Nei, 1987) diversities were estimated using Arlequin v. 3 (Excoffier et al., 2005). Intraspecific relationships among the mtDNA haplotypes were inferred using statistical parsimony with Tcs v. 1.13 (Clement et al., 2000) with indels coded according to Barriel (1994).

Historical demographic changes for *F. vesiculosus* were inferred using a generalized skyline plot (Strimmer and Pybus, 2001). The method derives from coalescence theory (Kingman, 1982), as the shape of a genealogy depends on the demographic history. More specifically, the generalized skyline plot uses a step function and the genealogy to represent estimated changes in effective population size (*Ne*) over time. The method provides a trend, rather than a detailed estimate, assumes clock-like evolution, and does not account for phylogenetic error. As a first step, a maximum-likelihood (ML) ultrametric tree (equal root-to-tip distance for all lineages) was constructed with the HKY85+G model of molecular evolution (Coyer et al., 2006a) and a molecular-clock assumption in Paup v. 4.0b10 (Swofford, 2002) as clock-like branch lengths are required. Next, a generalized skyline plot was generated from the ML tree using Genie v. 3.0 (Pybus and Rambaut, 2002) with a smoothing algorithm to reduce the noise in the data while simultaneously preserving the demographic signal. The smoothing parameter (ε) was estimated using the 'maximize optimization' option. A mitochondrial divergence rate of 2.0–3.4%/My was used based on an mtDNA-IGS molecular clock estimated for *Fucus* (Hoarau et al., 2007b).

2.10. The issue of heteroplasmy

The presence of more than one mitochondrial genotype in an individual (heteroplasmy) has been detected in animals, angiosperms, and heterokonts, including in the genus *Fucus* (Coyer et al., 2004; Kmiec et al., 2006). It can lead to mtDNA recombination (Hoarau et al., 2002), which would affect phylogeographic and demographic inferences (e.g., Schierup and Hein, 2000; Posada and Crandall, 2002). Heteroplasmy was verified in our data set by separate labeling of each DNA strand for SSCP, permitting an unambiguous identification of heteroplasmic individuals.

3. Results

3.1. Determination of genetic entities using microsatellites (F. spiralis)

Four groups were identified with the software Structure v. 2.2 (Pritchard et al., 2000) using 12 microsatellite loci among 91 individuals of *F. vesiculosus* and *F. spiralis* collected at Santec and Perharidy (Brittany) (Fig. 1). Three groups corresponded to *F. spiralis*: two previously identified by Billard et al. (2010) as Fsp-High and Low and a third reported here for the first time as Fsp-South (Fig. 1). A fourth and more variable cluster consisted of *F. vesiculosus* individuals.

The diagnostic subset of five microsatellites also resolved the three *F. spiralis* entities in locations throughout the distributional range, with Fsp-South dominating in the southern locations (Portugal, Morocco, Azores, Canary Islands) and Fsp-Low and Fsp-High dominating the northern locales (except for Perharidy) (Fig. 2). At sites sampled with Method 1, where two *F. spiralis* types were found, Fsp-High was generally found in the upper transect and Fsp-Low in the lower transect, but with several exceptions. Thus, there was predominance of Fsp-High at several locations along the range where High and Low co-occur (e.g., English and Spanish locations) (Fig. 2a and e) and the variable numbers of mismatched individuals in most locations (e.g., Fsp-High found in the lower transect) revealed some degree of vertical overlap in these genetic types (e.g., Fig. 2b and e).

Strong population differentiation was found among all (but one) comparisons between genetic types and locations, with F_{ST} values ranging between 0.544 (Port Lazo Low and High) and 0.982 (*F. spiralis* from Tromsø and Fsp-Low from Viana do Castelo) (Table 2). On average, the Fsp-South/Fsp-Low comparison revealed the highest values. All pairwise comparisons were significant ($p < 0.05$) except between Morocco and Albufeira (Portugal).

3.2. Genetic diversity (mtDNA and microsatellites)

Allelic richness at microsatellite loci was uniformly low at 1–2 alleles per locus for Fsp-High, Fsp-Low, and Fsp-South (Table 3) and expected heterozygosity (H_{exp}) never exceeded 0.394 (Port Lazo). Haplotypic (mtDNA) diversity (h) ranged from 0 to 0.6883 and the highest nucleotide diversity (π) was 0.00414, with the highest for both parameters measured for Fsp-High in Viana do Castelo (Table 4).

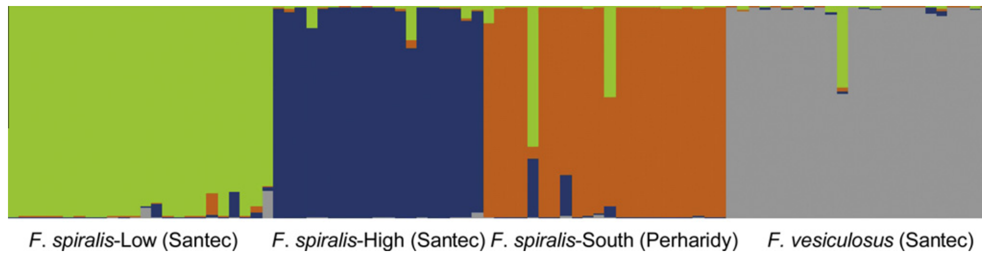

Fig. 1. Verification of four *Fucus* entities based on a Bayesian analysis with Structure v. 2.2 (Pritchard et al., 2000) based on 12 microsatellite loci. Each vertical bar is the multi-locus genotype of one individual and colors represent the proportion of the genotypes assigned to each genetic group. Green, Fsp-Low (Santec); blue, Fsp-High (Santec); orange, Fsp-South (Perharidy); grey, *F. vesiculosus* (Santec); parameters: $K = 4$; MCMC = 1000,000.

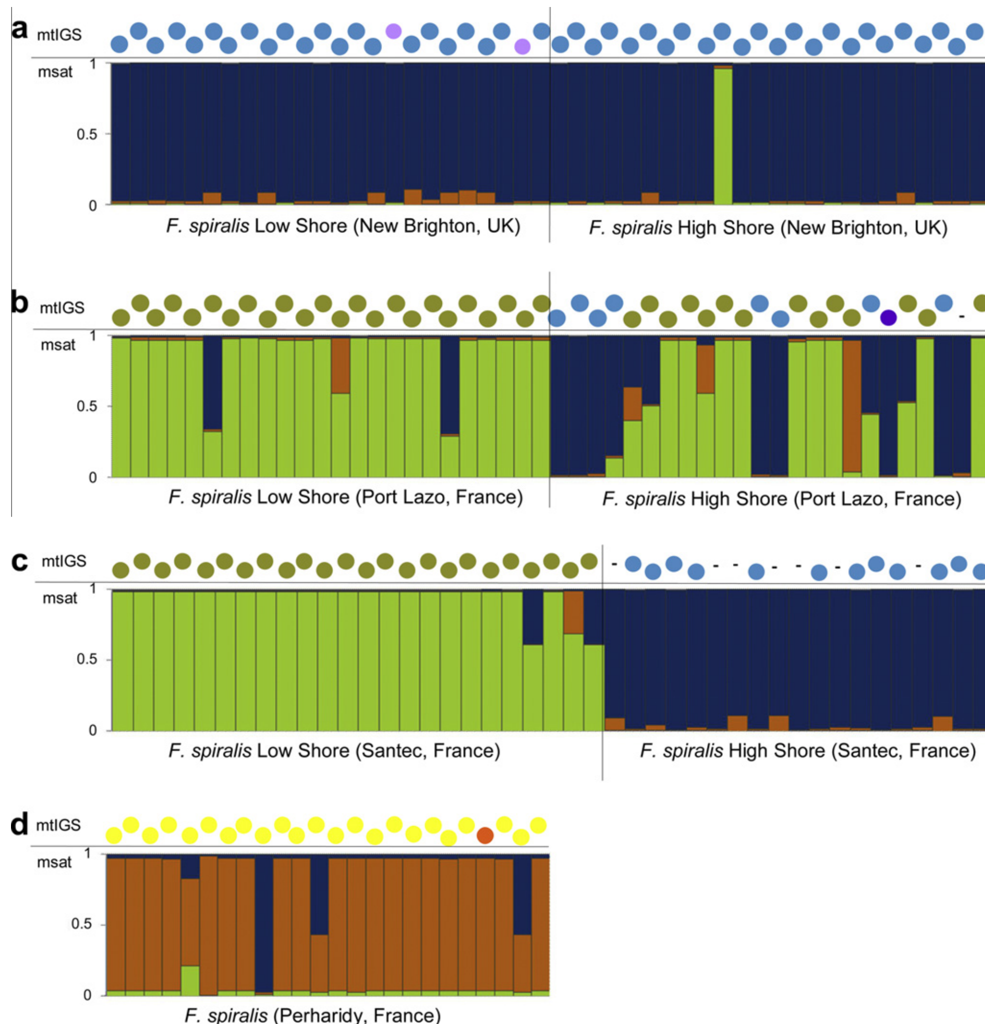

Fig. 2. Comparison of genetic identification based on microsatellites and the distribution of mtDNA haplotypes for *F. spiralis* at eight locations. Each vertical bar represents the multi-locus genotype (microsatellites) of one individual (colors represent the proportion of the genotypes assigned to each genetic group) with its mitochondrial haplotype (mtIGS) shown in the circle above. Circle colors represent distinct haplotypes as in Fig. 3. Individuals are divided and labeled according to their position on shore (*F. spiralis* Low/High Shore represents sampling location, not genetic type).

In these analyses, there was no evidence of heteroplasmy for the mtDNA spacer, as all 1539 samples of *F. vesiculosus* showed SSCP patterns characteristic of single sequences (one single peak for the forward sequences and one for the reverse) (data available on request). Overall mtDNA haplotype diversity (h) for *F. vesiculosus* was relatively high, ranging from 0 to 0.6000 but nucleotide diversity (π) was relatively low, ranging from 0 to 0.0270 (Table 4). Highest diversity was recorded in the Brittany/English Channel area followed by Scotland, Faroe, and Ireland. Diversity was very low from the Baltic to the White Seas (ranges; 0–0.1230 and 0–0.0006 for h and π, respectively) and populations from Iceland, Greenland, Canada and USA were monomorphic (fixed for *c3*) (Table 4).

3.3. mtDNA-IGS haplotype network

The haplotype network for *F. spiralis* revealed 13 haplotypes (*s1–s13*) in two distinct clusters. The North Cluster of *F. spiralis*

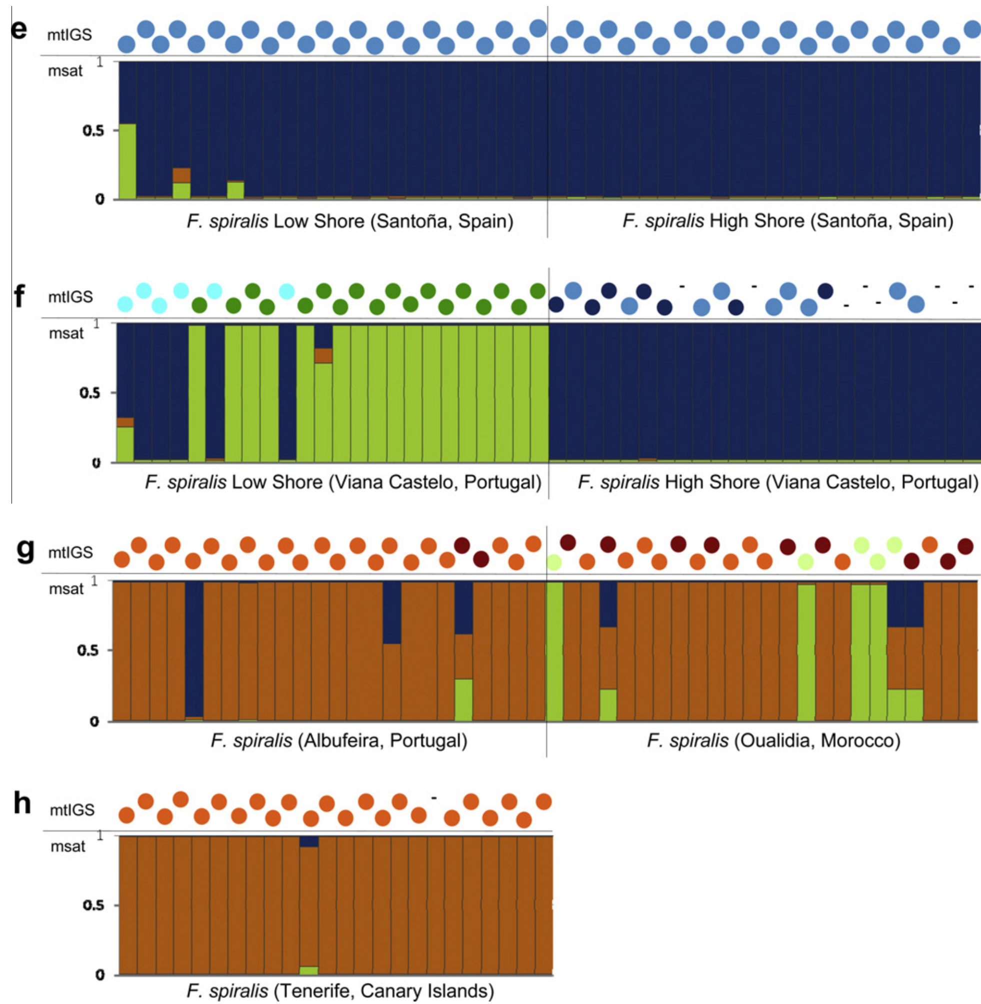

Fig. 2 (*continued*)

Table 2
Pairwise differentiation (F_{ST}) among *F. spiralis* entities. All F_{ST} values were significant except between MOR and ALB ($p < 0.05$). Codes as in Table 1.

	Fsp-Low			Fsp-High					Fsp-South			
	SAN	VIA	MOR	NBR	PLA	SAN	STA	VIA	PER	ABL	MOR	TEN
Fsp-Low												
PLA	0.488	0.693	0.463	0.623	0.544	0.734	0.756	0.815	0.586	0.725	0.718	0.846
SAN		0.836	0.843	0.692	0.702	0.814	0.845	0.900	0.867	0.905	0.910	0.964
VIA			0.761	0.740	0.703	0.805	0.806	0.954	0.922	0.923	0.925	0.982
MOR				0.654	0.455	0.691	0.704	0.946	0.845	0.843	0.835	0.970
Fsp-High												
NBR					0.140	0.250	0.390	0.331	0.508	0.545	0.569	0.711
PLA						0.217	0.225	0.575	0.547	0.593	0.596	0.733
SAN							0.435	0.695	0.721	0.698	0.701	0.734
STA								0.735	0.738	0.726	0.722	0.831
VIA									0.876	0.915	0.923	0.972
Fsp-South												
PER										0.677	0.687	0.912
ALB											0.023	0.909
MOR												0.917

(i.e., all Fsp-High and Fsp-Low) haplotypes all occurred from Viana do Castelo (Portugal) northwards, with the exception of a few individuals found in Morocco, and most of this cluster was shared with *F. vesiculosus*. The South Cluster included all haplotypes south of Viana do Castelo, plus those from a single location (Perharidy, where *F. vesiculosus* was absent) in Brittany (Fig. 3). Only three haplotypes (*c1*, *c2*, *c3*) were present in Fsp-High, Fsp-Low, and *F. vesiculosus*, with haplotype *c2* deemed the shared ancestral haplotype for the entire network. No Fsp-South haplotypes were shared with *F. vesiculosus*. The two most common haplotypes, *c1* (North Cluster) and *s11* (South Cluster), found in 52% and 18% of all samples, respectively, were never found together in the same

Table 3
Diversity measures based on five microsatellite loci in *F. spiralis.* L, Low; H, High; S, south; number of individuals of each entity determined by microsatellites used for diversity calculations is summed under each location category; *A*, allelic richness (standardized to $n = 15$); H_{exp}, expected heterozygosity, F_{IS} (1000 permutations, $* = p < 0.05$), ‡ = calculation of F_{IS} is not possible because of monomorphism in four loci and only one individual possessed one different allele for the fifth locus (indicates strong selfing).

Location	L	H	S	*A*	H_{exp}	F_{IS}
England						
New Brighton – L	0	23	0	–	–	–
New Brighton – H	0	24	0			
Summary		47		1.74	0.272	0.95363*
Brittany (FRA)						
Port Lazo – L	22	2	0			
Port Lazo – H	11	13	1			
Summary	33			2.40	0.166	0.52968*
Summary		15		1.88	0.394	0.66712*
Santec – L	24	0	0			
Santec – H	0	19	0			
Summary	24			1.37	0.049	1.00000*
Summary		19		1.96	0.281	0.66939*
Spain						
Santoña – L	0	24	0			
Santoña – H	0	24	0			
Summary		48		1.86	0.157	0.84211*
Canary Islands						
El Medano, Tenerife – S	0	0	24			
Summary			24	1.13	0.080	‡
Portugal						
Viana do Castelo – L	18	6	0			
Viana do Castelo – H	0	24	0			
Albufeira (S)	0	0	22			
Summary	18			1.29	0.022	1.00000
Summary		30		1.20	0.032	0.79433*
			22	1.38	0.052	1.00000*
Morocco						
Oualidia – L	4	0	20			
Oualidia – H						
Summary	4			1.20	0.086	1.00000
Summary			20	1.35	0.062	0.84298*

population and three haplotypes (*s2*, *s4*, *s8*) were unique. Haplotype *c1* was dominant throughout the North Atlantic and in the single North Pacific population (Fig. 3). The greatest haplotype diversity was evident in the Brittany region and along the Iberian Peninsula (Fig. 4).

A total of 14 mtDNA haplotypes were recovered (*v1*–*v14*) from 1045 *F. vesiculosus* individuals, with five (29%) being unique (*v7*, *v9*, *v10*, *v12*, *v14*) (Fig. 3). The most common haplotype (*c3*), shared with *F. spiralis*, was present in 62% of the samples, whereas the second most common haplotype (*c2*), and also shared, was found in 29% of the samples. Shared haplotype *c3* (and related haplotypes within Cluster 1) also was the most widespread, ranging from northern France to Scandinavia and to northeastern Atlantic shores (Fig. 4). In contrast, haplotype *c2* and closely related haplotypes (Cluster 2) showed a more limited distribution, from the Faroes to Portugal. The distribution of the two clusters overlapped only around the British and Irish Isles and the Faroes.

Each of the two distinct clusters in the *F. vesiculosus* haplotype network exhibited a starlike topography, with all haplotypes only one step from their central haplotypes (*c2* and *c3*) (Fig. 3). The minimum sequence divergence between the clusters (0.4%) corresponded to a minimum divergence time of 230,000–130,000 yrs (with a clock of 2–3.4%/Myr), clearly predating the LGM. The generalized skyline plot (Fig. 5) indicated exponential demographic growth from the mid-Pleistocene with a dramatic acceleration (1–2 orders of magnitude) 300,000–200,000 yrs BP (during the Cromer and/Holstein interglacial period in Northern Europe). There was no evidence of a post-LGM expansion, although the suitability of these data for such an analysis can be questioned if there are introgressed haplotypes (see discussion) mixed within a species whose coalescence history would, therefore, be distinct from the history of divergence between and within the taxa.

4. Discussion

At the most basic level, speciation is determined by the degree of gene flow between two entities and speciation processes reflect the degree of separation or barriers to the gene flow. Various categories of extrinsic barriers promoting species divergence have been used to define the speciation process: allopatric with a complete barrier, parapatric with a partial barrier, and sympatric with no barrier. However, both the species concept and the processes driving speciation represent sliding windows along continuums, rather than discrete categories (Butlin et al., 2008). For example, it is now recognized that speciation can occur despite incomplete reproductive isolation (=adaptive speciation); similarly, very low levels of gene flow are "allowed" to breach the "complete" barriers of allopatry (Schluter, 2001, 2009; Hey, 2006; Butlin et al., 2008; Fitzpatrick et al., 2008).

The intertidal brown alga *F. spiralis* and its interactions with the sister species *F. vesiculosus* provide excellent examples of the conceptual and philosophical challenges to understanding speciation and the processes of speciation. The morphological-based variety *F. spiralis* var. *platycarpus* (Batters, 1902) corresponds to the genetic-based entity identified as Fsp-Low (Billard et al., 2010), whereas Fsp-High corresponds to the typical *F. spiralis* morphology. Our genetic analysis of the *F. spiralis* complex, using a wider sampling base and a combination of microsatellites and mtDNA, not only confirmed the existence of Fsp-High and Fsp-Low, but also revealed a third entity, Fsp-South, and specific geographical patterns of the three entities. Fsp-High and Low were found in the northern portion of the distributional range and co-occurred with *F. vesiculosus*, whereas Fsp-South occurred primarily in the southern portion of the distributional range of the species and in the absence of *F. vesiculosus*.

4.1. Divergence of F. spiralis entities

How did the three entities of *F. spiralis* diverge and what is their relationship with *F. vesiculosus*? It is likely that the *Fucus* lineage comprised of these species (Lineage 2) (Serrão et al., 1999; Coyer et al., 2006a) arose and diverged in the North Atlantic, rather than the North Pacific where none of the species occurs naturally (*F. spiralis* is an introduction, see below).

In a recent study describing Fsp-Low and Fsp-High, Billard et al. (2010) suggested that the initial divergence of *F. spiralis* and *F. vesiculosus* coincided with evolution of mating system variation between the selfing hermaphrodite *F. spiralis* and the dioecious *F. vesiculosus*. Ecological factors reinforcing the divergence include limited dispersal capacities and temporal variation in the timing of gamete release between the two species (Ladah et al., 2008). Similarly, the subsequent divergence of *F. spiralis* into Fsp-Low and Fsp-High may have been facilitated by the high selfing rates in the *F. spiralis* complex, as well as differential tolerance to desiccation stress (Billard et al., 2010), a physiological distinction now confirmed experimentally (Zardi et al., submitted for publication).

Another mechanism explaining the Fsp-High/Low divergence, however, may be the degree of overlap with *F. vesiculosus* and the concomitant potential for hybridization. Intermediate genotypes (putative hybrids) between *F. spiralis* and co-occurring *F. vesiculosus* have been reported in the East and West Atlantic (Wallace et al., 2004; Engel et al., 2005; Coyer et al., 2006b; Mathieson et al.,

Table 4
Sampling locations and diversity measures for IGS haplotypes in *F. spiralis* and *F. vesiculosus*. *h*, haplotype diversity; π, nucleotide diversity.

Location	Code	*F. spiralis*					*F. vesiculosus*				
		N	N_h	H_{id}	h	π	N	N_h	H_{id}	h	π
Pacific											
San Juan, WA	SJI	48	1	*c1*	0.0000	0.00000	–	–	–	–	–
Western Atlantic											
Appledore	APP	18	2	*c1*, *c3*	0.1111	0.00106	31	1	*c3*	0.0000	0.0000
Woods Hole	WHO	–	–	–	–	–	29	1	*c3*	0.0000	0.0000
Inverness	INV	–	–	–	–	–	27	1	*c3*	0.0000	0.0000
St. Lunaire	STL	–	–	–	–	–	22	1	*c3*	0.0000	0.0000
Greenland											
Tasiluk	TAS	–	–	–	–	–	30	1	*c3*	0.0000	0.0000
Iceland											
Grindavik	GRI	22	1	*c1*	0.0000	0.00000					
Patreksfjörður	PAT	–	–	–	–	–	31	1	*c3*	0.0000	0.0000
Sandgerði	SDG	–	–	–	–	–	31	1	*c3*	0.0000	0.0000
Blönduós	BLO	–	–	–	–	–	32	1	*c3*	0.0000	0.0000
Breiðdalsvik	BRE	–	–	–	–	–	31	1	*c3*	0.0000	0.0000
Norway											
Tromsø	TRM	20	1	*c1*	0.0000	0.00000	25	1	*c3*	0.0000	0.0000
Trondheim	TRD	–	–	–	–	–	30	1	*c3*	0.0000	0.0000
Kirkenes	KIR	–	–	–	–	–	32	3	*c3*, *v12*, *v14*	0.1230	0.0006
Faeroes											
Suduroy	SUD	–	–	–	–	–	32	3	*c1*, *c2*, *c3*	0.5222	0.0270
Shetland											
Hamma Voe	HVO	21	1	*c1*	0.0000	0.00000					
Flora Tang	FTA	–	–	–	–	–	32	3	*c2*, *v3*, *v8*	0.3730	0.0045
East Voe of Quaff	EVQ	–	–	–	–	–	32	3	*c3*, *v6*, *v7*	0.1230	0.0012
Scotland											
Oban	OBA	23	2	*c1*, *s1*	0.4032	0.00192	32	3	*c2*, *c3*, *v4*	0.5222	0.0047
Aberlady Bay	ABB	–	–	–	–	–	32	1	*c3*	0.0000	0.0000
Northern Ireland											
Giants Causeway	GIC	–	–	–			32	2	*c2*, *v13*	0.1754	0.0017
England											
New Brighton	NBR	48	2	*c1*, *s5*	0.0816	0.00039					
Praa Sands	PRA	–	–	–	–	–	24	2	*c3*, *v4*		
Sennen Cove	SEN	–	–	–	–	–	24		*c2*		
Ireland											
Labasheeda	LAB	24	1	*c1*	0.0000	0.00000	31	2	*c3*, *v13*	0.3447	0.0017
Spanish Point	SPP	–	–	–	–	–	32	1	c2	0.0000	0.0000
Seafield	SEA	–	–	–	–	–	28	4	c2, *v4*, *v6*, *v8*	0.5159	0.0035
Galway	GAL	–	–	–	–	–	26	4	c2, *v6*, *v8*, *v9*	0.4031	0.0043
Kattegat											
Göteborg	GOT	24	2	*c1*, *c3*	0.0833	0.0079	32	1	*c3*	0.0000	0.0000
Egmont	EGM	–	–	–	–	–	32	1	*c3*	0.0000	0.0000
Tjärnö	TJA	–	–	–	–	–	32	1	*c3*	0.0000	0.0000
Hovs Hallar	HOH	–	–	–	–	–	32	1	*c3*	0.0000	0.0000
Baltic											
Askö	ASK	–	–	–	–	–	11	1	*c3*	0.0000	0.0000
Normandy											
Cap Gris Nez	CGN	24	2	*c1*, *s2*	0.0833	0.00039	31	3	*c1*, *c2*, *c3*	0.6000	0.0033
Sotteville	SOT	–	–	–	–	–	31	2	*c1*, *c3*	0.1247	0.0012
Brittany											
Port Lazo - L	PLA	33	1	*c2*	0.0000	0.00000	–	–	–	–	–
Port Lazo - H	PLA	14	3	*c1*, *c2*, *s4*	0.5824	0.00304	–	–	–	–	–
Santec – L	SAN	22	1	*c2*	0.0000	0.00000	–	–	–	–	–
Santec – H	SAN	13	1	*c1*	0.0000	0.00000	–	–	–	–	–
Perharidy	PER	24	2	*s11*, *s12*	0.0833	0.00039	32	3	*c2*, *v2*, *v6*	0.5585	0.0030
Menez-Ham	MEH	–	–	–	–	–	29	4	*c2*, *v1*, *v6*, *v11*	0.5000	0.0036
Aber Wrach	ABW	–	–	–	–	–	27	3	*c2*, *v1*, *v10*	0.4160	0.0025
Spain											
Santoña, Cantabria	STA	48	1	*c1*	0.0000	0.00000	–	–	–	–	–
Castello, Asturias	CAS	16	2	*s8*, *s12*	0.1250	0.00238	–	–	–	–	–
Canary Islands											
El Medano, Tenerife-S	TEN	23	1	*s11*	0.0000	0.00000	–	–	–	–	–
El Burrero, Gran Canaria	GCA	18	1	*s11*	0.0000	0.00000	–	–	–	–	–
Portugal											
Viana do Castelo – L	VIA	18	1	*s7*	0.0000	0.00000	32	2	*c2*, *v5*	0.1754	0.0008
Viana do Castelo – H	VIA	22	3	*c1*, *s1*, *s3*	0.6883	0.00414	–	–	–	–	–

(*continued on next page*)

Table 4 (*continued*)

Location	Code	*F. spiralis*					*F. vesiculosus*				
		N	N_h	H_{id}	*h*	π	*N*	N_h	H_{id}	*h*	π
Albufeira (S)	ALB	24	2	*s11*, *s13*	0.1590	0.00076	–	–	–	–	–
Aveiro	AVE	–	–	–	–	–	32	2	*c2*, *v1*	0.1247	0.0006
Ayamonte	AYA	–	–	–	–	–	32	1	*c2*	0.0000	0.0000
Azores											
São Miguel Island	AZO	23	3	*s9*, *s10*, *s11*	0.5020	0.00294	–	–	–	–	–
Morocco											
Oualidia – L	MOR	4	1	*s6*	0.0000	0.00000	–	–	–	–	–
Oualidia – H	MOR	44	3	*s6*, *s11*, *s13*	0.4746	0.00319	–	–	–	–	–

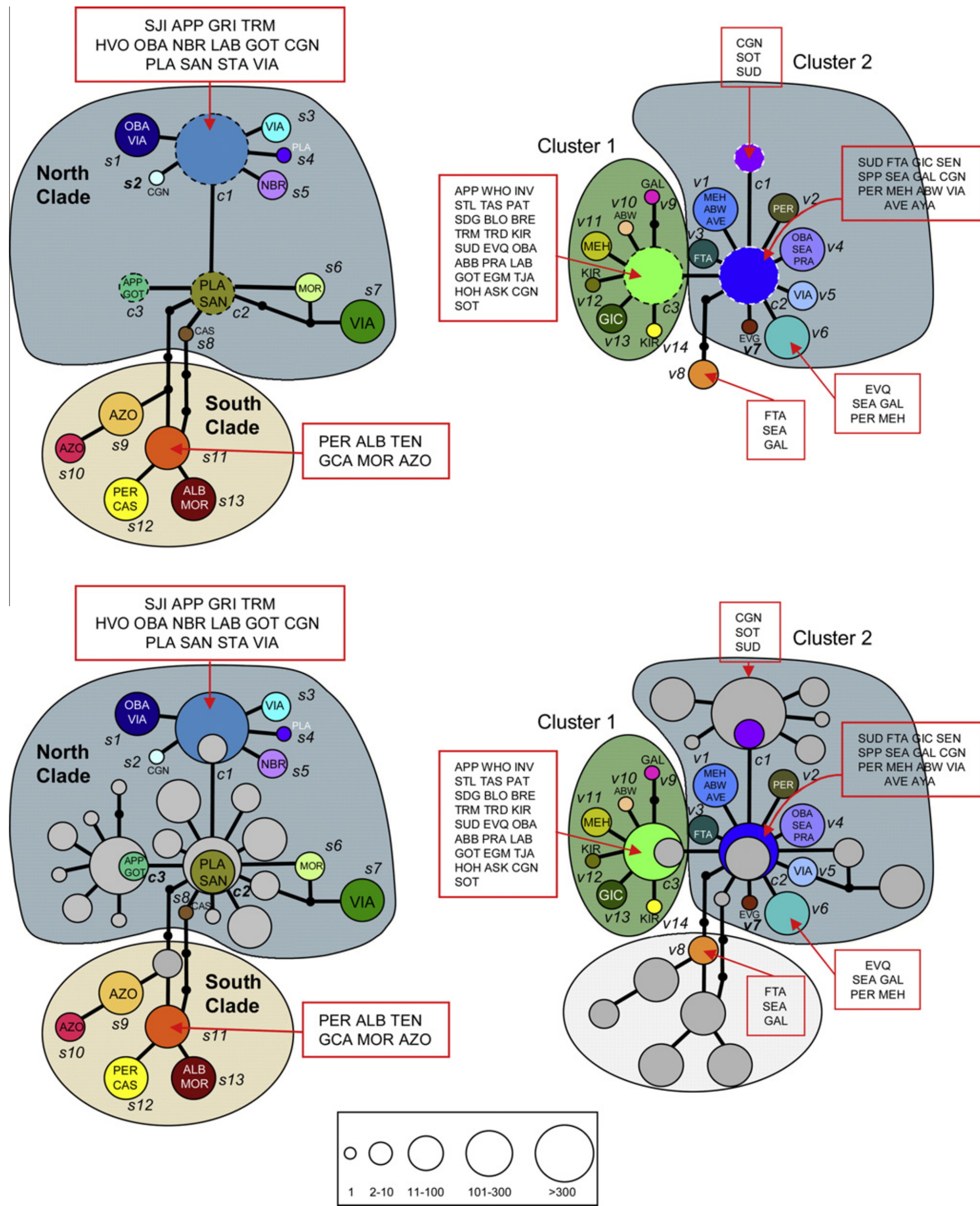

Fig. 3. Statistical parsimony network of *F. spiralis* and *F. vesiculosus* based on mtDNA-IGS haplotypes. Each circle represents one haplotype and size is proportional to the number of individuals with the given haplotype as shown in legend below. Each branch between two nodes represents one mutational step and small black nodes represent haplotypes with intermediate mutation steps not found. Abbreviations are given in Table 1 and haplotypes in Table 4. Colors can be compared within a species (e.g., Figs. 3 and 4), but cannot be compared among species. Three haplotypes (*c1*, *c2*, *c3*) are shared among *F. spiralis* and *F. vesiculosus* and are indicated with dotted lines in the upper panels and overlapped in the lower panels. Upper left: *F. spiralis* haplotypes are shown in color (*s1*–*s13*), lower left: *F. spiralis* haplotypes overlaid on *F. vesiculosus* haplotypes (grey, unlabeled). Upper right: *F. vesiculosus* haplotypes are shown in color (*v1*–*v14*), lower right: *F. vesiculosus* haplotypes overlaid on *F. spiralis* haplotypes (grey, unlabeled).

2006; Billard et al., 2010) and can be expected wherever *F. spiralis* and *F. vesiculosus* co-occur along rocky intertidal shores north of NW Iberia. However, the two species do not co-occur further south (Ladah et al., 2003).

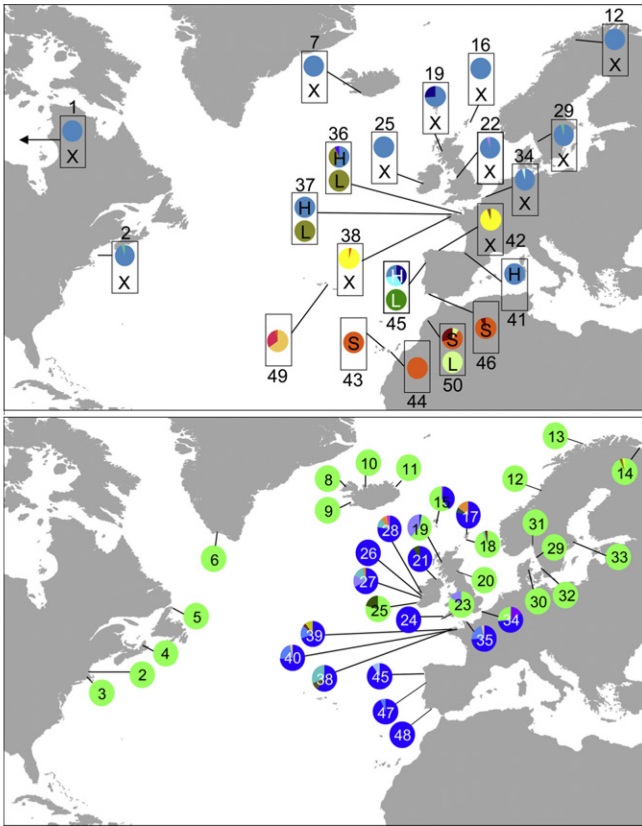

Fig. 4. Distribution of species. Top: *F. spiralis* mtDNA haplotypes based on identification using five microsatellite loci. Each box contains two circles representing genetic identifications as low (L) and high (H) shore – this microsatellite-based identification was only conducted on sites whose circles contain a letter, identifying the circle as high (H), low (L) or south (S) genetic types; boxes with one circle and an "X" represent a location where only the high shore was sampled and verified. Location numbers are given in Table 1 "X". Bottom: *F. vesiculosus* mtDNA haplotypes based on identification using 12 microsatellite loci. For both species, size of the circles is NOT proportional to the number of individuals with the given haplotype and location numbers are presented in Table 1.

The observed pattern of shared mtDNA-IGS haplotypes across *F. vesiculosus* and *F. spiralis* from Northern regions most likely resulted from lateral transfer and spread via hybridization. The alternative hypothesis of shared ancestral polymorphisms among these genetic entities that have not yet been sorted into lineages is unsupported if *F. spiralis* are a monophyletic entity, as *F. spiralis* South would have to have diverged earlier than the separation of the remaining *F. spiralis* from *F. vesiculosus*. If all *F. spiralis* entities are considered a monophyletic group, however, then our results could arise via lateral transfer and replacement of one species' organelles by those of the other, *following* divergence and resulting in paraphyly. Such a process could take place by hybridization and subsequent spread of introgressed organelles via a selective or neutral sweep, a process recently documented in *F. ceranoides* (Neiva et al., 2010).

Distinguishing between unsorted ancestral polymorphism and introgression (as an explanation for shared haplotypes across these species) also can be resolved by comparing the nuclear genes. Monophyly of all *F. spiralis* genetic types, including Fsp-South, is supported by a nuclear multi-gene phylogeny (Zardi et al., submitted for publication, Canovas-Garcia et al., unpub. data). The hybridization hypothesis is also supported by our data and several other reports revealing the presence of intermediate genotypes (Wallace et al., 2004; Billard et al., 2005; Engel et al., 2005; Coyer et al., 2006b; Mathieson et al., 2006). Thus, hybridization among *F. vesiculosus*, Fsp-High, and Fsp-Low is likely to be (or has been) fairly common.

The third and newly discovered Fsp-South entity (South clade) was at least three mutational steps away from the closest haplotype found in the North cluster (Fsp-High, Fsp-Low, *F. vesiculosus*) (Fig. 3). The separation between northern and southern entities almost certainly predates the LGM because it is unlikely that so many steps could have evolved in just 20,000 yrs, although divergence may be much higher in a selfing species characterized by smaller population size. One-step mutations based on modeling predictions for mitochondrial DNA suggest 50,000 yrs is minimal (Wares, 2002) and chloroplast DNA (both genes and introns) have an even slower mutation rate (Anderson et al., 2006).

Fsp-South was also dominant and almost exclusively found in the four locations sampled at the southern edge of *F. spiralis* distribution, a region where Fsp-South and *F. vesiculosus* populations only occur in allopatry. The pattern may be explained by physiological adaptations of the two species. Unlike more northern areas, *F. vesiculosus* is not present on open coastal shores in southern Portugal because of low recruitment and survivorship in exposed areas without canopy; instead, it is confined to relatively benign habitats such as lagoons and estuarine habitats (Ladah et al.,

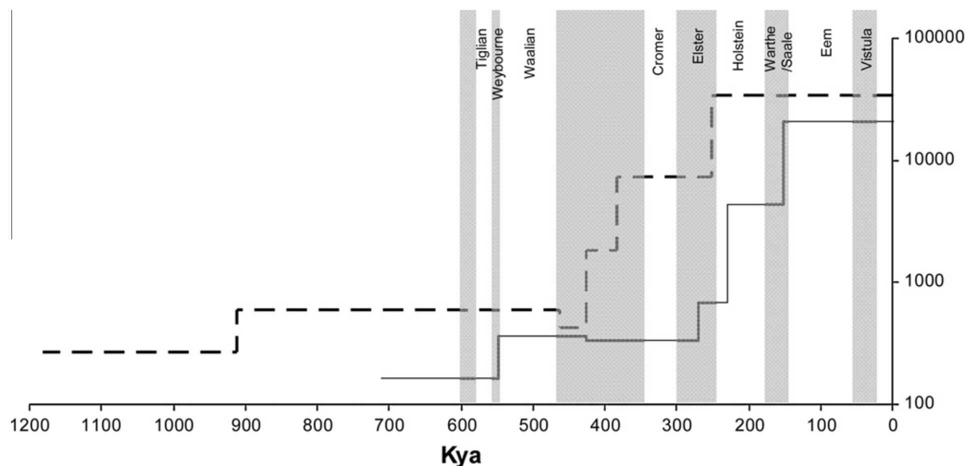

Fig. 5. Generalized skyline plot of population growth through time for *F. vesiculosus*. The lower (dashed line, 2%/Myr) and the upper (solid line, 3.4%/Myr) correspond to boundaries of the molecular clock. The *x*-axis represents time before present (thousand years ago) and the *y*-axis (log) the estimated effective population size (N_e). Glacial (grey) and interglacial (white) periods are indicated.

2003). Conversely, *F. spiralis* (=Fsp-South) is found in the mid-high intertidal along open coasts. Both habitats are, however characterized by high temperatures and desiccation stress.

Although the mtDNA haplotypes (this study) of Fsp-Low are more closely related to Fsp-High than to Fsp-South, thereby suggesting a common ancestor to these entities after divergence from Fsp-South, nuclear markers (three diagnostic microsatellites and SNPs in 14 protein-coding regions, Zardi et al., submitted for publication) do not support the common ancestry. The nuclear genome shows that Fsp-Low is not a phylogenetically distinct entity, mostly clustering with Fsp-South, but with some individuals clustering with *F. vesiculosus* and Fsp-High (Zardi et al., submitted for publication). Collectively, these data suggest that Fsp-South expanded northwards and hybridized with *F. vesiculosus* to form Fsp-Low, a scenario that also explains organellar capture by Fsp-Low. The different physiological adaptations of Fsp-South and *F. vesiculosus* in the southern regions, therefore, may have enhanced genetic divergence by maintaining physical separation (*F. vesiculosus* in estuaries versus Fsp-South on the open coast), thus reducing the opportunity for hybridization in the south. Allopatry explains why Fsp-South shows no traces of hybridization, whereas along the sympatric northern range, Fsp-Low has totally introgressed organelles (this study) and a highly introgressed nuclear genome (Zardi et al., submitted for publication).

Organellar capture via hybridization with *F. vesiculosus* has also been shown for the sister species *F. ceranoides*, which now has alien organelles throughout most of its present range along northern Europe (Neiva et al., 2010). Thus, hybridization/introgression of at least two species (*F. spiralis*, *F. ceranoides*) with *F. vesiculosus*, all in Lineage 2, has resulted in genetically distinct hybrid populations.

4.2. Phylogeography

4.2.1. F. spiralis – North Atlantic

In our study, the Fsp-High/Low entities were present and completely segregated in only two of the five northern locations specifically sampled for their presence: Santec (Brittany) and Viana do Castelo (Portugal) (same samples used in Billard et al., 2010). Their absence from the most northerly site sampled for their presence (New Brighton, UK) may reflect a colonization wave from the Hurd Deep area (Brittany), which served as a glacial refugium during the LGM and was a source of colonizers for several intertidal/shallow subtidal species, including *F. serratus* (reviewed in Maggs et al., 2008). The colonization scenario is supported by the low haplotype diversity and dominance of haplotype *s1* (characteristic of Fsp-High) throughout the North Atlantic and in the single site in the North Pacific. On the other hand, the exclusive presence of Fsp-High at Santoña (Northern Spain), an area unlikely to be affected by ice cover during the LGM, can only be explained by habitat or dispersal restrictions in this more estuarine area. The presence of Fsp-South in southern Portugal (Albufeira) and its near-absence north of northern Portugal (Viana do Castelo) brackets a well-described biogeographic boundary along the central Portuguese coast for diverse species such as red algae, ground fish, and seagrass (Alberto et al., 1999; Gomes, 2001; Diekmann et al., 2005), as well as the composition of intertidal communities (Boaventura et al., 2002; Lima et al., 2007). Abiotic dissimilarities such as changes in coastal morphology, precipitation patterns, outflow of Tagus River, the imposing submarine Nazaré Canyon, air temperatures, sea surface temperatures, and surface currents undoubtedly maintain the North–South break (Gomes, 2001; Boaventura et al., 2002; Martins et al., 2002; Ladah et al., 2003; Koho et al., 2007). The break is especially important for species with limited gamete dispersal, such as *F. spiralis* (Ladah et al., 2008). For example, eastern North Atlantic surface currents bifurcate at 41°N and the resulting south-flowing Portuguese Current and the northeast-flowing Portuguese Counter-Current (Martins et al., 2002) form an effective barrier for gametes with limited dispersal and floating reproductive thalli. Despite the strong biogeographic break, however, the finding of Fsp-South in Perharidy (Brittany), only one km from Santec where both Fsp-High and Fsp-Low were present and segregated, can be seen as further support for the important role of introgression among the Fsp entities. In the absence of *F. vesiculosus*, for example, Fsp-Low is effectively Fsp-South. The role of introgression also holds for the isolated individuals of Fsp-High (Albufeira) and Fsp-Low (Morocco) in the south.

Was Fsp-South historically present throughout the Atlantic, becoming extinct during the LGM and/or competitively excluded by Fsp-High and Fsp-Low in the northern areas? Or is Fsp-South endemic to the south and only under special circumstances able to colonize northern areas? We hypothesize that *F. spiralis* initially inhabited the entire Atlantic, and diverged into two entities in the northern and southern environments because of geographic separation during climatic shifts and/or local adaptation. The southern-adapted entity diverged to an Fsp-South endemic, whereas the northern-adapted entity diverged to Fsp-High. Hybridization of *F. vesiculosus* with occasional northward migrants of Fsp-South might have been at the origin of Fsp-Low given the high amount of haplotype sharing. We further hypothesize that the LGM forced the northern entities into at least one refugium (Hurd Deep, an inland marine lake off Brittany, see Maggs et al., 2008), which could have provided the opportunity for organelle introgression from *F. vesiculosus* into Fsp-South leading to Fsp-Low and posterior spread during postglacial re-colonization (e.g., as in Neiva et al., 2010), whereas the distribution of Fsp-South was relatively unaffected. Contemporary gene flow remains highly restricted because of the biogeographic break along the central Portuguese coast, but nevertheless does occur. Another example of introgression between closely related species in the Hurd Deep region of Brittany was recently documented in red algae (*Gracilaria* spp.) (Destombe et al., 2010) suggesting that the hybridization/introgression among algal species might be more widespread than suspected.

4.2.2. F. spiralis – North Pacific

The presence of a unique haplotype in the single Pacific population that was shared with Atlantic populations supports the hypothesis of a recent introduction of the Pacific by *F. spiralis* (see also Lüning, 1990; Serrão et al., 1999; Coyer et al., 2006a). The restriction of populations to the Washington State (USA)–British Columbia (Canada) border (as opposed to distribution throughout the North Pacific) further supports the introduction hypothesis (see Coyer et al., 2006a, and references therein). However, extensive sampling of other North Pacific populations may well reveal additional haplotypes and the possibility of multiple introductions. The alternative hypothesis that *F. spiralis* evolved in the North Pacific, colonized the North Atlantic, survived only in the southern portion of its Atlantic distribution (Azores, Canary Islands, Morocco) during an earlier glacial period, and then diverged to Fsp-Low and High, is not consistent with observed patterns of haplotype sharing.

4.2.3. F. vesiculosus – North Atlantic

The greatest demographic expansion (e.g., increase of effective population size) of *F. vesiculosus* occurred 300,000–200,000 yrs ago (during the Cromer and/Holstein interglacial period), well before the LGM (30,000–22,000 yrs ago). Refugia often are characterized by species with high allelic and/or haplotypic diversity and the presence of private alleles. Thus, post-LGM re-colonization of *F. vesiculosus* likely commenced from areas with high mtDNA haplotype diversity, possibly SW Ireland and the Hurd Deep, that were previously identified as glacial refugia for a number of marine

organisms (reviewed in Maggs et al., 2008). As the Hurd Deep was not reconnected to the open North Atlantic until 15,000–13,000 yrs ago (see references in Hoarau et al., 2007b), initial re-colonization either stemmed from the SW Ireland refugium or from another putative refugium in the Brittany region. Both of the dominant haplotypes (*c2*, *c3*) must have been present in SW Ireland and initially expanded north, as evident by their co-occurrence on the islands of Shetland and The Faroes. Only the *c3* haplotype expanded throughout Scandinavia, the Baltic, and Iceland/Canadian Maritimes/New England, however, whereas the *c2* haplotype colonized areas to the south.

A recent microsatellite-based study of additional *F. vesiculosus* populations in the New England/Canadian Maritimes region, as well as areas extending to the southern limit of the species near North Carolina (USA), revealed a high degree of fixation and lack of diversity, characteristics consistent with a recent colonization event (Muhlin and Brawley, 2009). Furthermore, examination of the mtDNA-IGS region (600–700 BP) revealed the presence of five haplotypes, each differing by 1 BP, further emphasizing a recent colonization event (Muhlin and Brawley, 2009). As Haplotype *c3* in our study was identical to the corresponding region of the most widespread haplotype (A) in Muhlin and Brawley (2009), populations of *F. vesiculosus* throughout its range along the western Atlantic shore originated from European colonizers from the SW Ireland glacial refugium. In this regard, the phylogeographic patterns of *F. vesiculosus* are similar to the patterns of the closely related species *F. serratus*, which also experienced demographic expansion during a pre-LGM interglacial (Eem, 128,000–67,000 yrs BP) and rapid recolonization by a single mtDNA-IGS haplotype (throughout the Northern European portion of its range) (Hoarau et al., 2007b). For both species, therefore, the LGM influenced population distribution, but not necessarily in a historical demographic context (i.e., populations remained large and, therefore, did not undergo a bottleneck followed by rapid population growth). The major expansions of the two species occurred during pre-LGM interglacial periods.

5. Conclusion

Despite hybridization/introgression, the three *F. spiralis* entities are maintained by local and global scale ecological adaptations. Fsp-South is mostly confined to continental shorelines south of the Targus River in central Portugal and the offshore Azores. *F. vesiculosus* also is found along the southern continental shores, but does not overlap (or hybridize) with Fsp-South because of different habitat preferences (estuarine vs open shore). When sympatric populations of Fsp-South and *F. vesiculosus* contacted along the NW Iberian coastlines and the Brittany coast of France, these resulted in the Fsp-Low lineage through hybridization. We further hypothesize that Fsp-Low was more adapted to the lower shoreline than the co-occurring *F. spiralis*, which subsequently diverged to Fsp-High. Thus, the Fsp-Low and Fsp-High lineages initially were (and continue to be) maintained by high selfing rates and limited dispersal of their respective gametes, as well as differential physiological adaptation to desiccation (Billard et al., 2010). The contemporary distribution of Fsp-Low appears to be restricted to a few areas in Brittany and only in sympatry with *F. vesiculosus*, whereas Fsp-High ranges throughout northern Europe, Iceland, and northeastern US–Canadian Maritimes. Hybridization occurs between Fsp-High and *F. vesiculosus*, but the dwarf hybrids inhabit a much different habitat (salt marsh grass) than either parental species (Wallace et al., 2004; Coyer et al., 2006b; Mathieson et al., 2006).

The forces driving reproductive isolation and the underlying basis of genetic divergence can best be examined in systems where isolation and divergence are maintained in the face of gene flow (Butlin et al., 2008). Our understanding of speciation and the processes of speciation will be enhanced by thinking more of sliding windows along continuums and by investigations of other species residing within the micro-environment-rich intertidal areas (Johannesson, 2009).

The rapidly evolving intertidal macroalgal genus *Fucus* provides an excellent species complex in which to test hypotheses of the environmental gradient as a force promoting diversification and speciation. For example, the common garden approach utilized by Zardi et al. (submitted for publication) can be used to examine fine-scale gene flow along the environmental garden (comparison between neutral and selected markers), as well as the use of reciprocal transplant experiments and controlled crosses to test for reproductive isolation (e.g., Engel et al., 2004; Schmidt et al., 2008; Johannesson, 2009; Pearson et al., 2009).

Acknowledgments

We thank F. Alberto, R. Araújo, B. Bahuaut, E. Boon, C. Daguin, C. Destombe, C. Engel, K. Johanesson, F. Kupper, C. Monteiro, J. Neiva, A. Neto, C. Perrin, A. Peters, and M. Skage for assistance with sample collecting; J. Veldsink and S. Boele-Bos for laboratory assistance; and J. van Schaik for useful discussions. The research was supported by FCT, Portugal, FEDER, and the European Union through the NEST-complexity project EDEN (043251) and the FP6 Networks of Excellence Marine-Genomics-Europe (GOCE-CT-2004-505403) and MARBEF-Marine Biodiversity and Conservation, GBIRM Response Mode Project (GOCE-CT-2003-505446).

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